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The Future of Pediatric Rheumatology: Many Questions Remain

Thomas J. A. Lehman

In a report published elsewhere in this issue of *Arthritis & Rheumatism*, Ruperto et al describe a randomized, placebo-controlled trial of infliximab plus methotrexate (MTX) for the treatment of polyarticular-course juvenile rheumatoid arthritis (JRA) (1). The trial resulted from the combined effort of 39 authors from 30 institutions in 13 countries, working together in an excellent example of multi-institutional, international collaboration. This study would be hailed as a hallmark of what pediatric rheumatologists can accomplish, except that the results failed to reach statistical significance. Infliximab plus MTX is effective therapy for RA (2), and the majority of pediatric rheumatologists believe it is effective for polyarticular-course JRA as well. What, then, should we conclude from the lack of statistical significance of the primary end point result in this study?

Should it be concluded that the combination of infliximab and MTX is ineffective for treatment of polyarticular JRA? JRA is clearly not the same disease as adult RA, and the findings of this randomized, placebo-controlled trial (1) do not support an evidence-based conclusion that the combined use of infliximab and MTX is beneficial in JRA. An alternative explanation for the negative results might be a questionable study design, the small number of subjects (despite the large number of authors and institutions), or simply the random nature of Type II statistical error.

The difficulty of organizing large, randomized, placebo-controlled trials and recruiting a sufficient number of subjects is well recognized. It is a problem for studies of adults with rheumatic disease and is compounded manyfold for studies of children with rheumatic disease, because of the smaller numbers of affected children and the concerns of physicians, parents, and institutions that children receive optimal care at all times, leading to unwillingness to commit patients to participation in trials in which they might receive placebo. If the study by Ruperto et al had been performed in adults, it could have been repeated with appropriate modification of the study design or increase in the number of subjects; many studies of the combined use of MTX and infliximab in adults with RA have been reported in the literature (3–5). However, because of the small numbers of available patients, this cannot be done easily for a study of a pediatric rheumatic disease.

The resources of the pediatric rheumatology community are clearly limited. Should these resources be used in attempts to replicate the findings in studies of adult patients, or would they be better utilized in other investigations? A strong argument for the former is that the rheumatic diseases of childhood are not identical to those of adults. However, there are currently no examples of therapies that have proven successful in adults with RA that have not been efficacious in JRA patients as well.

Not all drugs that are safely used in adults can be safely used in children, and every new therapy for rheumatic diseases must be carefully reevaluated before it is broadly applied to pediatric patients. The Food and Drug Administration has a responsibility to assure the safety of these regimens before sanctioning their use in children. Such studies can be accomplished with far fewer resources than are necessary for randomized, placebo-controlled trials of efficacy. Unless one is prepared to argue that the failure of Ruperto and colleagues’ study to prove the efficacy of combined inflix-
imab and MTX means that the combination should not be used in children, one must instead ask, “Could the resources of the pediatric rheumatology community be better used elsewhere?”

In an ideal world, every drug shown to be safe and effective in adults with rheumatic disease would undergo separate, specific testing in a pediatric population. However, with limited resources, the pediatric rheumatology community would be better served by efforts to understand and treat the conditions that are unique to pediatric rheumatology, such as systemic-onset JRA, linear scleroderma, juvenile dermatomyositis, and Kawasaki disease, rather than attempting to replicate in children well-established findings reported in the literature on adult patients. The scientific and academic value of pediatric rheumatology is more likely to derive from development of new information based on improved understanding of our patients’ diseases than from replication of adult studies, regardless of whether the results reach statistical significance.

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EDITORIAL

Mutations in Cryopyrin: Bypassing Roadblocks in the Caspase 1 Inflammasome for Interleukin-1β Secretion and Disease Activity

Charles A. Dinarello

Unlike the release of tumor necrosis factor α (TNFα), there are several roadblocks to the release of interleukin-1β (IL-1β), beginning with the transcription of the IL1B gene and ending with the exit of the active cytokine from the cell. There is also the additional roadblock of the high level of synthesis and secretion of the naturally occurring IL-1 receptor antagonist (IL-1Ra), with its tight binding to the IL-1 receptor type I and an affinity higher than that of either IL-1β or IL-1α. If these roadblocks are not enough to limit the activity of IL-1β, there is nature’s decoy mechanism, which consists of the preferential binding of the cell surface IL-1 receptor type II to IL-1β, preventing it from triggering the signal-transducing type I receptor. The soluble form of the type II receptor also prevents active IL-1β from engaging the signaling receptor by forming an inactive complex with the soluble IL-1 receptor accessory protein, which is constitutively produced in the liver. The facilitation of IL-1β processing by the caspase 1 inflammasome through ATP activation of the P2X7 receptor can also be viewed as a potential roadblock to the activity of IL-1β.

It seems clear that in the evolution of cytokines, some cytokines, such as IL-6 and IL-1Ra, are quickly driven from the cell by the Golgi machinery, whereas others encounter a series of roadblocks that limit their activity. The most revealing justifications for nature’s wisdom in placing several roadblocks to IL-1β activity are the consequences of the effects of IL-1β in the loss of insulin-producing pancreatic beta cells (1) and the manifestations of autoinflammatory syndromes, diseases that carry a portfolio of destructive inflammation (2–4). Familial Mediterranean fever (5) and hyper-IgD syndrome (6) are also classified as autoinflammatory diseases because of the poor control of the processing and secretion of IL-1β.

In this issue of Arthritis and Rheumatism (7), the study reported by Gattorno and coworkers offers important new lessons about these roadblocks. The investigators studied the release of IL-1β from primary blood monocytes obtained from patients with chronic infantile neurologic, cutaneous, articular (CINCA) syndrome (known in North America as neonatal-onset multisystem inflammatory disease [NOMID]) and from a patient with Muckle-Wells syndrome (MWS). These 2 syndromes, as well as familial cold-induced autoinflammatory syndrome (FCAS), are the primary examples of dysregulated processing and secretion of IL-1β. The investigators also studied the effects of in vivo treatment with anakinra, a recombinant human IL-1Ra. Specifically, the study examined the secretion of IL-1β from peripheral blood monocytes obtained from the patients before and during treatment with anakinra, a recombinant human IL-1Ra. Specifically, the study examined the secretion of IL-1β from peripheral blood monocytes obtained from the patients before and during treatment with anakinra. The study focused on the function of the IL-1β “inflammasome” (more appropriately termed the caspase 1 inflammasome) and the effect of mutations in cryopyrin, the protein of the NALP3 gene (8). The lessons learned from these studies provide new insights into the tight control of the processing and secretion of IL-1β. While the syndromes studied are rare, the mechanisms revealed by these studies have broad implications for how evolution favored the restriction of biologic responses to IL-1β by placing several roadblocks to its egress from the cell.

The clinical manifestations of these syndromes are characterized by recurrent fevers, neutrophilic leukocytosis, rashes, deforming arthritis, and high serum levels of acute-phase reactants. Progressive deafness,
leptomeningitis, and amyloidosis may be present in some patients. These syndromes are part of a larger group of diseases that are called “autoinflammatory” to distinguish them from those considered to be “autoimmune,” although there is clearly overlap between the 2 groups. Both the adult-onset and the juvenile-onset forms of Still’s disease (systemic-onset juvenile idiopathic arthritis) fall into the autoinflammatory group. The best criterion for identifying an autoinflammatory disease is that the clinical, biochemical, and hematologic manifestations are rapidly and impressively reversed upon initiation of treatment with IL-1Ra, with the IL-1 trap, or with monoclonal anti–IL-1β antibodies. Hence, the culprit in these diseases is IL-1β and not IL-1α; essentially, autoinflammatory diseases are diseases that occur as a result of poor control of the release of active IL-1β.

Compared with monocytes from healthy subjects, freshly isolated peripheral blood monocytes from patients with autoinflammatory diseases secrete more IL-1β when incubated in vitro. For example, peripheral blood mononuclear cells (PBMCs) from patients with NOMID secreted >1 ng/ml more IL-1β during a 24-hour incubation in the absence of any exogenous stimulus, as compared with <50 pg/ml secreted by cells from healthy controls (2). When stimulated with endotoxin, PBMCs from NOMID patients secreted 3–4 times more IL-1β than PBMCs from controls. In the Gattorno study (7), monocytes from patients with CINCA syndrome secreted even greater amounts of IL-1β when stimulated with lipopolysaccharide as compared with monocytes from controls. Although patients with FCAS exhibit dramatic reversal of disease manifestations upon treatment with anakinra, elevated levels of IL-1β in the circulation following cold exposure is not observed (4). Rather, the circulating cytokine is IL-6, which is under the control of IL-1.

These observations reinforce an important clinical lesson in cytokine-mediated diseases: causation is established with specific receptor blockade or specific cytokine neutralization, and not with elevated circulating levels. For example, many diseases are associated with elevated serum levels of IL-6 because this cytokine is readily secreted, but few are due to a pathologic role of IL-6. Although increased secretion of IL-1β by stimulated blood monocytes in vitro can be helpful in the diagnosis of an IL-1β–mediated disease, a few days of treatment with anakinra is safe and will unambiguously reveal the role of IL-1 in the disease in question. Despite this rather “easy IL-1 test of causation,” in the report by Gattorno, there is a lesson about the control of active IL-1β secretion and this lesson may apply to other cytokines, such as IL-18 and IL-33. Although IL-18 likely contributes to several autoinflammatory diseases, macrophage activation syndrome appears to be a disease that is attributable to IL-18 (9). IL-33 may mediate mast cell–related and other T helper type 2 diseases. Both cytokines are members of the IL-1 family and are caspase 1 dependent for secretion of the active cytokine.

The first lesson from the Gattorno study is that monocytes obtained from their patients secreted IL-1β without a second signal. What is meant by the expression “second signal”? There are 2 ways to study the secretion of IL-1β from fresh human blood monocytes. Most studies stimulate the cells with endotoxin (usually at concentrations of 1 ng/ml or lower) and, after 24 hours of incubation, measure the “mature” cytokine released into the supernatant, using a specific enzyme-linked immunosorbent assay. In the absence of cell leakage or cell death, starting after 4 hours and over the course of 24 hours, IL-1β is steadily released into the supernatant medium. The IL-1β that is released is called “mature” because it represents the “active” cytokine following cleavage of the inactive IL-1β precursor by caspase 1. As described below, activation of the intracellular cysteine protease caspase 1 is the primary function of the inflammasome. In general, most, if not all, Toll-like receptor (TLR) ligands, as well as microorganisms themselves, stimulate the secretion of IL-1β via a caspase 1–dependent processing of the IL-1β precursor. Like endotoxins from gram-negative bacteria (via TLR-4), microbial products from gram-positive bacteria (via TLR-2) will result in the progressive release of IL-1β from monocytes starting at 4 hours and continuing for the next 20–36 hours. The vast majority of studies on the secretion of IL-1β use a 24-hour stimulation period. Importantly, in animals or humans injected with endotoxins, the earliest detectable level of IL-1β in the circulation is at 3 hours, and peak levels are present between 4 and 8 hours.

The second way to study the secretion of mature IL-1β is to accelerate the process by incubating the monocytes with endotoxin for a short period of time (3 hours) and then triggering the rapid release of mature IL-1β within 15 minutes. This experimental approach is called the “two-signal” method. As shown in Figure 1A, 3 hours of exposure to endotoxin provides the first signal and results in transcription and translation of the IL-1β precursor. A second signal results in the rapid release of mature IL-1β. This so-called second signal is triggered by activation of the purinergic ion channel called P2X7.
Figure 1. Steps in the activation of the caspase 1 inflammasome and the secretion of interleukin-1β (IL-1β). A, Monocytes from unaffected, healthy controls. Activation of Toll-like receptor (TLR) (step 1) results in transcription (step 2) and translation of the IL-1β precursor (step 3). The IL-1β precursor remains diffusely in the cytosol (14). Upon activation of the P2X7 receptor (P2X7 R) (step 4), there is a rapid efflux of potassium from the cell (step 5a), resulting in a fall in intracellular potassium levels (step 5b). The fall in intracellular potassium levels triggers the assembly of the components of the caspase 1 inflammasome (step 6) and its association with procaspase 1. The caspase 1 inflammasome is composed of cryopyrin plus ASC (apoptosis-associated speck-like protein containing a caspase 1 recruitment domain [CARD]) and Cardinal (CARD inhibitor of NF-κB–activating ligands). Cryopyrin is a large protein with 4 domains: PYR (a pyrin domain), NACHT (a domain found in NAIP [neuronal apoptosis inhibitor protein], CIITA [class II major histocompatibility complex transcription activator], HET-E [incompatibility locus protein from Podospora anserina, a bacterial nucleotide triphosphatase protein], and TP-1 [telomerase-associated protein 1]), NAD (NALP-associated domain), and LRR (leucine-rich repeats). The processing of procaspase 1 results in the formation of the active caspase 1 heterodimer and the cleavage of the IL-1β precursor. The enzymatic processing of the IL-1β precursor by caspase 1 may take place in the cytosol (step 7a) or in the secretory lysosome (step 7b) or both. An influx of calcium into the cell (step 8), with an increase in intracellular calcium levels, provides a mechanism by which mature IL-1β is released from the cell (10,15) (step 9). Calcium influx activates 3 phospholipases; phosphatidylinositol-specific phospholipase C and calcium-dependent phospholipase A2 are required for secretion of IL-1β, with exocytosis of the lysosomal contents (10). B, Monocytes from subjects with mutations in cryopyrin. TLR activation and the synthesis of the IL-1β precursor take place (steps 1, 2, and 3) as in A. TLR triggering may also result in the assembly of the inflammasome (step 4). However, there is constitutive activation of caspase 1 in monocytes from subjects with mutations in cryopyrin, and activation of the inflammasome by a fall in intracellular potassium is not required. Similar to monocytes from healthy controls, the processing of the IL-1β precursor by caspase 1 may take place in the cytosol (step 5a) or in the secretory lysosome (step 5b) or both. Also similar to monocytes from healthy controls, an influx of calcium into the cell (step 6), with an increase in intracellular calcium levels, provides a mechanism by which mature IL-1β is released from the cell (10,15) (step 7). TIR = Toll/interleukin-1 receptor.
with millimolar concentrations of ATP. The activation of the P2X7 receptor by ATP results in the near-simultaneous efflux of potassium from the cell (see Figure 1A). The fall in intracellular potassium levels provides the signal for the cleavage of the IL-1β precursor by caspase 1 (10). The cleavage may take place in cellular organelles called secretory lysosomes, but also possibly in the cytosol. The cleavage likely takes place near the cell membrane. Within 15 minutes of ATP activation of the P2X7 receptor, potassium levels fall, and mature IL-1β is released from the cell. Activation of the P2X7 receptor and the fall in intracellular K+ concentrations, with the subsequent events leading to IL-1β processing and secretion, likely also take place in the “long incubation” method, but at lower levels, resulting in lower secretion of IL-1β. Naturally occurring peptides that activate the P2X7 receptor have been described (11).

The expected finding in the study by Gattorno was that after 3 hours of exposure to endotoxin, monocytes from the CINCA syndrome or MWS patients secreted significantly more IL-1β than did those from the healthy controls (7). The unexpected finding in the study was that monocytes from the CINCA syndrome and MWS patients did not respond to ATP with a burst of IL-1β release. The rapid release of IL-1β by monocytes from healthy donors is triggered by a short exposure to ATP. Unlike monocytes from healthy donors, the release of larger amounts of IL-1β did not require the second signal from ATP. Thus, the ATP activation step (step 4 in Figure 1A) was not needed in monocytes from patients with the cryopyrin mutations. One interpretation is that the caspase 1 inflammasome is constitutively active in these cells during the 3-hour incubation period and does not require the ATP-driven efflux of potassium to cleave the precursor (Figure 1B). But, there was yet another unexpected finding.

In monocytes from healthy controls, ATP activates the processing and the rapid secretion not only of mature IL-1β, but also of caspase 1. However, caspase 1 release did not take place following the addition of ATP to cells from the patients. Hence, the 2 ATP-driven effects are not needed in monocytes from these patients. Procaspa 1 is present in resting monocytes, whereas synthesis of the IL-1β precursor requires TLR activation. Therefore, one would expect to detect spontaneous secretion of caspase 1 in monocytes from patients with a constitutively activated caspase 1 inflammasome. The observation that caspase 1 secretion by the patients’ monocytes occurred only after TLR triggering suggests that the caspase 1 inflammasome is partially, but not fully, active in cells from patients with autoinflammatory syndromes. Therefore, one can interpret the effect of mutations in cryopyrin as bypassing one of the roadblocks that control the exit of active IL-1β from the cell.

Although all patients with CINCA syndrome exhibit the same spectrum of clinical, biochemical, and hematologic abnormalities, not all patients carry mutations in the CLASI1 gene. For example, in a study of 18 patients with NOMID (CINCA syndrome), only 67% carried the mutation (2). Nevertheless, monocytes from all of the patients released significantly more IL-1β, whether resting or after stimulation with endotoxin for 24 hours, as compared with monocytes from healthy controls. The study by Gattorno and colleagues (7) went one step further and discovered that the failure to require ATP for the 3-hour release of IL-1β was only observed in the CINCA syndrome and MWS patients who had CLASI1 mutations. Monocytes from the CINCA syndrome patient without CLASI1 mutations responded to ATP, as did monocytes from healthy donors. We can assume that this differential response to ATP also existed in the cohort of 18 NOMID patients whose cells were studied only for the release of IL-1β over 24 hours (2).

The Gattorno study draws attention to a unique property of the mutations, since one can now assign a molecular mechanism to the increase in IL-1β secretion relevant to the ATP-driven activation of caspase 1 by the complex of interacting proteins that comprise the caspase 1 inflammasome. It is a unique example of how a single mutation reveals a great deal of new information about a complex molecular mechanism. The assembly of proteins of the mutated inflammasome, although not requiring the fall in intracellular potassium, nevertheless still requires an initiating signal, and in the laboratory, endotoxin is used. In the patient, IL-1β itself likely functions as the initiator.

The function of the caspase 1 inflammasome is primarily to convert inactive procaspase 1 into the active enzyme. In the rapid release method, this activation is triggered by the efflux of potassium and is rapidly followed by the appearance of mature IL-1β in the supernatant together with the processed caspase 1. Gattorno provides evidence that the mutation in cryopyrin results in activation of caspase 1 without requiring a sudden fall in the level of intracellular potassium in order to activate the inflammasome. The authors propose that mutated cryopyrin allows for the assembly of the complex of interacting proteins that comprise the inflammasome in the presence of normal
intracellular levels of potassium. This explanation would, in fact, provide a molecular mechanism for the ATP trigger in the wild-type cryopyrin. In the wild-type inflammasome, ATP activation of the P2X7 receptor opens the potassium channel, and as potassium levels fall, caspase 1 is simultaneously activated by the inflammasome. Thus, cryopyrin, in which any one of several mutations may occur, is the molecular target and results in the activation of caspase 1. The model provides an explanation for the consistent observation that monocytes from patients with CINCA/NOMID or MWS secrete more IL-1β than do those from healthy controls. Although studied using an exogenous stimulant such as endotoxin, monocytes from these patients more likely experience background stimulation from endogenous inflammation in vivo. In fact, as discussed below, the most likely endogenous stimulant is IL-1β itself.

The model also supports the concept that the rate-limiting step in the secretion of IL-1β is activation of caspase 1. Any disease process that includes an increase in the steady-state levels of caspase 1, components of the inflammasome, or the IL-1β precursor carries the potential to be an “autoinflammatory” disease. Gattorno and colleagues report that in vivo therapy with anakinra in patients with CIAS1 mutations was associated with an in vitro reduction in the secretion of IL-1β from monocytes as compared with secretion before treatment. Monocytes from the patient without mutations, although secreting more IL-1β than those from controls, secreted the same amount of IL-1β before, as well as after, anakinra treatment. The reduction in the secretion of IL-1β following initiation of anakinra treatment was also reported by Goldbach-Mansky et al (2).

What accounts for these observations, whether assessed by the long (24-hour) release method (2) or the short (3 hours plus 15 minutes) release method (7)? There is no magic about these observations. IL-1β stimulates both its own gene expression as well as the translation of the messenger RNA into the precursor. The original observations were made 20 years ago (12,13). The most likely explanation for a down-regulation of secretion with IL-1 receptor blockade is that IL-1β stimulates the synthesis of caspase 1 and IL-1β precursor. In fact, in NOMID patients treated with anakinra, there was a down-regulation of both caspase 1 and the IL-1β precursor (2), suggesting that blocking the activity of IL-1β brings about a reduction in the further release of IL-1β. Thus, the rate-limiting step is IL-1β activity itself. In support of this concept is the observation that a single dose of a neutralizing anti-IL-1β monoclonal antibody in patients with MWS resulted in a rapid amelioration of disease that lasted 6 months, but within 24 hours after the infusion of the antibody, steady-state levels of IL-1β messenger RNA from PBMCs were decreased (Lachmann H: personal communication). It would therefore be of considerable interest to activate the caspase 1 inflammasome in healthy donor monocytes stimulated with IL-1α for 3 hours and then activate the P2X7 receptor with ATP. Such a study would address a model closer to the clinical reality, since IL-1 itself, rather than endotoxin, is the challenge encountered by patients who have autoinflammatory diseases.

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EDITORIAL

Systemic Humoral Autoimmunity but Joint-Specific Inflammation: The Syndrome of Rheumatoid Arthritis

Xiaobo Wu, Fei Shih, and John P. Atkinson

In our ongoing efforts to explain the immunopathogenesis of rheumatoid arthritis (RA), we will integrate and highlight two recent advances. The first advance is the identification of IgG autoantibodies to citrullinated peptides in a substantial (30–50%) portion of patients with RA and their role as a diagnostic marker for RA (1,2). Surprisingly, this antibody binds to a ubiquitous autoantigen, citrullinated peptide, yet leads to a joint-specific disease. The mechanism through which anti–citrullinated peptide antibodies mediate joint disease is unknown. However, clues to their pathogenesis in RA can be deduced from the K/BxN mouse model of arthritis. The second advance, the K/BxN mouse model, features IgG autoantibodies that likewise bind to a ubiquitous self antigen, glucose-6-phosphate isomerase (GPI), resulting in joint-centered pathology. Analogous to immune complex–mediated glomerulonephritis, we propose a disease paradigm in which joints serve as a repository for autoantibodies, culminating in immune complex formation and subsequent immune activation and synovitis.

The model

K/BxN, an established murine model of arthritis (3–11), is based on the KRN-TCR–transgenic mouse.* The KRN T cell receptor (TCR) recognizes bovine RNase peptide bound to the class II major histocompatibility complex (MHC) molecule IA	extsuperscript{8}. In the initial H-2	extsuperscript{b} background, these mice had no autoimmune phenotype. Remarkably, when the KRN-transgenic mouse was bred to the NOD mouse, all of the F	extsubscript{1} offspring (referred to as K/BxN) spontaneously developed a joint-specific, autoimmune, inflammatory synovitis at age 3–5 weeks (3–5), which faithfully mimicked many clinical, pathologic, and immunologic features of RA. Cartilage destruction and bone erosion occur in the later stages of the disease. By serendipity, the KRN TCR also recognizes a self antigen (GPI) bound to the IA	extsuperscript{27} molecule of the NOD-specific class II MHC. Thus, in K/BxN mice, KRN T cells are autoreactive.

Particularly important for dissecting immunopathologic mechanisms, transfer of serum from K/BxN mice into wild-type (WT) mice results in joint inflammation within a few days (6,7) (Figure 1). Disease is initiated by IgG and can be induced in lymphocyte-deficient recipients, indicating independence of T cells and B cells in this passive-transfer model. Similar to human RA, joint-specific immune complex formation occurs, with subsequent leukocyte recruitment and inflammation (6,7,10) (Table 1). However, the passive antibody-transfer model does not result in pannus formation.

The target antigen recognized in K/BxN mice was identified as GPI (3–5) (Table 2). This ubiquitous glycolytic enzyme catalyzes the interconversion of glucose-6-phosphate and fructose-6-phosphate in the second step of glycolysis. Purified anti-GPI IgG transfers the disease phenotype (6,7). Thus, in this model, KRN T cells recognize GPI and provide help to GPI-specific B cells to produce arthritogenic IgG. With 100% penetrance and rapid onset of the disease, both the sponta-

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*This model needs a new name, because this cumbersome nomenclature is unfortunately indecipherable and meaningless to most rheumatologists. Perhaps, similar to the collagen-induced model of arthritis, it should be called the “GPI-autoimmune model of arthritis.”
neous and passive transfer models are informative tools to study the immunopathogenesis of an organ-specific autoimmune disease arising from an adaptive immune response to a self antigen expressed by all cells.

Requirements for disease in the model

Initiation of arthritis in the K/BxN mouse is attributable to the breakdown of T cell tolerance, with collaboration of autoreactive B cells (3–7). Arthritis does not develop in the μMT mutant mouse, which lacks B cells (6,7). Further studies established that direct interaction of T cells and B cells through CD40/CD40L is necessary for the elaboration of arthritogenic anti-GPI antibodies (4). The subsequent effector phase of arthritis is predominated by cells of innate immunity, macrophages, and granulocytes, with a paucity of T cells. Neutrophils are the earliest infiltrating innate immune cells and play a critical role (8–10). Polymorphonuclear (PMN) cell–depleted mice are resistant to the serum-transfer model of arthritis (10). Further, anti-PMN treatment reverses disease progression (10). Mast cells are detectable in the joint and are activated in human RA. They are required for the GPI-autoimmune model of arthritis (11). Mast cell–deficient mouse strains (W/Wv or Sl/Sld) are resistant to arthritis induction by serum transfer; mast cell transfer restores susceptibility in the W/Wv strain (11). Proinflammatory cytokines, particularly interleukin-1 (IL-1), tumor necrosis factor (TNF), and IL-6 are up-regulated in arthritis (6,7). Whereas some TNFα-deficient mouse strains can develop robust disease in the K/BxN mouse serum-transfer model, IL-1−/− and IL-1 receptor (IL-1R)−/− mice are resistant (11). Intraperitoneal administration of IL-1β, but not TNFα, restores the arthritis susceptibility of W/Wv mice. This latter result indicates that mast cells contribute to the pathogenesis of arthritis through their production of IL-1β at the site of inflammation.

Table 1. Similarities between human RA featuring autoantibodies to citrullinated peptides and a mouse model of arthritis featuring autoantibodies to GPI

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Human RA</th>
<th>K/BxN mouse model</th>
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<tbody>
<tr>
<td>Arises spontaneously</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Transferred by autoantibodies</td>
<td>Possibly†</td>
<td>Yes</td>
</tr>
<tr>
<td>Ubiquitous antigen</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Joint centered</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Complement/Fc receptor</td>
<td>Likely</td>
<td>Yes</td>
</tr>
<tr>
<td>Mast cell triggering</td>
<td>Likely</td>
<td>Yes</td>
</tr>
<tr>
<td>Polymorphonuclear cell recruitment</td>
<td>Likely</td>
<td>Yes</td>
</tr>
<tr>
<td>Cytokine release</td>
<td>Yes</td>
<td>Yes</td>
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</tbody>
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† See ref. 27 for information regarding some of the first attempts to address this issue.

Table 2. Implications for rheumatoid arthritis based on the mouse model

1. Autoantibody-mediated process.
2. The autoantigen is ubiquitous, yet the disease process is joint centered.
3. Rheumatoid arthritis is a syndrome. Future investigators will likely stratify rather than generalize the syndrome of rheumatoid arthritis; that is, based on the patient’s autoantibody profile alone, there appear to be distinct diseases within what is currently labeled rheumatoid arthritis, e.g., rheumatoid factor positive versus rheumatoid factor negative, citrullinated peptide positive versus citrullinated peptide negative, and so on.
Antibodies to GPI are detectable in synovial tissue 20 minutes after intraperitoneal injection, and GPI/anti-GPI immune complexes are in the circulation of K/BxN mice (10). Because the pathologic activity of anti-GPI is in the IgG fraction, the role of the Fc receptor (FcR) was examined in the serum-transfer model, using FcR-deficient mouse strains (6). As expected, FcRγ-deficient mice are resistant. The high-affinity FcRI plays no role, but the low-affinity FcRIII is important. No inhibitory effect was observed in FcRIB-deficient mice.

The role of the complement cascade in the K/BxN mouse model of arthritis has also been elegantly described (6,7). By using C5 and C5a receptor–knockout mice, A/J congenic C5-deficient mice, and neutralizing antibodies to C5a, investigators established a critical role for C5a interacting with its receptor. C6 was not required, ruling out participation of the membrane attack complex. As expected, C3, being a gateway to the activation of C5, is essential for the induction of arthritis. Most informative and unexpected was the finding that the classical pathway of complement activation is dispensable, despite the disease being mediated by antibody and featuring immune complex formation. Instead, the alternative pathway is required for the activation of C5. Factor B, but not C4, is necessary, providing additional evidence for the participation of the alternative pathway. In the mouse model, the dominant subclass of the anti-GPI antibodies, IgG1, does not bind C1q and therefore cannot activate the classical pathway. Instead, antibody bound to antigen serves as a site for alternative pathway amplification: the so-called antibody-dependent activation of the alternative pathway (12).

Local synthesis of complement

In a field pioneered by Harvey Colten and his colleagues, many different cell types have been shown to synthesize complement components (13,14). Although the liver is responsible for supplying plasma with almost all (>90%) of the complement components of the activating cascades (two exceptions being factor D and properdin of the ancient alternative pathway), cells involved in the acute inflammatory reaction synthesize many components. This is especially true of cells of monocyte/macrophage lineage that reside in diverse organs (liver, spleen, lymph node, kidney, peritoneum, lung, skin, brain, breast, and placenta). Resident or elicited peritoneal and alveolar macrophages have been commonly used as a "factory" to assess biosynthesis, especially of the relatively more abundant C1q, C3, C4, and C5 components. Most of the early knowledge about the biosynthesis of complement components was gained using cells of monocyte/macrophage lineage. Local synthesis of complement components evolved for a purpose, presumably to provide host defense against infection. Local synthesis has been noted in all species examined to date, and has been evaluated extensively in mice, guinea pigs, and humans.

Complement components in body fluids (pleural, pericardial, gastrointestinal, peritoneal, spinal) represent a small percent of plasma concentrations. What part of this is derived from local synthesis versus a filtrate from plasma has not been well delineated. After an inflammatory reaction is under way, >90% of the components (e.g., in a pleural effusion caused by instillation of an irritant or bacteria) are derived from plasma (15). These data do not reveal how the response was initiated. Is local synthesis of components necessary, or is that derived from a filtrate of plasma sufficient?

What, then, is the role of local synthesis? There are two likely possibilities, which are not mutually exclusive, and evidence exists to support both. One possible role of local synthesis is to initiate the local inflammatory response, and the other is to facilitate the innate/adaptive immune response. A simple paradigm relative to the first possibility is that resting/resident monocyte/macrophages serve as a factory to secrete components (13–15). They maintain a sufficient level of complement-activating potential to alert the local environment to injury and infection. After an inflammatory response has been initiated, further complement activation via local components is no longer necessary. The local response team has now been augmented many-fold by the influx of plasma. For example, murine peritoneal macrophages placed in culture secreted a decreasing quantity of C4 over the first 24 hours (16,17). At the same time, they were becoming progressively more activated. Macrophages elicited with agents such as thioglycolate secrete minimal C4 in culture but are highly phagocytic. In sum, resident phagocytic cells appear to change their activity profile from that of a secretory cell–producing reagent to initiate an inflammatory response to that of a cell now primed to phagocytose and kill microorganisms. This is a logical and desirable sequence of events in response to an infectious challenge.

Complement component C3 is an abundant plasma protein (1–2 mg/ml). Hepatocytes synthesize a single-chain C3 precursor that is cleaved intracellularly to a two-chain, disulfide-linked mature form that is then secreted into the blood. C3 is an acute-phase protein,
the level of which increases up to two-fold in inflammatory conditions. More than 90% of circulating C3 is contributed by the liver. C3 biosynthesis has been studied in a wide range of human cells, monocyte/macrophages, T cells, and B cells, but also in endothelial cells and alveolar and renal tubular epithelial cells. Neutrophils, which play a key role in the initiation of arthritis in the K/BxN mouse model (10), constitutively synthesize low amounts of C3 but have the capacity to increase production in response to stimuli. In vitro stimulation of neutrophils with TNFα increased the production of both C3 messenger RNA (~50-fold) and protein (~6-fold) (18).

The availability of C3<sup>−/−</sup> mice has permitted performance of a series of interesting experiments to examine the role for extrahepatic C3 synthesis in immune responses (19–21). Bone marrow–derived cells provide sufficient local C3 synthesis for priming an adaptive antibody response. Evidence supporting this hypothesis has come from the bone marrow chimera studies of C3<sup>−/−</sup> mice (19–21). Radiation is used to eliminate the sensitive bone marrow cells in C3<sup>−/−</sup> mice. Bone marrow from WT mice, containing cells secreting C3, is transferred to create chimeric mice in which the level of circulating C3 is negligible. C3 synthesized by WT mouse bone marrow–derived cells restores the antibody response against intradermally administered herpes simplex virus (HSV) (21). In the reverse situation, in which C3<sup>−/−</sup> bone marrow reconstituted WT hosts, the chimeric mice failed to generate a sufficient B cell response to cutaneous HSV infection (even with a normal C3 level in the circulation). Interestingly, if HSV was administered intravenously, the deficient antibody response in the reverse chimeric (C3<sup>−/−</sup> bone marrow into an irradiated WT host) was normalized. The antibody response profiles from chimeric mice paralleled the size of germinal centers. Similar results were obtained with C4<sup>−/−</sup> mice. Together, these experimental data support the hypothesis that locally synthesized complement components, at least in the skin, play a critical role in initiating a robust adaptive antibody response.

No requirement for local synthesis of C3 in this mouse model

In a study reported in this issue of *Arthritis & Rheumatism*, Monach et al (22) used a clever experimental design to address the question of the role of local synthesis of complement components in the K/BxN-TCR mouse model of RA. Those investigators used the serum (antibody)–transfer model in the setting of C3<sup>−/−</sup> mice, bone marrow chimeras, and parabiotic animals. They conclusively demonstrated that local synthesis by monocyte/macrophages of complement components is *not* required to induce arthritis, i.e., the arthritis phenotype is not influenced by a lack of local synthesis of complement components.

A few caveats must be considered. As noted, Monach and colleagues performed their analysis in a transfer system in which pathogenic IgG antibodies reacting with GPI were injected into recipients. The primary function of local synthesis of complement may be to alert the host to the presence of an infectious agent. Injecting preformed antibodies to initiate joint inflammation is not likely to closely mimic HSV infection of the skin, which is the one situation in which local synthesis has been shown to facilitate an adaptive immune response to this pathogen (19–21). Thus, these data do not eliminate a role for local synthesis of complement components in facilitating a de novo immune response. Further, they do not tell us what would be the outcome at other tissue sites and in body cavities and fluids.

These data are reminiscent of those involving C4-deficient guinea pigs, from several decades ago (23). This time, the model under investigation was the Forssman shock model. Guinea pigs are Forssman antigen (a lipopolysaccharide antigen) positive, while rabbits are Forssman antigen negative. Therefore, antibodies to the Forssman antigen can be raised in rabbits. Upon intravenous injection of such antibodies into guinea pigs, they travel to the lung, bind the Forssman antigen to form immune complexes, and then activate the classical complement pathway. Pulmonary edema, leading to hypoxic death, occurs in a few minutes. C4-deficient guinea pigs are resistant to Forssman shock. Upon reconstitution, however, with just a small percentage of the normal concentration of plasma C4, they now behave similarly to C4-sufficient animals. Interestingly, if the Forssman antibodies are injected during the initial 12 hours after C4 reconstitution, the animals do not die. If one waits until 24 hours to inject the anti-Forssman antibodies, death occurs. The interpretation of these data is that it takes a few hours for the C4 introduced into plasma to

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**Table 3. Key questions about the immunology of rheumatoid arthritis**

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<th>Question</th>
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<td>1. How is tolerance to ubiquitous self antigens broken?</td>
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<td>2. Why is the process joint centered?</td>
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<tr>
<td>3. What perpetuates the immune response?</td>
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distribute to the interstitial space. A second lesson, analogous to what was reported by Monach et al, is that local synthesis is not required. Instead, sufficient complement components accumulate in the interstitial space, derived from plasma, to trigger the complement system.

The data in this report are an example of how the K/BxN mouse serum-transfer model can be used to explore questions of importance for rheumatologists trying to understand how autoantibodies contribute to the pathogenesis of RA (Table 2). Although an initial report claimed a high percentage of anti-GPI antibodies among patients with RA, this result was not confirmed in subsequent studies of patients with RA or those with juvenile RA (24–26). At this stage, the consensus is that antibodies to GPI are uncommonly found and are not specific for human RA. Nevertheless, they provide a mechanism for how autoantibodies such as those to anti–citrullinated peptide antibodies might cause RA (27).

**Conclusion**

Along this line of reasoning, we conclude by pointing out parallels between RA featuring IgG antibodies to citrullinated peptides and IgG antibodies to GPI. First, citrullinated peptides are produced in all organ systems. GPI is a ubiquitous enzyme. Second, despite this, in both cases the disease process is joint centered. Third, the autoantibodies are of the IgG class, and the disease is transferable with serum or with purified IgG. Fourth, how and why these antibodies arise are unclear. Fifth, the concept of scarcity (or absence) of complement regulatory proteins in cartilage is a prominent aspect of the discussion of disease pathogenesis in reports on the mouse model (6). In other words, the antibodies bind to many tissue sites, but the inflammatory reaction is “successful” only in triggering disease in a certain anatomic site—in this case, the joint. In the mouse model of RA, a peculiarity of the host’s distribution of regulators of complement has been proposed (6,7). It is our opinion that further analysis of this and related models, particularly in relation to the role of anti–citrullinated peptide antibodies in human RA, will be fruitful (Table 3). Hopefully, such investigations will continue to improve our understanding of the immunopathogenesis of RA.

**ACKNOWLEDGMENT**

We thank Paul Allen for his review of this commentary and discussions, over many years, about the immunopathogenesis of RA.

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Pre-B Cell Colony-Enhancing Factor/Visfatin, a New Marker of Inflammation in Rheumatoid Arthritis With Proinflammatory and Matrix-Degrading Activities

Fabia Brentano, Olivier Schorr, Caroline Ospelt, Joanna Stanczyk, Renate E. Gay, Steffen Gay, and Diego Kyburz

Objective. To study possible mechanisms that mediate induction of the recently described adipokine pre-B cell colony-enhancing factor (PBEF) in joints of patients with rheumatoid arthritis (RA), and to analyze whether levels of PBEF correlate with disease severity and whether PBEF itself has the potential to act as a proinflammatory and destructive mediator in RA.

Methods. RA synovial fibroblasts (RASFs) and monocytes were stimulated with Toll-like receptor (TLR) ligands, cytokines, and recombinant human PBEF or were transfected with PBEF expression constructs or with PBEF-specific small interfering RNA. Production of interleukin-6 (IL-6), IL-8, and tumor necrosis factor \( \alpha \) (TNF\( \alpha \)) was measured by enzyme-linked immunosorbent assay, and expression of matrix metalloproteinases (MMPs) was assessed by real-time polymerase chain reaction. PBEF expression in synovial tissue, synovial fluid, serum, and SFs was assessed by immunohistochemistry, in situ hybridization, Western blotting, and enzyme immunoassays.

Results. In RASFs, PBEF was up-regulated by TLR ligands and cytokines that are characteristically present in the joints of patients with RA. In synovial tissue, RASFs were the major PBEF-expressing cells. A predominance of PBEF was found in the synovial lining layer and at sites of invasion into cartilage. Levels of PBEF in serum and synovial fluid correlated with the degree of inflammation and clinical disease activity. Moreover, PBEF itself activated the transcription factors NF-kB and activator protein 1 and induced IL-6, IL-8, MMP-1, and MMP-3 in RASFs as well as IL-6 and TNF\( \alpha \) in monocytes. PBEF knockdown in RASFs significantly inhibited basal and TLR ligand–induced production of IL-6, IL-8, MMP-1, and MMP-3.

Conclusion. Our findings establish PBEF as a proinflammatory and destructive mediator of joint inflammation in RA and identify PBEF as a potential therapeutic target.

Rheumatoid arthritis (RA) is a chronic inflammatory disease that ultimately leads to the progressive destruction of joint cartilage and bone. Synovial fibroblasts (SFs) and inflammatory cells such as macrophages and T cells play key roles in this process. There is mounting evidence for an important function of innate immunity in the pathogenesis of RA (1). Activation of cells by microbial components and also by endogenous molecules via pattern recognition receptors, such as Toll-like receptors (TLRs), results in the production of a variety of cytokines, chemokines, and matrix metalloproteinases (MMPs), some of which are characteristically observed in patients with RA (2). We previously reported induction in SFs of the proinflammatory cytokine interleukin-6 (IL-6) and the chemokines IL-8, granulocyte chemotactic protein 2, macrophage chemotactic protein 2, and RANTES by the TLR-2 ligand bacterial peptidoglycan (3,4). In a subsequent study, we demonstrated overexpression of TLR-3 in RA synovial tissue and established that RNA released by necrotic synovial
fluid cells can act as endogenous ligand for TLR-3 on cultured RASFs (5). In the current study, we performed subtractive hybridization experiments with untreated and poly(I-C)-treated RASFs to investigate novel TLR-3–dependent gene regulation. We observed that pre-B cell colony-enhancing factor (PBEF) was up-regulated by the TLR-3 ligand poly(I-C).

PBEF was originally cloned from a complementary DNA (cDNA) library of activated human peripheral blood mononuclear cells (PBMCs) and identified as a secreted protein that enhances the effect of stem cell factor and IL-7 on pre–B cell colony formation (6). Later, it became evident that PBEF is a multifunctional protein, having nicotinamide phosphoribosyltransferase, adipokine, and cytokine activities. In smooth muscle cells (SMCs), intracellularly located PBEF regulates NAD+-dependent reactions and promotes the acquisition of a mature SMC phenotype (7,8). Fukuhara et al reported the expression of PBEF in visceral fat adipocytes and noted that its insulin-like effect is dependent on binding to the insulin receptor (9). Because of its presence in visceral fat, PBEF is also referred to as visfatin. Furthermore, antiapoptotic effects of PBEF are documented in neutrophils (10). Regarding cytokine activities, PBEF induces IL-6 and IL-8 in amniotic cells (11), whereas down-regulation of PBEF results in inhibition of the thrombin-stimulated increase of IL-8 secretion in pulmonary artery endothelial cells (12).

Levels of PBEF in serum and synovial fluid are elevated in patients with RA (13,14). Furthermore, Nowell et al (13) demonstrated increased synovial expression of PBEF in antigen-induced arthritis in mice. The up-regulation of PBEF was shown to be regulated by IL-6 trans-signaling via STAT-3. However, the role of PBEF in joint inflammation remains to be determined.

We investigated whether PBEF is involved in the inflammatory and destructive processes in the rheumatoid joint. The expression of PBEF was examined in synovial tissue, serum, and synovial fluid obtained from patients with RA. We showed that levels of PBEF correlate with the degree of inflammation and clinical disease activity in patients with RA. Stimulation of RASFs and primary monocytes with recombinant human PBEF (rHuPBEF), as well as PBEF overexpression and knockdown experiments, revealed that PBEF acts as a proinflammatory mediator by triggering the release of cytokines, chemokines, and destructive enzymes that are characteristically observed in the inflamed joints of patients with RA. Thus, our findings indicate that PBEF is a marker of inflammation, and that PBEF itself promotes inflammatory and destructive processes in the joints of patients with RA.

**Patients and Methods**

Patients and tissue preparation. Synovial tissue specimens were obtained during synovectomy or joint replacement surgery from patients with RA and patients with osteoarthritis (OA), after informed consent had been obtained (Department of Orthopedic Surgery, Schulthess Clinic, Zurich, Switzerland). RASFs and OASFs were isolated from synovial tissue, digested by collagenase, and used after passages 4–8, as previously described (5). To obtain tissue sections, synovial specimens were fixed in paraformaldehyde and embedded in paraffin. Sera and synovial fluid from patients with RA and patients with OA were collected, centrifuged, and stored at −80°C until analyzed. Before analysis, synovial fluid samples were pretreated for 1 hour at 37°C with 1 mg/ml of hyaluronidase (Fluka, Buchs, Switzerland). All patients with RA fulfilled the American College of Rheumatology (formerly, the American Rheumatism Association) criteria for the classification of RA (15).

Stimulation assays. RASFs and OASFs were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Basel, Switzerland) supplemented with 10% fetal calf serum (FCS) and stimulated with the following agents: poly(I-C) (20 μg/ml; Invitrogen, San Diego, CA), lipopolysaccharide (LPS) from *Escherichia coli* (100 ng/ml; List Biological Laboratories, Campbell, CA), palmitoyl-3-cysteine-serine-lysine-4 (bacterial lipoprotein [bLP]) (300 ng/ml; Invitrogen), IL-1β (1 ng/ml; R&D Systems, Abington, UK) and TNF-α (10 ng/ml; R&D Systems).

PBMCs were isolated by standard ficoll density gradient centrifugation from blood samples from healthy donors. CD14+ monocytes were separated using CD14 MACS MicroBeads according to the manufacturer’s protocol (Miltenyi Biotec, Gladbach, Germany). The purity of the CD14+ cell fraction, as assessed by flow cytometry, was consistently >90%. CD14+ cells were cultured in RPMI 1640 supplemented with 5% FCS. RASFs and CD14+ cells were stimulated with rHuPBEF (Phoenix Pharmaceuticals, Belmont, CA) in the presence of polymyxin B sulfate (5 μg/ml) (Sigma, Basel, Switzerland).

Real-time polymerase chain reaction (PCR). Quantification of specific PBEF and MMP messenger RNA (mRNA) was performed by SYBR Green and TaqMan real-time PCR, respectively. The primer sequences used are as follows: for PBEF, forward 5′-AACACCCACCAACACAAAGC-3′, reverse 5′-TCACGGCATCCTAAAGTAAAG-3′; for MMP-1, forward 5′-GGCTGACCTGAGAACAGG-3′, reverse 5′-CACGGATCCTGAGAACAGG-3′; for MMP-3, forward 5′-GGCCCATCAGAGGAAATGAG-3′, reverse 5′-GGCCCATCAGAGGAAATGAG-3′; for MMP-9, forward 5′-AGCTGC- TCAACTCTTGCAGTATAATGTACCC-3′; for MMP-3, forward 5′-GACACCCCGGAACTTATGGG-3′, reverse 5′-GACACCCCGGAACTTATGGG-3′. The endogenous control 18S cDNA was used for correcting the results with the comparative threshold cycle method for relative quantification, as described by the manufacturer.

In situ hybridization. PBEF sense and PBEF antisense probes for in situ hybridization were prepared according to

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**PATIENTS AND METHODS**

**Patients and tissue preparation.** Synovial tissue specimens were obtained during synovectomy or joint replacement surgery from patients with RA and patients with osteoarthritis (OA), after informed consent had been obtained (Department of Orthopedic Surgery, Schulthess Clinic, Zurich, Switzerland). RASFs and OASFs were isolated from synovial tissue, digested by collagenase, and used after passages 4–8, as previously described (5). To obtain tissue sections, synovial specimens were fixed in paraformaldehyde and embedded in paraffin. Sera and synovial fluid from patients with RA and patients with OA were collected, centrifuged, and stored at −80°C until analyzed. Before analysis, synovial fluid samples were pretreated for 1 hour at 37°C with 1 mg/ml of hyaluronidase (Fluka, Buchs, Switzerland). All patients with RA fulfilled the American College of Rheumatology (formerly, the American Rheumatism Association) criteria for the classification of RA (15).

**Stimulation assays.** RASFs and OASFs were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Basel, Switzerland) supplemented with 10% fetal calf serum (FCS) and stimulated with the following agents: poly(I-C) (20 μg/ml; Invitrogen, San Diego, CA), lipopolysaccharide (LPS) from *Escherichia coli* (100 ng/ml; List Biological Laboratories, Campbell, CA), palmitoyl-3-cysteine-serine-lysine-4 (bacterial lipoprotein [bLP]) (300 ng/ml; Invitrogen), IL-1β (1 ng/ml; R&D Systems, Abington, UK) and TNF-α (10 ng/ml; R&D Systems).

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**In situ hybridization.** PBEF sense and PBEF antisense probes for in situ hybridization were prepared according to
methods previously described (16). In situ hybridization was performed using the method described by Kriegsmann et al (17).

**Immunohistochemical analysis.** Synovial tissue sections were deparaffinized and pretreated with trypsin (1 mg/ml; Sigma). After blocking endogenous peroxidase and non-specific binding, slides were incubated overnight at 4°C with anti-human PBEF antibodies (5 μg/ml; Bethyl Laboratories, Montgomery, TX). Sections were then incubated with biotinylated goat anti-rabbit IgG (Jackson Immunoresearch, Soham, UK) in Tris buffered saline with 3% bovine serum albumin for 30 minutes at room temperature, followed by incubation for 30 minutes with horseradish peroxidase (HRP)–conjugated streptavidin complex at room temperature (ABC kit; Vector, Peterborough, UK). HRP-labeled cells were visualized using aminoethylcarbazole substrate–chromogen (Dako, Glostrup, Denmark). Nuclei were counterstained with hematoxylin. To identify subsets of synovial cells expressing PBEF, slides were additionally incubated with monoclonal mouse anti-human CD68 or anti-human vimentin antibodies (2 μg/ml; Dako), respectively. Bound mouse primary antibodies were detected using alkaline phosphatase–conjugated goat anti-mouse IgG antibodies (Jackson Immunoresearch). Alkaline phosphatase–labeled cells were visualized using Fast Blue B reagent. In control experiments, rabbit IgG and isotype-matched mouse IgG were used instead of the primary antibodies.

**Western blotting.** Protein preparation from supernatants. Cultured SFs were grown in T75 culture flasks (7 × 10^5 cells/flask) in DMEM supplemented with 0.5% FCS and were subsequently stimulated for 24 hours with poly(I-C) or were left untreated. Supernatants were collected and concentrated using an Amicon Ultra-15 centrifugal filter device (Millipore, Billerica, MA).

**Protein preparation from tissue.** Protein was extracted by resuspending the crushed snap-frozen tissue in extraction buffer, with subsequent acetone precipitation overnight at −20°C. Proteins were separated on 10% sodium dodecyl sulfate–polyacrylamide gels and blotted on Protran nitrocellulose transfer membranes (Schleicher & Schuell, Dassel, Germany). Membranes were probed with anti-PBEF antibodies (0.5 μg/ml; Bethyl Laboratories) and detected with HRP-conjugated secondary antibodies, using an enhanced chemiluminescence Western blotting detection system (Amer sham Pharmacia Biotech, Little Chalfont, UK). Blots were stripped and reprobed with monoclonal mouse anti-human α-tubulin antibodies (Sigma) to confirm similar loading of the gels.

**Construction of PBEF plasmids.** Total RNA from RASFs was extracted, and 1 μg RNA was transcribed to first-strand cDNA using the Moloney murine leukemia virus reverse transcriptase system (Invitrogen). The cDNA was amplified with the upstream primer, 5’-GGGATCCGGGATCCGGATCTCCG-3’, containing a Bam H1 restriction site and a Kozak sequence for eukaryotic translation. The downstream primer was 5’-CCTCTGCAGGCGTGAGATGACATCGTTC-3’, containing an Xho I restriction site. Full-length PBEF fragments, including the signal sequence and fragments encoding the mature PBEF peptide sequence, were cloned into pcDNA3.1/myc-His vector. The recombinant plasmids were transfected into DH5α-competent cells (Invitrogen), and colonies were identified by restriction enzyme digestion and sequencing.

**Transfection of PBEF small interfering RNA (siRNA) into RASFs.** PBEF stealth siRNAs were designed based on the human PBEF cDNA reference sequence (NM_005746.1), using the BLOCK-it RNAi Designer (Invitrogen). Stealth106 siRNA PBEF 5’-AAUAAACUUUGCUUGUGGGUGG-3’ and stealth 106 scrambled PBEF 5’-CCACACACAAACAGUUGAUCCAUU-3’ were used. One day before transfection, RASFs were plated in DMEM/10% FCS without antibiotics, in a 24-well plate (30,000 RASFs per well). For each transfection, 50 nM PBEF stealth siRNA was diluted in 50 μl Opti-MEM I (Invitrogen) without serum and mixed with 1 μl Lipofectamine 2000 in 50 μl Opti-MEM I. After incubation for 20 minutes at room temperature, PBEF stealth siRNA–Lipofectamine 2000 complexes were added to each well. Transfected cells were further incubated at 37°C for 48 hours before culture medium was replaced and RASFs were used for further experiments.

**Enzyme-linked immunosorbant assay (ELISA) and enzyme immunoassay (EIA).** IL-6 and TNFα proteins were detected by ELISA with the OptiEIA Kit (BD PharMingen, San Diego, CA), and PBEF/visfatin protein was detected using a human EIA kit (Phoenix Pharmaceuticals), according to the manufacturer’s instructions. Absorption was measured at 450 nm, and data were analyzed using Revelation version 4.22 software (Dynex Technologies, Denkendorf, Germany).

**Electrophoretic mobility shift assay (EMSA).** For EMSA, RASFs were cultured to 80–90% confluency in culture flasks (75 cm²) and incubated with 100 ng/ml of rHuPBEF. Cells were collected by scratching in ice-cold phosphate buffered saline, at different time points (0, 10, 30, and 90 minutes after stimulation). DNA-binding proteins were extracted from RASFs according to the method described by Andrews and Faller, which utilizes hypotonic lysis followed by high-salt extraction of nuclei (18). The binding EMSA was carried out using a Panomics EMSA Gel Shift kit, according to the manufacturer’s instructions (Panomics, Redwood City, CA).

**Statistical analysis.** The Mann-Whitney U test, Wilcoxon’s test, and the nonparametric Spearman’s correlation were used, as appropriate, for statistical evaluation of the data by SPSS software (SPSS, Chicago, IL). P values less than 0.05 were considered significant.

**RESULTS**

Induction of PBEF in synovial fibroblasts by TLR ligands and inflammatory cytokines. In order to investigate novel TLR-3–dependent gene regulation, we performed subtractive hybridization between cDNA prepared from poly(I-C)–stimulated and unstimulated RASFs. PBEF was one of the transcripts shown to be up-regulated by TLR-3 activation. The induction of PBEF mRNA in RASFs was validated at various time points after poly(I-C) treatment, by real-time PCR (Fig-
ure 1A). The expression of PBEF was significantly up-regulated after 5, 10, and 24 hours of poly(I-C) stimulation, with a peak at 10 hours after stimulation. To investigate the regulation of PBEF expression by other TLR ligands and proinflammatory cytokines present in RA synovial fluid, SFs were additionally treated with the TLR-2 ligand bLP, the TLR-4 ligand LPS, IL-1β, TNFα at optimal concentrations, and PBEF itself. IL-1β potently up-regulated PBEF mRNA, to levels similar to those reached with poly(I-C) stimulation. The stimulatory effects of TNFα, LPS, bLP, and PBEF on the expression of PBEF mRNA were less prominent but were significant 10 hours following stimulation.

To analyze the expression of PBEF at the protein level, cell lysates from RASFs and OASFs treated with the indicated stimuli were subjected to Western blot analysis (Figure 1B). PBEF was found to be constitutively expressed by SFs, and its expression was further increased following stimulation with bLP, poly(I-C), LPS, TNF, IL-1β, or rHuPBEF. Additionally, we observed a tendency toward higher PBEF levels in RASFs compared with OASFs.

In previous studies, PBEF was shown to be a secreted protein, despite the lack of the typical signal peptide that is common to other secreted proteins (10). To analyze whether SFs have the potential to secrete PBEF protein as a cytokine, we performed Western blot analyses for PBEF using supernatants of unstimulated and poly(I-C)–stimulated RASFs and OASFs. Cultured RASFs released PBEF protein constitutively, as shown in the supernatants of unstimulated RASFs (Figure 1C). In the supernatants of unstimulated OASFs, PBEF protein was not detectable. However, in response to stimulation with poly(I-C), both RASFs and OASFs clearly showed up-regulated secretion of PBEF. Collectively, the results indicate that SFs secrete PBEF protein, and that the production of PBEF is up-regulated by TLR ligands, most notably by poly(I-C), as well as by the cytokines IL-1β and TNFα.

Figure 1. Pre-B cell colony-enhancing factor (PBEF) induction in rheumatoid arthritis synovial fibroblasts (RASFs) by Toll-like receptor (TLR) ligands and cytokines. A, RASFs (n = 5) were treated for 5, 10, and 24 hours with the indicated stimuli or were left untreated. Bars show the mean and SEM. * = P < 0.05, treated versus untreated cultures. B, Western blot analysis of PBEF expression in RASF and osteoarthritis SF (OASF) cell lysates, 24 hours following stimulation with the indicated TLR ligands and cytokines (α-tubulin served as a loading control). Protein levels were evaluated using densitometry. Results are representative of 3 individual experiments. C, Western blot analysis of secreted PBEF protein in supernatants of RASFs (n = 2) and OASFs (n = 2) stimulated for 24 hours with poly(I-C). bLP = bacterial lipoprotein; LPS = lipopolysaccharide; IL-1β = interleukin-1β; TNFα = tumor necrosis factor α; OD = optical density.

Predominant expression of PBEF in RASFs in the synovial lining and at sites of invasion. Next, we analyzed the expression of PBEF in synovial tissue from patients with RA. In situ hybridization revealed pronounced expression of PBEF mRNA predominantly in the synovial lining and at sites of attachment and invasion of RASFs into cartilage or bone (Figure 2A). The expression of PBEF protein in RA synovium was also confirmed by immunohistochemistry, documenting abundant expression of PBEF in RA synovium (Figure 2B). PBEF expression was found to be highest at sites of invasion and in the synovial lining layer, and it was detected to a lesser extent in the sublining and perivascular regions. Double-labeling revealed that PBEF
was more frequently expressed in vimentin-positive SFs than in CD68-positive monocyte/macrophages (Figure 2C).

To study the association of PBEF levels with chronic joint inflammation, we compared the expression of PBEF protein in RA and noninflammatory OA synovial tissue. Analysis of OA synovial tissue sections revealed reduced expression of PBEF protein compared with that in RA synovial tissue, with PBEF being mainly expressed around small vessels (Figure 2D). Western blot analysis of total protein extracted from synovial tissue from 3 individual patients with RA and 3 patients with OA confirmed increased expression of PBEF protein in synovial tissue from the joints of patients with RA compared with patients with OA (Figure 2E).

Positive correlation of PBEF with the C-reactive protein (CRP) level and clinical disease activity in patients with RA. Because of the abundant expression of PBEF in the joints of patients with RA compared with that in the joints of patients with OA, we investigated whether serum and synovial fluid levels of PBEF might also reflect the severity of inflammation. Levels of PBEF were significantly higher in serum and synovial fluid samples from patients with RA compared with those in samples from patients with OA (Figure 3A). However, high variability in PBEF levels was observed among individual patients with RA. Using correlation analysis with the CRP level and PBEF, we analyzed whether these varying levels of PBEF might be associated with the degree of inflammation (Figure 3B). A significant
positive correlation between the CRP level and PBEF in serum and in synovial fluid samples was observed. Additionally, the levels of PBEF in serum and synovial fluid and the Disease Activity Score in 28 joints (DAS28) and PBEF concentrations in serum and synovial fluid from patients with RA. Therefore, our results demonstrated that PBEF is associated with serum markers of inflammation as well as clinical disease activity in RA.

**PBEF-induced production of IL-6, MMP-1, and MMP-3 in RASFs.** To investigate the functional role of PBEF secreted in joints of patients with RA, RASFs were stimulated with increasing amounts of rHuPBEF for 24 hours. Recombinant human PBEF induced a dose-dependent increase in the levels of IL-6, MMP-1, and MMP-3. Already after stimulation with physiologic concentrations of rHuPBEF (50–200 ng/ml), IL-6 production was significantly up-regulated (Figure 4A). Similarly, RASFs treated with rHuPBEF showed significantly up-regulated expression of MMP-1 and MMP-3 mRNA upon incubation with 100 and 200 ng/ml of rHuPBEF (Figure 4B). To ascertain that the stimulatory effect was not attributable to contamination with endotoxin, RASFs were cultured in the presence of polymyxin. Polymyxin neutralized the stimulatory effect of 10 ng/ml of LPS, whereas the effect of PBEF on IL-6, MMP-1, and MMP-3 production remained unchanged (data not shown).

We additionally analyzed whether comparable results could be obtained by overexpressing PBEF in RASFs, using a eukaryotic expression vector. When a pcDNA3.1/PBEF/His expression construct was transfected into RASFs, the resulting protein could be demonstrated in the cell lysate by Western blotting (Figure 4C). Supernatants from RASFs obtained 48 hours after transfection with the PBEF/His construct showed significantly higher levels of IL-6 compared with that in RASFs transfected with the empty pcDNA3.1/His vector (Figure 4D). Additionally, levels of MMP-1 and MMP-3 were significantly higher in PBEF/His vectortransfected RASFs compared with those in controls (Figure 4E). These results demonstrated that PBEF induces proinflammatory cytokines and destructive enzymes in RASFs.

**Reduction of basal and TLR ligand-induced IL-6, MMP-1, and MMP-3 levels by PBEF knockdown.** PBEF is expressed in unstimulated RASFs, and we demonstrated that PBEF regulates IL-6, MMP-1, and MMP-3 production. Therefore, we investigated whether PBEF knockdown inhibits the characteristically high basal production of IL-6, MMP-1, or MMP-3 in RASFs. For this purpose, RASFs were transfected with PBEF-specific siRNA (siPBEF) or with nonspecific scrambled siRNA (scrambled) as a control. To ensure that responses to siPBEF transfection reflected PBEF knockdown, PBEF protein in cell lysates was quantified 72 hours after transfection. In 3 different RASF cultures analyzed, the inhibition of PBEF by specific siRNA was 69%, 59%, and 74%, respectively, as compared with that in controls (Figure 5A). Basal secretion of IL-6 was measured in the culture supernatants of siRNA-transfected RASFs (n = 6), 4, 6, and 24 hours after medium replacement. The basal production of IL-6 was
significantly lower in RASFs with down-regulated PBEF compared with controls at all time points analyzed. The most pronounced effect was seen 6 hours following medium replacement, with a mean ± SEM inhibition of 48.8 ± 7.2% (Figure 5B). Additionally, basal levels of MMP-1 and MMP-3 mRNA were down-regulated 4.7 ± 1.6-fold and 2.8 ± 0.6-fold, respectively, 48 hours following siPBEF transfection (Figure 5C).

In previous studies, we demonstrated that TLR-2, TLR-3, and TLR-4 ligands induce high amounts of cytokines and matrix-degrading enzymes in RASFs (3,5,20). In order to investigate whether PBEF is involved in the up-regulation of these effector molecules, we treated siPBEF-transfected RASFs with bLP, poly(I-C), and LPS, and analyzed the induction of IL-6, IL-8, MMP-1, and MMP-3, 24 hours after TLR ligand stimulation. PBEF knockdown significantly inhibited the up-regulation of all measured effector molecules (Figures 5D and E). These data indicated that PBEF is implicated in basal as well as TLR ligand-induced production of proinflammatory cytokines and matrix-degrading enzymes.

**PBEF induction of TNFα and IL-6 in primary human monocytes.** It has been demonstrated that primary blood monocytes express PBEF (10). However, it has not been determined whether monocytes are responsive to PBEF. Because we observed high levels of PBEF in serum obtained from patients with RA, we analyzed whether human primary blood monocytes are activated either by rHuPBEF or by overexpression of PBEF. Monocyte cultures treated with 50 ng/ml of rHuPBEF, a concentration that corresponds to the level of PBEF in
serum from patients with RA, showed significantly higher production of IL-6 and TNFα as compared with that in untreated cultures. The stimulatory effect of rHuPBEF on monocytes was dose dependent (Figures 6A and B). Similarly, overexpression of PBEF in monocytes resulted in a 5.0 ± 1.5-fold increase in TNFα secretion (Figure 6C). Thus, PBEF is a potent activator of human monocytes, inducing the production of key proinflammatory cytokines such as IL-6 and TNFα.

**PBEF stimulation of RASFs via NF-κB and AP-1.** Activation of the transcription factors NF-κB and AP-1 is a principal step in the initiation and maintenance of inflammatory responses. Therefore, we studied whether rHuPBEF activates NF-κB and AP-1 signaling pathways in RASFs. Nuclear extracts of RASFs stimulated with rHuPBEF (100 ng/ml) were subjected to EMSA. Ninety minutes following stimulation, the activation of RASFs resulted in translocation of NF-κB and AP-1 from the cytoplasm to the nucleus (data not shown). Therefore, PBEF has the capacity to activate 2 major transcription factors, NF-κB and AP-1.

**DISCUSSION**

In the present study, we demonstrated induction of the adipocytokine PBEF in RASFs via TLR stimulation as well as by stimulation with IL-1β and TNFα. Furthermore, we observed potent proinflammatory and matrix-degrading activities of PBEF and showed that in patients with RA, levels of PBEF correlate with the severity of inflammation.

Using a subtractive hybridization assay of RASFs stimulated with poly(I:C), we found PBEF to be upregulated. However, PBEF induction in RASFs is not restricted to TLR-3 activation, as shown by stimulation experiments with other TLR ligands and proinflamma-
tory cytokines that can be found in the joints of patients with RA (1). Interestingly, PBEF itself induces its own production, indicating the existence of a positive feedback-regulating mechanism. Expression and up-regulation of human PBEF has previously been documented in neutrophils by IL-1/β and LPS, in amniotic cells by TNFα, in monocytic cells by nitric oxide, in adipocytes by hypoxia, and in RASFs by IL-6 trans-signaling (10,11,13,21–23). Taken together, these data show that signaling pathways of the innate immune system have a strong regulatory effect on the expression of PBEF in a variety of cell types.

Our study demonstrates accumulation of PBEF in the joints of patients with RA and identifies SFs as the major PBEF-producing cells in the rheumatoid synovium. PBEF expression is predominantly localized at the site of invasion into cartilage and in the synovial lining. This staining pattern may reflect the local availability of PBEF-stimulating agents such as cytokines and TLR ligands in the lining layer. Additionally, hypoxic conditions that are most marked at the site of invasion into cartilage may drive local PBEF expression, as has been shown previously in adipocytes (22).

It is known that IL-6 exerts stimulatory effects on T cells and B cells, thus favoring chronic inflammatory responses, whereas MMPs have been closely linked to the progressive destruction of articular cartilage in rheumatoid joints. A hallmark of RASFs is the high basal production of IL-6 and MMPs (24). We showed that PBEF has an important role in the regulation of these key proinflammatory and matrix-degrading molecules. Recombinant human PBEF induces the expression of IL-6 and MMPs in RASFs, suggesting that secreted PBEF contributes to the local inflammatory and destructive processes in arthritic joints. Moreover, in our experiments, down-regulation of PBEF in RASFs by siRNA not only decreased basal IL-6, MMP-1, and MMP-3 levels but also significantly inhibited TLR ligand–induced production of cytokines and destructive enzymes. TLRs were shown to be key players in inflammatory and destructive processes in RA (3,4,20,25). TLR ligands of microbial origin as well as endogenous TLR ligands were demonstrated to be present in RA synovial fluid as possible drivers of inflammatory processes (1,26). Our findings suggest that the stimulation of RASFs by TLR ligands is at least partially dependent on PBEF expression. Therefore, targeting PBEF not only might counteract its direct stimulatory effect on RASFs but also reduces TLR-driven proinflammatory and destructive responses.

Peripheral blood monocytes have been shown to be a source of PBEF in the blood circulation. Our study shows that primary human monocytes are responsive to PBEF, suggesting that PBEF acts in an autocrine manner to increase serum levels. Notably, activation of monocytes by PBEF results in the production TNFα, a key cytokine in the pathogenesis of RA (27). Moreover, PBEF present in synovial fluid might trigger chronic inflammation not only via induction of proinflammatory cytokine production by RASFs but also by a direct antiapoptotic effect on neutrophils (10). Neutrophils are abundant in the synovial fluid of patients with RA and are less susceptible to TNFα-induced apoptosis than are blood neutrophils. In this regard, it has been shown that synovial fluid from patients with RA exhibits an anti-apoptotic effect on neutrophils (28).

The possible mechanisms by which PBEF exerts its proinflammatory effects in the arthritic joint are incompletely understood. The identification of PBEF in
the visceral fat added PBEF to a growing list of adipocytokines with potent effects on immunity and inflammation in addition to their metabolic activity (9). Leptin, adiponectin, resistin, and most recently PBEF have been shown to be up-regulated in patients with RA compared with healthy control subjects (14,29,30). PBEF mimics insulin signaling by binding to the insulin receptor with an affinity similar to that of insulin but does not share the binding site with insulin on the receptor. In contrast, previous studies have shown intracellular expression of PBEF and have demonstrated that PBEF is a nicotinamide phosphoribosyltransferase (7,8). Overexpression of PBEF in human vascular SMCs induced enhanced survival by its regulatory effect on NAD-dependent deacetylase activity. Moreover, it has been shown that subcellular localization of PBEF is dependent on the cell cycle, suggesting a role for PBEF in cell cycle regulation (31). Using immunohistochemical analysis, we observed nuclear and cytoplasmic expression of PBEF. Whether the effects provoked by PBEF are dependent on binding to the insulin receptor or an as yet unknown receptor, or alternatively by its enzymatic activity, needs to be determined in further studies.

Elevated levels of PBEF in serum and synovial fluid from patients with RA have been observed in this and previous studies (13,14). Our new finding of a strong correlation of PBEF with markers of inflammation such as the CRP level provides support for an important role of this cytokine in inflammatory reactions. This is further underscored by the correlation of PBEF concentrations with scores for clinical disease activity (comprising the tender and swollen joint counts and the erythrocyte sedimentation rate) (32). These findings suggest that PBEF is a marker of the severity of inflammation in patients with RA.

Taken together, our results suggest that PBEF plays a key role as a mediator of innate immune pathways in chronic synovial inflammation and joint destruction, and identify this adipocytokine as a possible therapeutic target for the treatment of RA.

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AUTHOR CONTRIBUTIONS

Dr. Kyburz had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

REFERENCES


Protein Isoprenylation Regulates Secretion of Matrix Metalloproteinase 1 From Rheumatoid Synovial Fibroblasts

Effects of Statins and Farnesyl and Geranylgeranyl Transferase Inhibitors

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Objective. To determine whether protein prenylation (farnesyl/geranylgeranylation) regulates matrix metalloproteinase (MMP) secretion from rheumatoid arthritis (RA) synovial fibroblasts (RASFs), and whether MMP-1 secretion can be regulated by statins or prenyltransferase inhibitors via effects mediated by ERK, JNK, and NF-κB.

Methods. RASFs obtained from patients during elective knee replacement surgery were assessed by immunoblotting and/or enzyme-linked immunosorbent assay for secretion of MMP-1 and MMP-13 in the presence of tumor necrosis factor (TNFα), interleukin-1β (IL-1β), statins, the farnesyl transferase (FT) inhibitor FTI-276 and geranylgeranyl transferase inhibitor GGTI-298, and prenyl substrates (farnesyl pyrophosphate [FPP] and geranylgeranyl pyrophosphate [GGPP]). Activities of JNK and ERK were determined by phosphoimmunoblotting, and NF-κB activation was determined by nuclear translocation of the p65 component.

Results. FTI-276, but not statins, inhibited RASF secretion of MMP-1, but not MMP-13, following induction with TNFα (P = 0.0007) or IL-1β (P = 0.006). Loading RASFs with FPP to promote farnesylation enhanced MMP-1 secretion. FTI-276 inhibited activation of JNK (P < 0.05) and NF-κB (P = 0.02), but not ERK. In contrast, GGTI-298 enhanced, while GGPP inhibited, MMP-1 secretion. FTI-276 and GGTI-298 together had no effect on MMP-1 secretion. Stimulation of RASFs with TNFα or IL-1β led to increased expression and activity of FT.

Conclusion. Protein farnesylation is required for expression and secretion of MMP-1 from RASFs, via effects on JNK and NF-κB. The ability of cytokines to stimulate the expression and activity of FT suggests that FT may be increased in the rheumatoid joint. In contrast, geranylgeranylation down-regulates MMP-1 expression. Statins simultaneously inhibit farnesylation and geranylgeranylation, and in consequence do not inhibit MMP-1 secretion. The ability of FTI-276 to inhibit MMP-1 secretion suggests a potential therapeutic strategy in RA.

Rheumatoid arthritis (RA) causes joint pain, swelling, destruction of cartilage and bone, and increased morbidity and mortality (1–3). RA synovium (pannus) acquires features of both inflamed/immune tissue and hyperplastic tissue (4). Whereas the interior of RA pannus is infiltrated with immune and inflammatory cells, the surface of pannus adjacent to cartilage is...
predominantly populated by synovial fibroblasts (SFs; also known as fibroblast-like synovial cells). SFs comprise the normal resident stromal cell population of the joint, but are present in increased numbers in RA and play essential roles in RA propagation and progression (5). RASFs define pannus architecture, secrete cytokines, and recruit and activate both immune cells and osteoclasts that contribute to bone destruction (4,6). Importantly, RASFs are the primary effectors of marginal cartilage erosion in the RA joint, via cytokine-stimulated secretion of matrix metalloproteinases (MMPs) (7,8).

When secreted, MMPs digest connective tissue components. MMPs secreted by RASFs include MMPs 1, 3, 9, and 13 (8,9). MMP-1 degrades type II collagen (the main component of articular cartilage) as well as types I and III collagen and other matrix components (e.g., aggrecan, versican, and serpins) (10), and may be particularly important for cartilage destruction in RA. Secretion of individual MMPs is differentially regulated (11). Tumor necrosis factor α (TNFα) and interleukin-1β (IL-1β) stimulate the synthesis and secretion of MMP-1 from RASFs in a manner dependent on the activation of both NF-κB and the MAPKs ERK and JNK (12–15). Neutralization of IL-1β and, particularly, TNFα improves the clinical picture in RA, partly by reducing the RASF response (16–20). However, many patients with RA do not respond adequately to available RA treatments (18–20), and alternative therapies are needed.

Statins inhibit hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase, blocking synthesis of mevalonic acid and the accumulation of cholesterol synthetic intermediates (21,22). In addition to their effects on serum lipids, statins have antiinflammatory effects that are useful in cardiovascular disease and may be useful in systemic inflammatory diseases as well (23). Results from a recent double-blind, placebo-controlled study suggest that statins may provide benefit in the treatment of RA (24). The antiinflammatory actions of statins are likely derived from their ability to deplete 2 isoprenyl cholesterol pathway intermediates, farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP). FPP and GGPP are enzymatically attached to, and determine the physicochemical properties of, signaling proteins with important functions in inflammation, including Ras-related, low molecular weight GTPases (small GTPases) and the larger, heterotrimeric GTP-binding proteins (G proteins) (25).

Small GTPases and G proteins share a canonical, carboxy-terminal amino acid sequence known as the CAAX box, which renders proteins susceptible to isoprenylation (tagging with FPP or GGPP) (26). The specific “A” (aliphatic) and “X” (any) amino acids of the CAAX box determine isoprenylation with either a farnesyl or geranylgeranyl chain (27). G proteins and Ras proteins (H-Ras, N-Ras, Ki-Ras) are farnesylated, whereas small GTPases of the Rho family (RhoA, RhoB, Rac1, Rac2, and Cdc42hs, among others) are geranylgeranylated. Specific enzymes (farnesyl transferase [FT] and geranylgeranyl transferase [GGT]) catalyze attachment of FPP or GGPP to their respective targets (28). In the absence of appropriate isoprenylation, CAAX proteins mislocalize, leading to disruption of proper functioning (29).

Small GTPases regulate critical cellular processes, including cytoskeletal organization, transcriptional regulation, and assembly of the NADPH oxidase system (30,31). They also participate in cytokine-dependent MAPK signaling. Ras family proteins are best recognized as mediators of ERK activation, and Rho family proteins as mediators of JNK activation, although significant cross-talk occurs. Both protein families also mediate the signaling for NF-κB activation (32–36). Thus, farnesylation of Ras proteins and geranylgeranylation of Rho proteins may modulate pathways known to regulate MMP-1 secretion in RASFs.

By depleting the levels of both FPP and GGPP, statins can alter the activity of both the Ras family and Rho family GTPases (25). Alternatively, protein farnesylation and geranylgeranylation can be specifically blocked by inhibitors of these enzymes. FT inhibitors (FTIs), currently being used in phase III trials for neoplastic disease, were originally designed as chemotherapeutic inhibitors of Ras (37,38), but have also been reported to have antiinflammatory properties both in vitro and in vivo (39,40). FTIs inhibit both MAPK and NF-κB in macrophages (39,41), and were beneficial in a mouse model of arthritis (39). GGT inhibitors (GGTIs) have antiinflammatory properties in cell cultures (42); however, they have been lethal in animal models and are not currently being used in clinical trials (43).

The effects of FTIs and GGTIs on RASFs have not been previously determined. In the present study we tested the hypothesis that inhibition of farnesylation, geranylgeranylation, or both regulates the cytokine-stimulated secretion of MMP-1 from RASFs.

**PATIENTS AND METHODS**

**Preparation of fibroblasts.** Human RASFs or osteoarthritis SFs (OASFs) were prepared from synovial tissue ob-
tained from patients at the time of total joint arthroplasty, as previously described (44). The collection and use of human RASFs and OASFs were reviewed and approved by the Institutional Board of Research Associates, New York University School of Medicine (New York, NY). Synovium was minced and incubated with 1 mg/ml type VIII collagenase (Sigma-Aldrich, St. Louis, MO) in serum-free RPMI 1640 (Invitrogen Life Technologies, Carlsbad, CA) for 1 hour at 37°C, and then filtered, washed extensively, and cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen Life Technologies) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. After overnight incubation, cells were cultured in DMEM with 10% FBS to 70–80% confluence, and then split in a 1:3 ratio and recultured and/or frozen for future use. For the experiments reported herein, RASFs were used between passages 3 and 7. Primary adult human dermal fibroblasts were obtained from Cascade Biologics (Portland, OR) and maintained as previously described (45).

**Determination of MMP-1 secretion, and levels of other proteins of interest.** MMP-1 secretion was determined as previously described (46). Briefly, RASFs grown to near confluence were serum-starved for 24 hours, treated with simvastatin, lovastatin (EMD Biosciences, Darmstadt, Germany), sqalestatin, FTI-276, GGTI-298, FPP, or GGPP (Sigma-Aldrich) for various time periods, or with pertussis toxin (PT; Calbiochem, La Jolla, CA) for 2 hours, and then stimulated for 24 hours (a time span selected to permit easily measurable MMP-1 secretion) with TNFα or IL-1β (Sigma-Aldrich) (each at 20 ng/ml). Cells were lysed (with 20 mM EGTA, 2 mM sodium vanadate, 25 mM sodium fluoride, 0.5% [volume/volume] Triton X-100, and protease inhibitor cocktail; Sigma-Aldrich) for 20 minutes at 4°C, and the lysates were analyzed for FT by SDS-PAGE, electrophoretic transfer to nitrocellulose paper, and immunoblotting using an anti-FT antisera (catalog no. sc137; Santa Cruz Biotechnology), with imaging and quantitation of FT as described above.

**Determination of MMP-1 messenger RNA (mRNA) in RASFs.** RNA from RASFs was isolated using TRI Reagent (Sigma-Aldrich) according to the manufacturer’s instructions. One microgram of total RNA was subjected to real-time polymerase chain reaction (PCR) using a proprietary primer mixture (TaqMan Gene Expression Assay, identification no. Hs00233958_m1; Applied Biosystems, Foster City, CA), and run on an ABI Prism 7300 sequence detection system (Applied Biosystems). The PCR was carried out in a reaction mix comprising 1 µl complementary DNA (cDNA) and 8 µl deionized water mixed with 10 µl Master Mix in 2× buffer (Applied Biosystems) and 1 µl TaqMan primer in a final volume of 20 µl. The PCR cycles were 10 minutes at 95°C followed by 40 amplification cycles at 95°C for 15 seconds and 60°C for 60 seconds. A standard amplification curve for an RNase P calibration plate (Applied Biosystems) was plotted to evaluate the sensitivity of the real-time quantitative PCR analysis. Samples were made with 4 series of halfed dilutions of RNase P, ranging from 20,000 to 1,250 copies of cDNA. The relative expression levels of MMP-1 mRNA were calculated using Sequence Detection software, version 2.0 (Applied Biosystems). Specific mRNA levels were normalized using G3PDH as a housekeeping gene.

**Determination of FT activity.** After 12 hours of preincubation with or without FTI-276, the RASFs were stimulated for an additional 24 hours with TNFα or IL-1β, followed by lysis as described above. The activity of FT was determined in lysates of cells using a commercially available kit (catalog no. TRKQ7010; Amersham Biosciences), as previously described (47).

**Measurement of ERK and JNK activation.** The activation of ERK-1, ERK-2, and JNK was measured as described previously (48). Briefly, RASFs were serum-starved for 24 hours, incubated with FTI-276 (10 µM) for 18 hours, and then stimulated with TNFα or IL-1β for 30 minutes. Cells were lysed as described above, and lysates were analyzed by 10% Tris–glycine SDS-PAGE with transfer to nitrocellulose and immunoblotting with an antibody recognizing total ERK or phospho-ERK (Sigma-Aldrich) (1:2,000; Santa Cruz Biotechnology) or antibody recognizing phospho-JNK antibodies (1:2,000; Cell Signaling, Danvers, MA). Activity was determined in pixel counts using Quantity One (version 4.0.3; Bio-Rad, Hercules, CA). Secretion of IL-6 was measured using a commercially available kit according to the manufacturer’s instructions (Quantikine Human IL-6 Immunoassay, catalog no. D6050; R&D Systems, Minneapolis, MN). For determination of intracellular FT, cells were incubated overnight with or without FTI-276, followed by additional overnight incubation with or without TNFα or IL-1β (each at 20 ng/ml). Cells were lysed (with 20 mM Tris, pH 7.4, 1 mM EGTA, 2 mM sodium vanadate, 25 mM sodium fluoride, 0.5% [volume/volume] Triton X-100, and protease inhibitor cocktail; Sigma-Aldrich) for 20 minutes at 4°C, and the cell lysates were analyzed for FT by SDS-PAGE, electrophoretic transfer to nitrocellulose paper, and immunoblotting using an anti-FT antisera (catalog no. sc137; Santa Cruz Biotechnology), with imaging and quantitation of FT as described above.

**Measurement of NF-κB activation.** After treatment of RASFs with FTI-276 (10 µM) for 18 hours followed by stimulation with TNFα (20 nM) for 30 minutes, nuclear and cytoplasmic extracts were prepared using NE-PER nuclear and cytoplasmic extraction reagents (Pierce Biotechnology, Rockford, IL) according to the manufacturer’s instructions. Protein content was determined with a MicroBCA protein assay (Pierce Biotechnology). Equal amounts of protein (5–10 µg)
were analyzed by 10% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk in phosphate buffered saline–0.1% Tween 20 at 4°C and incubated overnight with anti-p65 antibody (Santa Cruz Biotechnology). After visualization and quantitation of immunoblots, nuclear p65 was calculated as a percentage of total cellular p65.

Statistical analysis. All statistical analyses were performed using SigmaStat (SPSS, Chicago, IL). Results of experiments were analyzed by one-way analysis of variance, followed by the Holm-Sidak or Student-Newman-Keuls post hoc test for pairwise comparisons.

RESULTS

Noninhibitory effect of statins on MMP-1 secretion from RASFs. As previously reported (46), stimulation of RASFs with TNFα for 24 hours resulted in increased MMP-1 secretion ($P < 0.001$) (Figure 1A). Basal levels of MMP-1 secretion varied somewhat between experiments (Figures 1, 2, and 3), which is consistent with the previously reported heterogeneity of RASFs (49). Incubation of RASFs with simvastatin (10
Inhibitors of protein farnesylation and cholesterol biosynthesis had no effect on TNFα-stimulated secretion of MMP-1 (P = 0.79 with simvastatin; P = 0.78 with lovastatin, versus no statin). To confirm that the failure of statins to inhibit MMP-1 secretion was not due to inadequate dosing, we also tested simvastatin at concentrations up to 200 μM, and again no inhibitory effect was observed (Figure 1D). Incubation of RASFs with simvastatin or lovastatin for intervals shorter than 24 hours also did not inhibit MMP-1 secretion (results not shown). Similarly, simvastatin and lovastatin had no effect on MMP-1 secretion from RASFs stimulated with IL-1β (Figures 1A, B, and C, bottom) (P = 0.86 with simvastatin; P = 0.78 with lovastatin, versus no statin). Thus, either statins do not regulate MMP-1 secretion from RASFs or they have a complex effect on multiple pathways that results in no net change in MMP-1 secretion.

Requirement of farnesylation for MMP-1 secretion. To determine whether individual, statin-sensitive signaling pathways independently regulate MMP-1 secretion, we next tested the effects of specific inhibitors of distinct, mevalonic acid–dependent pathways, including inhibitors of protein farnesylation (FTI-276) and cholesterol biosynthesis (squalestatin, an inhibitor of biosynthesis of cholesterol, but not of FPP or GGPP). Incubation of RASFs with FTI-276 (10 μM) for 18 hours prior to TNFα stimulation inhibited secretion of MMP-1 (P = 0.0007) (Figures 2A and B, top). Inhibition of MMP-1 secretion by FTI-276 was dose dependent (50% inhibitory concentration 11 μM) (Figure 2C) and not due to general toxicity, since, as demonstrated by lactate dehydrogenase (LDH) release assay, FTI-276 did not diminish cell viability (results not shown). The effect of FTI-276 was delayed, since inhibition of MMP-1 secretion was not observed until at least 8 hours after exposure to FTI-276 (Figure 2D).
FTI-276 also inhibited MMP-1 secretion in response to IL-1β (*P* = 0.006) (Figures 2A and B, bottom). FTI-277, a related, more hydrophobic FTI (50), also inhibited MMP-1 secretion (results not shown). These data demonstrate that inhibition of farnesylation blocks MMP-1 secretion from RASFs, suggesting that at least one farnesylation-dependent protein mediates the secretion of MMP-1 in these cells. In contrast, the specific cholesterol synthesis inhibitor squalestatin did not inhibit MMP-1 secretion in response to either TNFα or IL-1β (Figures 2A and B), confirming that MMP-1 secretion is independent of cholesterol.

To further examine the role of protein farnesylation in the regulation of MMP-1 secretion, we tested the consequences of increasing farnesylation by supplying the cells with FPP (51) prior to stimulation. In contrast...
to the effects of FTI-276, FPP (10 μM for 18 hours) enhanced TNFα-stimulated MMP-1 secretion (P = 0.007) (Figures 2E and F, top). FPP also enhanced MMP-1 secretion in response to IL-1β (P = 0.01) (Figures 2E and F, bottom). In the absence of TNFα or IL-1β, FPP alone did not stimulate MMP-1 secretion (results not shown), suggesting that protein farnesylation is necessary, but not sufficient, to regulate MMP-1. FTI-276 alone also had no effect on the basal extracellular levels of MMP-1 (results not shown).

FTI-276 functions as both a peptidomimetic analog of CAAX and a competitive inhibitor of CAAX, but not of FPP, binding to FT (50,52,53). Consistent with these contrasting mechanisms of action, coinubcation of RASFs with FPP failed to reverse the effect of FTI-276. Instead, coinubcation with FTI-276 and FPP resulted in inhibition of stimulated MMP-1 secretion (P = 0.04 for FTI-276/FPP in TNFα-stimulated cells; P = 0.04 for FTI-276/FPP in IL-1β-stimulated cells) (see supplementary Figure 1 at http://www.abelesetalfti.com).

In contrast to its effects on MMP-1 secretion, FTI-276 failed to inhibit MMP-13 secretion from RASFs and also had no effect on IL-6 secretion from RASFs (see supplementary Figure 1). Thus, rather than having nonspecific effects, the effects of FTI-276 on MMP-1 secretion reflect the inhibition of one or more specific signaling pathways.

Inhibition of MMP-1 secretion by geranylgeranylation. Because geranylgeranylated proteins are implicated in cellular signaling in the processes of inflammation, we next examined the consequences of inhibiting geranylgeranylation. Surprisingly, incubation of RASFs with GGTI-298 enhanced TNFα-stimulated MMP-1 secretion (P = 0.002) (Figures 3A and B, top). GGTI-298 also enhanced IL-1β-stimulated MMP-1 secretion (P = 0.008) (Figures 3A and B, bottom). The effect of GGTI-298 on MMP-1 secretion was dose dependent (Figure 3C). Moreover, GGTI-298 was not lethal to RASFs, in that it did not significantly increase LDH levels in the LDH release assay (results not shown).

In contrast to the effect of GGTI-298, loading RASFs with GGPP to promote geranylgeranylation inhibited secretion of MMP-1 in response to either TNFα or IL-1β (P < 0.001 for GGPP in TNFα-stimulated cells; P = 0.001 for GGPP in IL-1β-stimulated cells, versus no GGPP) (Figures 3D and E). Thus, in contrast to the effects of farnesylation, geranylgeranylation downregulates the secretion of MMP-1.

In the absence of other stimuli, incubation of RASFs with GGTI-298 alone stimulated extracellular MMP-1 secretion (P = 0.03 versus no treatment) (see supplementary Figure 2 at http://www.abelesetalfti.com). Conversely, incubation of RASFs with GGPP alone suppressed the basal levels of MMP-1 (P = 0.01 versus no treatment) (see supplementary Figure 2). Therefere, geranylgeranylation of at least one protein is both necessary and sufficient to tonically inhibit both the basal and stimulated secretion of MMP-1.

The ability of FTI-276 to inhibit, but GGTI-298 to enhance, MMP-1 secretion suggests that the failure of statins to alter extracellular MMP-1 levels might be related to their ability to inhibit both farnesylation and geranylgeranylation in a simultaneous manner. To examine this possibility, we tested the effects of simultaneous inhibition with specific inhibitors of both FT and GGT. Treatment with FTI-276 and GGTI-298 together for 18 hours prior to stimulation of RASFs with TNFα or IL-1β neither inhibited nor enhanced secretion of MMP-1 (Figure 3F) (P = 0.65 for FTI-276/GGTI-298 in TNFα-stimulated cells; P = 0.4 for FTI-276/GGTI-298 in IL-1β-stimulated cells, versus untreated RASFs). Thus, dual inhibition of farnesylation and geranylgeranylation by statins would also be expected to have no net effect on MMP-1, as was observed.

Effect of modulation of farnesylation and geranylgeranylation on MMP-1 mRNA expression in RASFs. To test whether the effects of farnesylation and geranylgeranylation on MMP-1 secretion are related to alterations in the expression of MMP-1 at the level of transcription, we exposed RASFs to FTI-276, FPP, GGTI-298, or GGPP prior to TNFα stimulation, and measured the resultant intracellular MMP-1 mRNA levels (Figure 4A). Concordant with the effects of these agents on MMP-1 secretion, FTI-276 and GGPP each inhibited MMP-1 mRNA expression. In contrast, MMP-1 mRNA message levels were enhanced by both FPP and GGPP. These data confirm that the opposing effects of farnesylation and geranylgeranylation on MMP-1 synthesis are regulated, at least in part, at the level of mRNA expression.

Effects of FT and GGT inhibition on MMP-1 secretion from cells other than RASFs. To determine whether regulation of MMP-1 secretion by prenylation is a unique feature of RASFs, we measured the secretion of MMP-1 from primary cultures of OASFs and dermal fibroblasts, in the presence of TNFα (Figure 4B). Both OASFs and dermal fibroblasts demonstrated MMP-1 secretion under these conditions, although the amount of MMP-1 secretion from OASFs was lower than that observed in either RASFs or dermal fibroblasts. In contrast, we were unable to observe MMP-1 secretion from either NIH3T3 cells or A237 cells (pulmonary
fibroblast cell line), in the presence of TNFα (results not shown).

Interestingly, whereas TNFα stimulated MMP-1 secretion from OASFs, it inhibited MMP-1 secretion from dermal fibroblasts relative to that in unstimulated cells (Figures 4C and D). In both OASFs and dermal fibroblasts, FTI-276 inhibited, and GGTI-298 enhanced, MMP-1 secretion (Figures 4C and D). Thus, although various fibroblast types may respond differently to TNFα, MMP-1 secretion from both OASFs and dermal fibroblasts is regulated by farnesylation and geranylgeranylation in a manner congruent with the effects of these processes on RASFs.

Up-regulation of FT in RASFs in response to IL-1β or TNFα. We next examined whether the role of FT in RASFs is related to increased expression and/or activity of the enzyme. FT was constitutively expressed in unstimulated RASFs, with the results showing that the levels were not increased relative to those in either OASFs or dermal fibroblasts (Figure 5A). The enzymatic activity of FT in unstimulated RASFs was also not significantly higher than that found in the other fibroblast types (Figure 5B). Interestingly, however, we observed a significant increase in FT expression in RASFs stimulated overnight with either TNFα (P = 0.049) or IL-1β (P = 0.03) (Figures 5C and D), and these stimulated increases in expression were accompanied by concomitant increases in FT activity (Figure 5E) (P = 0.05, versus unstimulated cells).

As would be expected from previous observations of direct enzyme inhibitors, FTI-276 inhibited the activity of FT (Figure 5E) (P = 0.005, versus TNFα alone; P = 0.015, versus IL-1β alone), but did not inhibit the expression of FT (Figure 5D), whereas GGTI-298 affected neither the expression nor the activity of FT (results not shown). These data suggest that FT may play both a constitutive and a stimulus-dependent role in regulating the signaling in inflammation pathways.

Inhibition of JNK and NF-κB by FTI in RASFs. Because farnesylated proteins that participate in inflammation include heterotrimeric G proteins, we next tested whether FTI-276 acts via effects on G proteins. Pretreatment of RASFs with PT to inhibit Gi protein or Go protein activity inhibited neither the TNFα-stimulated nor the IL-1β-stimulated secretion of MMP-1 (Figure 6A). On the contrary, PT moderately enhanced MMP-1 secretion, suggesting the presence of G proteins that could tonically inhibit MMP-1 secretion. IL-1β- and TNFα-stimulated MMP-1 secretion is therefore not mediated by G proteins, and therefore it is likely that FTI-276 acts on another target (most likely, a small GTPase) in regulating the expression of MMP-1.

Since cytokine-induced MMP-1 secretion is regulated by the MAPks ERK and JNK, and because ERK and JNK are regulated by small GTPases, we next tested whether FTI-276 disrupts MMP-1 secretion by inhibiting the activities of ERK and/or JNK. FTI-276 did not inhibit TNFα-stimulated activation of ERK (P = 0.96,
versus TNFα alone) (Figure 6B), but did inhibit activation of JNK ($P < 0.05$, versus TNFα alone; $P$ not significant versus unstimulated cells) after overnight incubation (Figure 6B). Consistent with the effects of FTI-276 on MMP-1 secretion, an exposure time of at least 8 hours was required in order to achieve the

Figure 5. Expression and activity of farnesyl transferase (FT) in RASFs and other types of fibroblasts. Lysates of unstimulated RASFs, OASFs, or dermal (Derm) fibroblasts were assessed by immunoblotting for FT expression (A) or by enzymatic assay for FT activity (B). RASFs were also incubated with or without FTI-276 prior to stimulation with or without TNFα or interleukin-1β (IL-1β) (C–E), and lysates were assessed for FT expression (C and D) (levels of FT protein were normalized to β-actin) and FT activity (E). Immunoblots are representative of 4 experiments in A and 5 experiments in C. Results are the mean and SEM from 4 experiments in B or 5 experiments in D and E. See Figure 4 for other definitions.

Figure 6. Effects of FTI-276 on signaling pathways regulating MMP-1 secretion. A, RASFs were incubated with or without pertussis toxin (PT) (100 ng/ml) for 1 hour, prior to stimulation with or without TNFα or interleukin-1β (IL-1β) (each at 20 nM) for 24 hours, followed by determination of MMP-1 levels in the supernatants. B and C, RASFs were incubated with or without FTI-276 (10 μM) for 18 hours, prior to stimulation with or without TNFα (20 nM) for 30 minutes, and analyzed for activation of the MAPKs ERK and JNK (B) or NF-κB (C) as described in Patients and Methods. Results are the mean and SEM and representative of 4 experiments in A and B or 3 experiments in C. See Figure 4 for other definitions.
inhibition of JNK activation in response to FTI-276 (results not shown). Thus, it is likely that farnesylation regulates MMP-1 secretion by, at least in part, permitting the activation of JNK.

Cytokine-stimulated secretion of MMP-1 also depends on activation of NF-κB, a process that may be regulated by small GTPases (54). We therefore tested whether inhibition of farnesylation could abrogate NF-κB activation, which was determined as translocation of the NF-κB p65 subunit from the cytosol to the nucleus (Figure 6C). In unstimulated RASFs, ~31 ± 11% (mean ± SEM) of the total cellular p65 was nuclear. In response to TNFα, the nuclear proportion of p65 increased to 74 ± 10% (P = 0.02 versus unstimulated control cells). Incubation with FTI-276 reduced the nuclear accumulation of p65 to 42 ± 6%, which was within the range of unstimulated levels (P = 0.02, versus TNFα alone; P not significant versus unstimulated cells). These data indicate that inhibition of NF-κB activation is another mechanism through which the inhibition of FT may regulate MMP-1 expression.

**DISCUSSION**

Because statins are antiinflammatory and have been suggested to be of therapeutic benefit in RA, we tested whether they inhibit MMP-1 secretion from RASFs. Neither simvastatin nor lovastatin inhibited the secretion of MMP-1, indicating either that statins do not affect MMP-1 regulatory pathways or that multiple countervailing effects result in no net change in MMP-1 secretion. Since HMG-CoA reductase inhibition by statins has 3 well-recognized consequences (inhibition of cholesterol biosynthesis, depletion of cellular FPP, and depletion of cellular GGPP), we tested whether these effects could individually regulate MMP-1 secretion. Blocking attachment of FPP to its protein targets via inhibition of FT significantly reduced cytokine-stimulated MMP-1 secretion. Conversely, promoting farnesylation by providing RASFs with FPP enhanced MMP-1 secretion. These observations suggest a novel way in which MMP-1 secretion is regulated in RASFs, whereby at least one protein may promote the expression and/or release of MMP-1, with dependence on farnesylation for their activity.

The findings showing that FTI-276 inhibited MMP-1 secretion after long (8–24-hour) but not short (1–4-hour) exposures confirms that farnesylation does not regulate signaling directly, but rather, is a posttranslational modification required for appropriate signal function of a CAAX protein (27). Although CAAX proteins undergo prenylation rapidly after synthesis (55), inhibition of farnesylation would not be expected to have an immediate effect on the functioning of CAAX proteins, since 1) following farnesylation, CAAX proteins must complete a series of additional posttranslational modifications, including proteolytic cleavage of the AAX motif, methylation of the C-terminal cysteine, and, in some cases, palmitoylation before they are functionally mature (55–58); 2) modified CAAX proteins must undergo controlled, directed migration to their appropriate cellular compartments in order to function, an event that appears to occur over a period of hours (29,57); and 3) at the time of farnesylation blockade (i.e., addition of FTI), the cell already possesses a full complement of CAAX proteins that must at least partially turn over before the effect of the inhibitor will become apparent, a process that may require several hours (57).

Thus, the kinetics of inhibition of MMP-1 secretion by FTI-276 are broadly consistent with the known biologic processes in farnesylated proteins. The fact that farnesylation regulates MMP-1 secretion, at least in part, at the level of mRNA expression suggests another reason that the effect of FTI-276 on MMP-1 secretion may be delayed, since a longer interval would be required in order for the effects of a farnesylated protein on MMP-1 message regulation to become apparent at the level of protein expression/secretion.

In contrast to the effects on farnesylation, inhibition of geranylgeranylation enhanced MMP-1, suggesting that one or more geranylgeranylated proteins act to inhibit MMP-1 secretion. Consistent with this observation, repleting cellular GGPP inhibited the secretion of MMP-1. Thus, statins, which deplete both FPP and GGPP, may fail to regulate MMP-1, not because they are inactive, but because they produce simultaneous but opposing effects on farnesylation and geranylgeranylation-dependent pathways. Such a model is supported by a recent study indicating that simvastatin is active in inhibiting NF-κB in RASFs (54), and by the present results indicating that simultaneous inhibition of farnesylation and geranylgeranylation by FTI-276 and GGTI-298 resulted in no net effect on MMP-1 secretion, essentially duplicating the effect of a statin on these pathways.

Our findings show that the expression and activity of FT are constitutive and in the range of values found in other fibroblast cell types. However, we also observed increases in both the expression and activity of FT in response to overnight stimulation of RASFs with either TNFα or IL-1β. These observations should be
interpreted with caution, since studies addressing the regulation of FT expression and/or activity are presently limited (59). Indeed, the genes for the FT \(\alpha\)- and \(\beta\)-chains lack known response elements for inflammatory transcription factors, including NF-\(\kappa\)B and activator protein 1, in the 5'-untranslated region (Ahearn I: personal communication). Moreover, the degree of increase in FT expression and activity that we observed in response to cytokine stimulation (30–100%) may be insufficient to account for the many-fold increase in RASF MMP-1 secretion. Nonetheless, our data suggest that the expression and activity of FT may respond to the inflammatory milieu of the RA synovium, and that increased FT activity may support the requisite posttranslational modification of CAAX box proteins that participate in RASF proinflammatory signaling. Additional studies will be needed to evaluate the mechanisms and significance of FT up-regulation.

The specific farnesylated and geranylgeranylated proteins that regulate MMP-1 secretion in RASFs were not determined in the present study. Proteins requiring farnesylation include the 3 isoforms of Ras, which act in diverse signaling pathways. Non–Ras family proteins requiring farnesylation for function include heterotrimeric G proteins, which transduce signals from 7 transmembrane-domain receptors. Farnesylation of G proteins is unlikely to play a role in RASF MMP-1 secretion, since neither TNF\(\alpha\) nor IL-1\(\beta\) are known to signal through these molecules. Indeed, in our studies, the G protein inhibitor PT increased, rather than inhibited, MMP-1 secretion, suggesting that a G\(\alpha\) or G\(\beta\) protein may constitutively suppress MMP-1 secretion from RASFs. Other known farnesylated proteins, including nuclear lamins, cannot be rigorously excluded as possible effectors in signaling for MMP-1 secretion, but these have primarily structural functions and are less likely to play a role in inflammatory responses (60).

In contrast to the pool of farnesylated proteins, which is circumscribed, there are hundreds of proteins undergoing geranylgeranylation. Geranylgeranylated proteins that are known to participate in inflammation, and which are therefore candidates for GGT regulation of MMP-1 secretion, include Rho family proteins, which regulate JNK (13,33,34). Rab family proteins, which regulate vesicular trafficking, are unlikely to have been involved in the effects seen in our studies, since such proteins are modified by the enzyme GGT-II, and the inhibitor that we used, GGTI-298, is specific for GGT-I (61). Since FTI-276 and GGTI-298 had opposite effects, our results do not exclude the possibility that a single protein may positively or negatively regulate MMP-1 secretion, depending on its prenylated state. One such candidate may be RhoB, which exists in both farnesylated and geranylgeranylated forms, each of which has its own cellular localization and function (62–64). Further studies will be required to elucidate the roles of specific small GTPases in regulating MMP-1.

Kinases regulating RASF MMP-1 secretion include JNK and ERK (13,46). In our study, FTI-276 inhibited JNK activation, consistent with a report that another FTI (FTI-744,832) prevented JNK activation in lipopolysaccharide-stimulated monocytes (41). Thus, FTIs likely abrogate MMP-1 secretion by, at least in part, inhibiting elements of a pathway leading to JNK. FTIs have also been reported to inhibit ERK activation in some cell types (41,65), by inhibiting Ras transduction of growth factor signals to the RAF–MEK–ERK pathway (66,67). However, FTI-276 did not inhibit ERK activation in RASFs stimulated with TNF\(\alpha\) or IL-1\(\beta\). Thus, it appears that FTIs cannot inhibit cytokine-stimulated MMP-1 secretion from RASFs via ERK inhibition.

Signaling pathways from cytokine receptors to ERK are not as well elucidated as those originating from growth factor receptors; the failure of FTI-276 to inhibit ERK suggests that cytokine stimulation of ERK may proceed wholly or in part through Ras-independent pathways. Alternatively, it is possible that Ras proteins mediate TNF\(\alpha\)- and IL-1\(\beta\)-stimulated ERK activation, but that one or more of them escaped the FTI blockade. Of the 3 Ras subtypes, Ki-Ras and N-Ras have been shown to undergo geranylgeranylation, with potential salvage of function, when cellular FPP levels are low (68), suggesting that one of these subtypes may have been resistant to the effects of FTI-276 in our experiments. Indeed, geranylgeranylation salvage of Ras appears to be a mechanism through which FTIs fail when they are utilized as antitumor therapies (69).

FTI-276 also inhibited NF-\(\kappa\)B activation in cytokine-stimulated RASFs, with results determined as translocation of p65 from the cytosol to the nucleus. NF-\(\kappa\)B is a family of transcription factors regulating cellular responses to inflammatory signals. Resting NF-\(\kappa\)B complexes are cytosolic dimers (most commonly, p65/p50), complexed with a regulatory subunit (I\(\kappa\)B). Engagement of cytokine and/or other receptors induces phosphorylation and degradation of I\(\kappa\)B, via activity of an I\(\kappa\)B kinase (IKK) (70). The functional dimers migrate to the nucleus and ligate specific transcriptional response elements. FTIs inhibit NF-\(\kappa\)B in cells other than RASFs by acting on farnesylated proteins upstream of IKK (39,40). NF-\(\kappa\)B has been implicated in regulating
MMP-1 secretion (15,71,72), and our results suggest that one or more farnesylated proteins regulate the levels of MMP-1 via effects on NF-κB. Since NF-κB regulates the synthesis of more than 40 inflammatory proteins (73) and is overexpressed in RASFs (74,75), FTIs likely exert a wider range of antiinflammatory effects in these cells.

The results of this study thus demonstrate that protein farnesylation is necessary for cytokine-stimulated MMP-1 secretion from RASFs, in part via mediation of JNK and NF-κB activation. In contrast, geranylgeranylation of one or more proteins provides an inhibitory signal for MMP-1 secretion. FTIs, now in clinical trials to test their effects on malignancy, appear to be relatively well-tolerated, and may hold promise for controlling joint destruction in RA. In contrast, statins have antiinflammatory effects that may be useful in RA, their ability to inhibit both farnesylation and geranylgeranylation suggests that they may have no net benefit in regulating MMP-1 secretion in the rheumatoid joint.

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**AUTHOR CONTRIBUTIONS**

Dr. Abeles had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

**Study design.** Abeles, Marjanovic, Pillinger.

**Acquisition of data.** Abeles, Marjanovic, Park, Attur, Al-Mussawir, Dave, Fisher, Stuchin, Pillinger.

**Analysis and interpretation of data.** Abeles, Marjanovic, Abramson, Pillinger.

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Regulation of Apoptosis in Fibroblast-like Synoviocytes by the Hypoxia-Induced Bcl-2 Family Member Bcl-2/Adenovirus E1B 19-kd Protein–Interacting Protein 3

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Objective. Rheumatoid arthritis (RA) synovial hyperplasia is related in part to a resistance to apoptosis exhibited by fibroblast-like synoviocytes (FLS). Since hypoxia is a regulator of apoptosis, and since RA synovium is hypoxic, we conducted this study to examine the effects of hypoxia on the Bcl-2 pathway and the role this may play in regulating apoptosis in FLS.

Methods. Synovium samples from RA patients, osteoarthritis (OA) patients, and normal subjects were used for immunohistologic assessments and for generating FLS lines in vitro. FLS were stimulated under conditions of hypoxia (1% O2) and using 100 μM CoCl2 to simulate the effects of severe hypoxia. Changes in the gene expression profile of FLS were evaluated using microarrays and were confirmed by quantitative polymerase chain reaction (PCR). Changes in protein expression were detected by Western blotting. The effect of transient transfection with a BNIP3 plasmid on the apoptosis of FLS was evaluated in the presence and absence of cytokines.

Results. Gene expression profiling demonstrated that BNIP3 was unique among the BCL2 family, in that it was induced by hypoxia in FLS. Quantitative PCR indicated a 2–3-fold induction of BNIP3 messenger RNA, and Western blotting showed a 3–5-fold increase in the 30-kd Bcl-2/adenovirus E1B 19-kd protein–interacting protein 3 (BNIP-3) monomer. BNIP-3 was widely expressed in RA synovium and was prominent in FLS from the lining layer. Overexpression of BNIP3 increased FLS apoptosis under hypoxic conditions, an effect that was inhibited by tumor necrosis factor α and interleukin-1β.

Conclusion. The proapoptotic protein BNIP-3 is induced in FLS by hypoxia and is widely expressed in RA synovium, but its proapoptotic effects may be inhibited in vivo by proinflammatory cytokines. Since overexpression of BNIP3 in FLS increases apoptosis, this may provide a novel approach for controlling synovial hyperplasia in RA.

Rheumatoid arthritis (RA) is a chronic inflammatory disease that leads to fundamental changes in the cellular composition and function of the synovial membrane (1). One aspect of this is that the resident fibroblast-like synoviocytes (FLS) that normally populate the thin synovial lining layer appear to increase in number and change their phenotype, becoming locally invasive to the adjacent cartilage and bone. This transformation of FLS, which resembles that seen in tumor cells, is evident in vitro as anchorage-independent growth, loss of contact inhibition, and in the expression of protooncogenes (2). Studies evaluating coimplantation of RA FLS and articular cartilage into SCID mice have clearly demonstrated the invasive nature of these cells (3). Although in vivo indices of proliferation in RA synovium have generally been found to be low, the FLS derived from these tissues have a survival advantage when cultured in vitro. This has led to the hypothesis that the survival advantage may be related, at least in part, to an increase in the levels of several antiapoptotic...
proteins, a reduction in the function of proapoptotic proteins, a protective effect of high levels of tumor necrosis factor α (TNFα), or a combination of these factors (4,5).

Cellular apoptosis is induced by 2 principal pathways: an extrinsic pathway mediated by activation of cell surface receptors, such as TNF receptor type I and Fas, that feature a “death domain” and an intrinsic pathway regulated by pro- and antiapoptotic members of the Bcl-2 family, with this latter pathway acting primarily on the mitochondria. Both pathways serve to ultimately activate caspase 8 and caspase 3, and result in a stereotyped set of morphologic changes that include nuclear condensation, DNA fragmentation, and increased plasma membrane permeability.

Hypoxia is a potent apoptotic stimulus and is an important cause of cell death in cancer and ischemia. Although hypoxia is generally proapoptotic, in some situations, brief or repetitive exposure to hypoxia may result in cells becoming resistant to apoptosis through the mitochondrial Bcl-2 pathway, as well as aggressive cancers becoming highly resistant to hypoxia-induced apoptosis (6). In the context of ischemia, recurrent hypoxia results in a related phenomenon that has been called preconditioning, which may serve to protect tissues from ischemic damage (7).

We and other investigators have demonstrated that chronic and repetitive episodes of hypoxia play a role in the pathogenesis of RA synovitis (8–10). Since apoptosis is impaired in RA synovium and FLS, we evaluated the effects of hypoxia on the Bcl-2 family members. Our data indicate that the proapoptotic protein Bcl-2/adenovirus E1B 19-kd protein–interacting protein 3 (BNIP-3) is unique among the members of the Bcl-2 family, in that it is induced by hypoxia in FLS. BNIP-3 was found to be widely expressed in RA synovium, and overexpression of wild-type BNIP-3 resulted in a marked induction of FLS apoptosis, a phenomenon that was inhibited by proinflammatory cytokines.

PATIENTS AND METHODS

Synovial tissue samples. Samples of RA and OA synovium were obtained at the time of arthroplasty of the knee or metacarpophalangeal joints. All patients gave their informed consent. All RA patients met the American College of Rheumatology (formerly, the American Rheumatism Association) criteria for the disease (11). Samples of normal synovium were obtained from cadavers.

Isolation of FLS. FLS were isolated from RA and OA synovial tissue samples as previously described (8). Briefly, fresh synovial tissues were digested in collagenase and hyaluronidase. After washing, adherent cells were cultured in Dulbecco’s modified Eagle’s medium and 10% fetal bovine serum. When the cells reached 80% confluence, they were treated with trypsin, washed, and then recultured. The FLS cell lines that were generated from the synovial tissues were then used in experiments after 3–5 cell passages.

Stimulation of FLS by hypoxia and CoCl2-simulated hypoxia. FLS were used for hypoxia-stimulation experiments when they reached 80% confluence. Prior to stimulation, FLS were serum starved for 48 hours, and after placement in fresh medium, the cells were incubated for up to 72 hours in a hypoxia chamber with a gas mixture containing 1% O2 (Medigas, Winnipeg, Manitoba, Canada), and the cells were harvested for protein and messenger RNA (mRNA) analysis. In some experiments, 100 μM CoCl2 was used to mimic the effects of severe hypoxia.

Affymetrix gene expression microarray analysis of hypoxia-stimulated FLS. Four FLS lines (2 RA and 2 OA) were maintained in serum-free media under normoxia, 1% hypoxia, and CoCl2 conditions (1 × 106 cells per condition) for 6 hours, then the cells were harvested for RNA extraction. We previously demonstrated that this time point is optimal for evaluating the effects of hypoxia at the mRNA expression level (8). Total RNA was extracted using RNeasy kits (Qiagen, Mississauga, Ontario, Canada), then Affymetrix microarray analysis using human genome U133 Plus 2.0 GeneChip array (Affymetrix, Santa Clara, CA) was performed on 10 μg of high-quality RNA (28S:18S ratio >1). Data from the scanned chips were normalized and analyzed using the MAS 5.0 algorithm, then imported into ArrayAssist software (Stratagene, La Jolla, CA) and analyzed by t-tests and hierarchical clustering techniques. Expression data relating to the BCL2 family genes were extracted and analyzed independently.

Quantitative polymerase chain reaction (PCR). Quantitative PCR was performed with a 7300 real-time PCR system using reagents from Applied Biosystems (Foster City, CA) as previously described (12). Briefly, total RNA was extracted from 6 separate FLS lines (4 RA and 2 OA) under normoxic conditions and hypoxic conditions (after exposure to 1% O2 or 100 μM CoCl2) for 6 hours. The expression level of 5 BCL2 family members was evaluated using the following primer/probe sets: for BNIP3, Hs00969293; for PUMA, Hs00248075; for LIP2, Hs00357350; for BAX, Hs00180269; and for BCL2, Hs00236808 (Assays-on-Demand; Applied Biosystems). The raw expression levels for each gene were normalized for GAPDH expression as previously described (12). The fold change in the GAPDH-normalized expression level of each of the above genes was calculated, comparing hypoxia and CoCl2 stimulation to basal normoxic conditions. The mean fold change in the 6 FLS lines was determined, and comparisons were statistically tested using Student’s paired t-test. For experiments that compared BNIP3 mRNA levels in RA and OA, a total of 6 FLS lines from RA patients and 6 FLS lines from OA patients were processed and analyzed as described above.

Western blot analysis. For detection of BNIP-3, whole cell extracts from 4 lines of RA or OA FLS were prepared using radioimmunoprecipitation assay–modified buffer plus a cocktail of inhibitors (Sigma-Aldrich, Oakville, Ontario, Canada). The protein content was determined using BCA reagent (Pierce, Rockford, IL). A total of 20 μg of samples was loaded.
on a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel, and after electrophoresis, was blotted on nitrocellulose membranes. The anti-BNIP-3 primary antibody (Sigma-Aldrich) was used at 1 μg/ml. The secondary antibody was an anti-mouse antibody coupled to horseradish peroxidase and was used at a 1:5,000 dilution (Sigma-Aldrich). An enhanced chemiluminescent Western blotting system (American GE Healthcare Biosciences, Baie d’Urfé, Quebec, Canada) was used to detect proteins. The same blot was stripped and reprobed with anti-ERK protein (Cell Signaling Technology, Danvers, MA) to control protein loading. Bands were quantified using Image-Pro software (Media Cybernetics, Silver Spring, MD), and the mean fold change detected with hypoxia or CoCl2-simulated hypoxia over basal normoxia was calculated.

**Transient transfection of FLS.** FLS were cultured in chamber slides coated with 5 μg/ml of fibronectin. After 24 hours, the cells were transiently transfected using 10 μg of the plasmid pcDNA3-BNIP3-Wt, which localizes to the cytoplasm, as detected by a hemagglutinin (HA) tag as described previously (13). A control pcDNA3 plasmid without BNIP3 was used for comparison. After 72 hours of transfection, cells were fixed and permeabilized for use in immunofluorescence microscopy or immunocytochemistry studies. An anti-HA antibody (Sigma-Aldrich) was used to detect pcDNA3-BNIP3-Wt expression. We used a biotin–avidin–horseradish peroxidase system to detect BNIP-3 protein expression. A minimum of 500 cells was counted, and the percentage of transfected cells was then determined.

**Detection of apoptosis.** Transfected FLS, as described above, were assessed for apoptosis by DNA fragmentation, condensation, and release of histones. DNA fragmentation is considered as a late event in apoptosis and was assessed using the TUNEL staining assay (Roche Applied Science, Laval, Quebec, Canada). The proportion of apoptotic cells was estimated by counting TUNEL-positive and TUNEL-negative cells in 10 high-power fields.

The early stages of apoptosis were assessed by evaluating the degree of DNA condensation and the nucleus shape. Sytox orange (Invitrogen, Burlington, Ontario, Canada), a DNA dye emitting red fluorescence, was used for this purpose. The proportion of cells demonstrating DNA condensation using this method was estimated by counting the number of Sytox orange–positive and Sytox orange–negative cells in 10 high-power fields.

To assess the effects of proinflammatory cytokines on BNIP-3 induced apoptosis, FLS were transfected as described above and exposed to 1% O2 for 24 hours in the presence or absence of 10 μM concentrations of TNFα and interleukin-1β (IL-1β). In these experiments, apoptosis was assessed using a Cell Death Detection Enzyme-Linked Immunosorbent Assay Plus histone release kit (catalog no. 11774425 001; Roche Applied Science) according to the manufacturer’s specifications. Apoptosis was measured using arbitrary units.

**Immunohistology and immunocytochemistry.** Fresh samples of RA and OA synovium were snap-frozen in TissueTek OCT (Miles, Elkhart, IN), and stored at −80°C. Immunoperoxidase staining was performed as previously described (8). For double-immunofluorescence staining experiments, 5-μm frozen sections were fixed in cold acetone for 5 minutes at 4°C, dried, and then blocked with 1% bovine serum albumin in phosphate buffered saline for 30 minutes. After incubation of the first primary antibody (mouse anti-CD68 or anti-CD55) for 1 hour, a secondary goat anti-mouse antibody conjugated with Cy3 (green stain) was then added to the section for 1 hour. The sections were then blocked with normal mouse serum. The second primary antibody (mouse anti-human BNIP3) was labeled with Alexa Fluor 555 (red stain), was then added. Photographs were taken using a SensiCam High Performance Camera (Arcturus, Mountain View, CA).
Immunocytochemistry was used to evaluate the expression of BNIP-3 in FLS lines. Cells were seeded at a density of 10^4 onto glass slides. After 48 hours of culture, cells were fixed with paraformaldehyde for 15 minutes, then permeabilized with 0.2% Triton X-100 for 5 minutes then rehydrated in Tris buffered saline. All slides were blocked with 1% bovine serum albumin for 1.5 hours at room temperature. One microgram of diluted BNIP-3 primary antibody was applied, and the slides were incubated for 2 hours at room temperature. A fluorescein-labeled goat anti-mouse secondary antibody was then applied for 1 hour, and staining was detected using fluorescence microscopy. Control cells were treated with an irrelevant isotype-matched murine antibody.

**RESULTS**

**Identification of hypoxia-responsive BCL2 family genes in FLS by Affymetrix microarray.** RNA from 4 lines of FLS (2 RA and 2 OA) was analyzed using Affymetrix human genome U133 Plus 2.0 GeneChip arrays as described in Patients and Methods. After normalization of the raw expression data using the MAS 5.0 algorithm, we determined the average expression levels of the full spectrum of BCL2 family members for the 4 FLS lines treated under normoxic conditions and hypoxic conditions (hypoxia and CoCl₂) for 6 hours. The average expression levels for these conditions were compared using t-tests after correction for the false discovery rate (FDR). Table 1 summarizes the mean fold change induced by hypoxia and CoCl₂ over the basal normoxic levels for the members of BCL2 gene family. These data indicate that only BNIP3 and, to a lesser extent, the closely related BNIP3L were significantly induced by hypoxia and CoCl₂-simulated hypoxia. On this basis, BNIP3 expression in synovium and the effects of hypoxia on the expression of BNIP3 by FLS and apoptosis of FLS were explored further.
Expression of BNIP-3 in synovium. We used immunohistologic techniques to determine the expression of BNIP-3 in normal, RA, and OA synovial tissue samples. These data are shown in Figure 1. Normal synovium expressed minimal amounts of BNIP-3 in the lining layer and in the microvasculature (data not shown). In contrast, both RA and OA synovium demonstrated widespread cytoplasmic staining for BNIP-3 in the lining cells. Expression was particularly intense in synovium from patients with early RA, demonstrating high levels of inflammation and infiltration with mononuclear cells (Figure 1A). In these samples, staining was evident both in the lining cell layer and in the sublining mononuclear cells. In contrast, the OA samples demonstrated minimal BNIP-3 expression in the sublining areas, despite expression in the lining cell layer (Figure 1B).

We sought to determine whether FLS in the lining layer express BNIP-3. To achieve this, we used double-immunofluorescence staining with BNIP-3 and either CD55 (FLS) or CD68 (macrophages). As shown in Figure 1D, there was clearly overlap between BNIP-3 staining and CD55 staining, consistent with FLS expression of BNIP-3 in the lining layer. Although there was also overlap of staining with BNIP-3 and CD68, this was not as intense (data not shown).

Quantitative PCR for hypoxic induction of BNIP3 in FLS. To more accurately evaluate the hypoxic induction of BNIP3 and to compare this with other representative members of the BCL2 family, we analyzed the expression of BNIP3, PUMA, IAP2, BAX, and BCL2 under normoxic conditions and under hypoxic conditions (after 6 hours of hypoxia and CoCl2-simulated hypoxia) using TaqMan quantitative PCR. These data are shown in Figure 2A, and represent the mean fold change seen in 6 separate lines of FLS (4 RA and 2 OA). Consistent with the Affymetrix microarray analysis, these data indicated a significant 2–3-fold induction of BNIP3, which was not observed with any of the other BCL2 family members tested. Indeed, there was a trend toward down-regulation by hypoxia of all the other genes tested, particularly BAX and BCL2.

To determine whether there were any differences between RA and OA FLS in the hypoxic induction of BNIP3, we compared BNIP3 mRNA expression levels under conditions of normoxia, CoCl2, and hypoxia in 6 lines of RA FLS and 6 lines of OA FLS. These results are summarized in Figure 2B and indicate that there were no consistent differences in hypoxic induction of BNIP3 between FLS derived from RA and OA synovium.

Figure 2. Hypoxic induction of BCL2 family member BNIP3. A, Expression of BNIP3, PUMA, IAP2, BAX, and BCL2 was assessed by TaqMan quantitative polymerase chain reaction (PCR). Values are the mean and SEM fold change in mRNA expression levels induced by hypoxia (open bars) and CoCl2-simulated hypoxia (solid bars) over basal normoxic conditions in 4 rheumatoid arthritis (RA) and 2 osteoarthritis (OA) fibroblast-like synoviocyte (FLS) lines after the raw expression data were normalized for GAPDH expression. * = P < 0.05; ** = P < 0.01 versus normoxic conditions, by Student’s t-test for paired samples. B, Comparison of the expression of BNIP3 in RA FLS (solid bars) and OA FLS (open bars) under normoxic, hypoxic, and CoCl2-simulated hypoxic conditions, as determined by TaqMan quantitative PCR. Values are the mean and SD of the GAPDH-adjusted ratio of BNIP3 mRNA levels in 6 RA and 6 OA FLS lines. There were no reproducible differences in BNIP3 expression between the RA and OA FLS lines under the 3 conditions tested.

Induction of BNIP-3 protein expression in FLS by hypoxia and CoCl2. We evaluated the expression of BNIP-3 protein in whole cell lysates of FLS exposed to hypoxia and CoCl2-simulated hypoxia. These data are
shown in Figure 3. A total of 4 separate FLS lines were exposed to 100 μM CoCl₂ for up to 96 hours and were examined at several time points for BNIP-3 expression, as determined by Western blotting. The levels of a 30-kd BNIP-3 monomer increased in a time-dependent manner, reaching a maximum at 24 hours. The mean (±SEM) fold increase in the 30-kd BNIP-3 protein seen in the 4 RA FLS lines after 24 hours of hypoxia was 4.3 ± 1.05. Thereafter, the levels were sustained for the subsequent 72 hours. We also detected a parallel increase in the levels of a 60-kd dimer (data not shown). No increase in total ERK protein levels were seen in the samples.

Effects of hypoxia and CoCl₂-simulated hypoxia on the localization of BNIP-3 in RA FLS. The biologic effects of BNIP-3 in mediating cell death are dependent on the localization of this protein to the mitochondria. Interestingly, nuclear localization of BNIP-3 protein has been observed in specific cell lines and tissues (14). The role of nuclear BNIP-3 remains unclear. We analyzed the localization of BNIP-3 protein in FLS by immunocytochemistry. As shown in Figure 4, staining for BNIP-3 protein was detected in the cytoplasm of RA FLS in a perinuclear distribution, and the intensity of staining increased with exposure to hypoxia or 100 μM CoCl₂.

Effects of BNIP-3 overexpression on FLS apoptosis. Since it has been shown that overexpression of BNIP-3 induces cell death (15), we evaluated the effects of BNIP-3 overexpression on the apoptosis of FLS. We used the pcDNA3-BNIP3-Wt plasmid, which has previously been shown to localize the BNIP-3 protein to the cytoplasm (13), as well as an empty control vector. Transfection efficiency was evaluated using specific antibodies for each plasmid and exceeded 80% in these experiments (data not shown).

We evaluated the effects of BNIP-3 transfection on FLS apoptosis using the TUNEL assay, which detects late apoptotic events by measuring DNA fragmentation. These data are shown in Figure 5A. The studies indicated that the pcDNA3-BNIP3-Wt–transfected cells demonstrated a mean of 27% TUNEL-positive cells,
Figure 5. Induction of apoptosis of fibroblast-like synoviocytes (FLS) by overexpression of BNIP3 and inhibition of the BNIP3-induced apoptosis by cytokines. FLS from rheumatoid arthritis (RA) patients were transiently transfected with a hemagglutinin-tagged pcDNA3-BNIP3-Wt (BNIP3-Wt) plasmid encoding the wild-type BNIP3 gene or with the empty control plasmid pcDNA3. A, Percentage of TUNEL-positive apoptotic cells following transfection of RA FLS. TUNEL-positive apoptotic cells were significantly increased in pcDNA3-BNIP3-Wt-transfected FLS (27%) as compared with control plasmid–transfected FLS (5%). B, Percentage of RA FLS undergoing DNA condensation, as determined by staining of cell nuclei with Sytox orange. FLS transfected with the pcDNA3-BNIP3-Wt plasmid demonstrated a significantly higher proportion of Sytox orange–positive cells compared with FLS transfected with the control empty plasmid. The proportion of cells demonstrating DNA condensation increased further to almost 80% with the addition of the hypoxia mimic CoCl2. C, Apoptosis index in RA FLS treated with tumor necrosis factor α (TNFα) and interleukin-1β (IL-1β) (10 μM each). TNFα and IL-1β inhibited the apoptosis of FLS that had been transiently transfected with the pcDNA3-BNIP3-Wt plasmid and then exposed to hypoxia for 24 hours. Values are the mean and SD of 2 different RA FLS lines.
compared with 5% TUNEL-positive cells in those transfected with the empty pcDNA3 vector ($P < 0.01$).

To evaluate the effects of BNIP-3 overexpression on the early events of programmed cell death, we used a DNA condensation assay. As shown in Figure 5B, the percentage of cells undergoing DNA condensation increased significantly after overexpression of BNIP-3. Exposure of the transfected cells to 100 $\mu$M CoCl$_2$ further increased the percentage of cells undergoing DNA condensation to almost 80%.

In order to assess the potential impact of proinflammatory cytokines on the proapoptotic effects of BNIP-3, we evaluated apoptosis in pcDNA3-BNIP3-Wt–transfected FLS under hypoxic conditions in the presence and absence of 10 $\mu$M TNF$\alpha$ and IL-1$\beta$ (Figure 5C). These data indicate both TNF$\alpha$ and IL-1$\beta$ reduced the proapoptotic effects of BNIP-3 overexpression in FLS under hypoxic conditions.

**DISCUSSION**

Synovial hyperplasia is a characteristic feature of RA and is responsible in part for the persistence and local destructiveness of this disease. Although it is likely that a number of mechanisms contribute to RA synovial hyperplasia, a substantial body of evidence has suggested that impaired FLS apoptosis is an important factor in this process (4). The mechanisms underlying this impaired apoptosis remain to be defined and may involve alterations in the level and function of a number of proteins that regulate the extrinsic and intrinsic apoptotic pathways.

Since hypoxia plays an important role in modulating the apoptotic responses of tumor cells, we evaluated the effects of hypoxia in regulating the expression of the Bcl-2 family of proteins and, in turn, the potential role that this may play in modulating FLS apoptosis. Our results indicated that the proapoptotic protein BNIP-3 and, to a lesser extent, its homolog BNIP-3L are the only members of the Bcl-2 family that are significantly regulated by hypoxia at the mRNA and protein levels. Our study further demonstrated that BNIP-3 is widely expressed in RA and OA synovium and that overexpression of wild-type BNIP-3 in RA FLS significantly increases their apoptosis, particularly in combination with hypoxia and CoCl$_2$-simulated hypoxia. Importantly, we demonstrated that TNF$\alpha$ and IL-1$\beta$ both inhibit the proapoptotic effects of BNIP-3 in the presence of hypoxia.

A number of lines of evidence have suggested that the RA synovial microenvironment is chronically hypoxic (9). It has long been established that inflamed RA synovial fluid is typically hypoxic and acidotic and shows evidence of anaerobic metabolism. Although direct measurements of RA synovial tissue $P_O_2$ are lacking, several studies, including our own, have demonstrated the presence of hypoxia-inducible factor 1$\alpha$ (HIF-1$\alpha$), particularly in association with the synovial lining cell layer, where RA FLS are believed to originate (9,16–19). Moreover, we demonstrated that the expression of HIF-1$\alpha$ in fresh whole synovial tissues can be dramatically augmented by exposing the tissues to hypoxic conditions (8). Since this key transcription factor is essentially undetectable under normoxic conditions, but is stabilized and translocated to the nucleus under hypoxic conditions, these data have provided compelling, albeit indirect, evidence of chronic hypoxia in RA synovium.

In response to hypoxia, the stabilization and accumulation of HIF-1$\alpha$ is now known to regulate a constellation of genes that feature hypoxia-responsive elements in their promoter; these collectively serve to increase the cell’s capacity to survive in low oxygen conditions (9). The effects of hypoxia on cellular apoptosis are complex and involve both direct and indirect effects (20). Hypoxia induces apoptosis by increasing the permeability of the mitochondrial membrane, leading to the release of cytochrome $c$ into the cytosol, which in turn, leads to activation of the caspase pathway (21,22). This process has been shown to be dependent on the activation of Bax/Bak, since mice lacking these genes are resistant to hypoxia-induced apoptosis (23). Two mechanisms potentially mediate the effects of hypoxia on Bax/Bak-induced apoptosis. First, hypoxia increases the levels of p53, which in turn activates Bax/Bak. This is achieved through stabilization of p53 by inhibiting p53 ubiquitin ligase Mdm2 (24) or by a direct interaction between HIF-1$\alpha$ and p53 (25). The second mechanism by which hypoxia may affect Bax/Bak-mediated apoptosis is by directly regulating 1 or more members of the Bcl-2 family.

In the current study, data generated from a comprehensive microarray analysis of hypoxia-stimulated FLS and confirmed by quantitative PCR indicated that the only member of the Bcl-2 family to be significantly up-regulated by hypoxia was BNIP-3. The potent induction of these genes by CoCl$_2$ further confirmed that this process is HIF-1$\alpha$ dependent, since CoCl$_2$ directly stabilizes HIF-1$\alpha$. Hypoxic regulation of BNIP-3 has previously been demonstrated in tumor cells, endothelial cells, and macrophages in an HIF-1$\alpha$–dependent manner (26,27). The promoter region of the BNIP-3 gene contains a functional hypoxia-responsive element that is responsible for up-regulating the expres-
sion of this molecule in response to stabilization and nuclear translocation of HIF-1α (27). We found a modest induction of the closely related BNIP-3L by hypoxia and CoCl₂, and it has been suggested that the hypoxic induction occurs indirectly through p53 (28).

BNIP-3, a proapoptotic Bcl-2 homology 3 (BH3)–only protein, induces apoptosis by binding to the prosurvival multidomain protein Bcl-2/Bcl-xL and, thus, inhibits the antiapoptotic effects of the latter protein on Bax/Bak activation. This has been shown in studies of the overexpression of BNIP-3 (29). Our data indicate that overexpression of wild-type BNIP-3 in RA FLS substantially increased DNA condensation and fragmentation, 2 characteristic features of apoptosis. Moreover, hypoxia and CoCl₂-simulated hypoxia further augmented the apoptotic effects of BNIP-3 overexpression.

In the current study, we demonstrated widespread expression of BNIP-3 in the lining layer of RA synovium, as well as proapoptotic effects of BNIP-3 and hypoxia on RA FLS in vitro. In view of these observations, it is paradoxical that multiple studies have demonstrated that there is little evidence of FLS apoptosis in vivo. A number of possibilities may reconcile these observations. First, it is possible that hypoxia induces the expression of 1 or more antiapoptotic Bcl-2 family members in parallel with BNIP-3. It has been shown that severe hypoxia (near 0% O₂) dramatically up-regulates the transcription of inhibitor of apoptosis protein 2 (IAP-2; or, BIRC-3) in an HIF-1α–independent manner (30,31). Our microarray and quantitative PCR studies did not demonstrate any evidence of induction of this protein in FLS. This may be related to the fact that we used less severe hypoxic conditions (1% O₂). Moreover, in view of the fact the CoCl₂ mimics hypoxia by directly stabilizing HIF-1α, it is not surprising that this stimulus did not up-regulate IAP-2 in FLS.

Another, more likely, possibility is that factors present in the inflamed RA synovium affect the intrinsic apoptosis pathway and modulate the proapoptotic effects of BNIP-3 on FLS. This possibility is supported by our observation that the FLS apoptosis induced by BNIP-3 overexpression was inhibited in vitro by physiologically relevant concentrations of TNFα and IL-1β, 2 cytokines that are critical proinflammatory mediators found abundantly in RA synovitis. The proapoptotic effect of TNFα on RA FLS has been shown to be mediated by the phosphatidylinositol 3-kinase (PI 3-kinase)/Akt pathway, which in turn, activates NF-κB (32). Furthermore, IL-15 and vascular endothelial growth factor both have an antiapoptotic effect on FLS by inducing Bcl-2 expression, the latter also functioning in a PI 3-kinase/Akt–dependent manner (33,34). Thus, it can be proposed that despite a hypoxic microenvironment and abundant in vivo expression of BNIP-3 in RA synovium, the resident FLS are resistant to apoptosis because of increased Bcl-2 expression induced by inflammatory cytokines, likely in a PI 3-kinase/Akt–dependent manner.

It is also possible that 1 or more antiapoptotic proteins act through the extrinsic death-domain pathway and synergistically inhibit the proapoptotic effects of BNIP-3 on FLS. These antiapoptotic proteins include the Fas-associated death domain–like IL-1β–converting enzyme–inhibitory protein FLIP and sentrin 1 (4,35).

Finally, the proapoptotic effects of BNIP-3 may be modulated in the presence of low levels of a critical downstream effector. It has been shown that the levels of p45 up-regulated modulator of apoptosis (PUMA; or, Bcl-2–binding component 3 [BBC-3]), which is a downstream effector of p53-mediated apoptosis, are low in FLS, and this has been proposed to contribute to the low apoptosis in the synovial intimal lining (36). As with BNIP-3 (29), PUMA is also a BH3 proapoptotic Bcl-2 family member that interacts with the antiapoptotic Bcl-2/Bcl-xL, this in turn, promoting mitochondrial dysfunction and cell death through Bax/Bak. It is not clear whether BNIP-3 acts upstream of PUMA or whether they both act synergistically on the multidomain Bcl-2/Bcl-xL. Of note, we did detect a modest down-regulation of PUMA by CoCl₂, but not by hypoxia (see BBC3 in Table 1).

In summary, we have demonstrated that the proapoptotic protein BNIP-3 is widely expressed in RA synovium and that its expression in FLS is further induced by hypoxia, both at the mRNA and protein levels. Overexpression of wild-type BNIP-3 in FLS resulted in a marked increase in apoptosis, which was further augmented by hypoxia and CoCl₂-simulated hypoxia, but was inhibited by TNFα and IL-1β. These data suggest that manipulation of BNIP-3 in RA FLS may increase the susceptibility of these cells to the apoptotic effects of hypoxia, particularly if proinflammatory cytokines such as TNFα and IL-1β are blocked. In turn, this may serve to inhibit the synovial hyperplasia which sustains this disease.

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AUTHOR CONTRIBUTIONS

Dr. El-Gabalawy had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Firestein, El-Gabalawy.

Acquisition of data. Kammouni, Wong, Ma, Firestein, El-Gabalawy.

Analysis and interpretation of data. Kammouni, Ma, Firestein, Gibson, El-Gabalawy.


Statistical analysis. El-Gabalawy.

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Differential Regulation of Matrix Metalloproteinase 2 and Matrix Metalloproteinase 9 by Activated Protein C

Relevance to Inflammation in Rheumatoid Arthritis

Meilang Xue, Lyn March, Philip N. Sambrook, and Christopher J. Jackson

Objective. To investigate the in vitro effect of activated protein C (APC), a natural anticoagulant and novel antiinflammatory agent, on the regulation of the gelatinases matrix metalloproteinase 2 (MMP-2) and MMP-9.

Methods. Synovial fibroblasts and peripheral blood monocytes isolated from patients with rheumatoid arthritis (RA) or osteoarthritis (OA) and Mono Mac6 cells were used in this study. After treatment, cells and culture supernatants were collected for zymography, enzyme-linked immunosorbent assay, reverse transcription–polymerase chain reaction, and Western blot analysis.

Results. Fibroblasts and monocytes from RA patients produced substantially more MMP-9 than did those from OA patients; however, there was no difference in MMP-2 production. The addition of recombinant APC markedly reduced MMP-9 at the gene and protein levels. In contrast, APC up-regulated and activated MMP-2. Using a blocking antibody to the endothelial protein C receptor (EPCR), we showed that the inhibition of MMP-9 by APC was EPCR-dependent. Furthermore, APC directly suppressed the production of tumor necrosis factor (TNF) and the activation of NF-κB and MAP kinase p38, and inhibitors of NF-κB or p38 reduced the production of MMP-9, suggesting that APC inhibits MMP-9 by blocking TNF, NF-κB, and p38. Thus, APC acts on MMP-9 by binding to EPCRs on the cell surface and, subsequently, inhibiting the intracellular activation of the proinflammatory signaling molecules NF-κB and p38.

Conclusion. APC appears to be the first physiologic agent to inhibit the production of proinflammatory MMP-9, yet increase antiinflammatory MMP-2 activity. Our results provide the initial evidence that APC may be beneficial in the prevention of inflammation and joint destruction in RA.

Rheumatoid arthritis (RA) is an inflammatory disease that predominantly affects the joints and is characterized by inflammatory cell infiltrates and hyperplasia of fibroblasts, resulting in the destruction of cartilage and periarticular bone (1). In RA, synovial fibroblasts manifest an abnormal phenotype, which is distinguished by increased proliferation, resistance to apoptosis, and invasiveness of adjacent tissues. These changes are often referred to as those of a tumor-like transformation (2,3). Like synovial fibroblasts, macrophages are normal constituents of synovial tissue. Circulatory monocytes migrate into tissues to become resident macrophages, which can also differentiate into dendritic cells and osteoclasts (4). Monocyte/macrophage numbers are dramatically increased in RA synovium (5). Once activated, synovial fibroblasts and macrophages produce a variety of cytokines, chemokines, and matrix-degrading enzymes and are responsible for the progressive destruction of articular cartilage and bone (5–8). RA synovial fibroblasts also release large amounts of RANKL (9), which mediates the differentiation of bone-resorbing osteoclasts from their macrophage precursors.

Degradation of articular cartilage is one of the
early features of RA and is mediated by increased proteolytic activity. An important aspect of RA synovial fibroblasts and macrophages is an increased production of members of the matrix-degrading enzyme family (10–12) the matrix metalloproteinases (MMPs). MMPs are a family of zinc-dependent endopeptidases that are capable of degrading all major components of the extracellular matrix. A number of studies have demonstrated that MMPs are important mediators in inflammatory and connective tissue diseases such as RA (11,12). Of considerable importance in arthritis are the gelatinase subfamily of MMPs, consisting of MMP-2 (gelatinase A) and MMP-9 (gelatinase B). Although gelatinases have similar substrate specificities, there are differences in their mechanism of regulation. MMP-2 is constitutively expressed by most cell types and is usually not induced by cytokines or growth factors. In contrast, basal levels of MMP-9 are usually low; its expression can be induced by different cytokines/chemokines including tumor necrosis factor α (TNFα), and it is mainly secreted by inflammatory cells. In addition to degradation of the extracellular matrix, gelatinases can regulate inflammation by processing cytokines/chemokines, with MMP-9 having stimulatory effects and MMP-2 having inhibitory effects on inflammation (13–16).

MMP-9 is markedly elevated in the sera and joints of RA patients (7,17,18) and correlates positively with disease progression and severity (7,18). MMP-9–knockout mice show reduced severity of antibody-induced arthritis (10). Although MMP-2 is elevated in the arthritic joint, MMP-2–knockout mice have exacerbated antibody-induced arthritis, indicating that MMP-2 may play a suppressive role in the progression of arthritis (10). The differential regulation of MMP-2 and MMP-9, therefore, may be a useful therapeutic target in the treatment of RA.

Activated protein C (APC) is a natural anticoagulant derived from its vitamin K–dependent plasma precursor protein C. Activation of protein C occurs on the endothelial cell surface and is triggered by a complex formed between thrombin and thrombomodulin. The conversion to APC is augmented in the presence of the specific receptor endothelial protein C receptor (EPCR) (19), which is expressed on the surface of endothelial cells, keratinocytes (20), and some leukocytes, including neutrophils and monocytes (21). In addition, APC is now recognized as playing a key role in the regulation of inflammation by down-regulating leukocyte activation and inflammatory cytokine production (22). Many of the antiinflammatory properties of APC are mediated through EPCR, which itself is antiinflammatory (23).

APC stimulates the expression and activation of MMP-2 in human keratinocytes, fibroblasts, and endothelial cells. A recent report has shown that APC down-regulates MMP-9 in the ischemic brain and inhibits tissue plasminogen activator–induced brain hemorrhage (24). We have shown that APC is elevated in the joints of patients with RA, particularly in the lining layer, where it colocalizes with MMP-2 (25). However, little is known about the role of APC in RA synovium.

In the present study, using synovial fibroblasts from RA patients, we found that APC inhibits the expression of MMP-9, yet stimulates the expression and activation of MMP-2. This differential effect was confirmed in MonoMac6 (Mac6) cells. Using Mac6 cells and RA monocytes, we further elucidated that APC acts through EPCR and suppression of NF-κB and p38 to inhibit MMP-9.

**MATERIALS AND METHODS**

**Monocyte and fibroblast isolation.** Peripheral blood samples (20 ml per patient) were obtained from 19 patients with RA and 14 patients with osteoarthritis (OA). Monocytes were isolated from whole blood using OptiPrep (Sigma, St. Louis, MO) according to the manufacturer’s instructions. Viability of monocytes was determined by the trypan blue exclusion test. Purity of monocytes was determined by immunostaining using anti-CD68 antibody (Dako, Carpinteria, CA). Cells were used for further experiments if both viability and purity were >95%.

Human synovial fibroblasts were isolated from synovial fluid that had been aspirated from the joints of patients with RA and OA. Briefly, fluid was collected in heparinized syringes and centrifuged at 300g for 15 minutes. The cell pellet was resuspended and cultured in growth medium (Dulbecco’s modified Eagle’s medium [DMEM] with 10% fetal calf serum [FCS]). Normal fibroblasts isolated from human foreskins were also prepared. Cells from passage 1 through passage 3 were used in the experiments. These cells were morphologically identified as fibroblasts.

Usage of human tissues was in accordance with the Ethics Committee of the Royal North Shore Hospital. All patients fulfilled the American College of Rheumatology (formerly, the American Rheumatism Association) criteria for RA (26) or OA (27) and gave their written informed consent.

**Cell culture and treatment.** Human monocytes and Mac6 cells, a cell line characteristic of mature monocytes (28), were maintained in RPMI 1640 containing 10% FCS and were supplemented with 2 mM L-glutamine, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. Before treatment, cells were preincubated for 2 hours in either DMEM or RPMI 1640 with 2% MMP-free FCS (basal medium) and then transferred to a fresh basal medium. Cells were treated with recombinant human APC (Xigris; Eli Lilly, Indianapolis, IN), RCR252 (EPCR blocking antibody), RCR92 (EPCR nonblocking antibody) (both anti-EPCR antibodies provided by Professor K. Fukudome, Department of Immunology, Saga Medical School,
Expression of matrix metalloproteinase 2 (MMP-2) and MMP-9 by rheumatoid arthritis (RA), osteoarthritis (OA), and normal neonatal foreskin fibroblasts. Passage 1 (P1) or passage 3 (P3) fibroblasts from RA and OA patients and normal neonatal foreskins were treated with activated protein C (APC; 1, 10, or 20 μg/ml) for 24 hours. MMP-2 and MMP-9 were detected in culture supernatants by A, zymography and B, enzyme-linked immunosorbent assay. Gel images are from 1 representative experiment of 3 experiments performed using cells from different patients. Values in B are the mean and SD of 5 patients. * = P < 0.05; ** = P < 0.01 versus passage 1 RA fibroblasts without APC treatment.
membranes were incubated for 1 hour with appropriate horse-radish peroxidase–conjugated secondary antibody. Immunoreactivity was detected using the enhanced chemiluminescence detection system (Amersham, Piscataway, NJ). Anti-human β-actin antibody was used to normalize the equal protein loading.

Statistical analysis. Significance was determined using one-way analysis of variance and Student-Newman-Keuls test. P values less than 0.05 were considered statistically significant.

RESULTS

Differential regulation of MMP-2 and MMP-9 in RA fibroblasts after APC treatment. Gelatinase secretion by fibroblasts was measured by zymography and ELISA, and the results are shown in Figure 1. Under normal growth conditions, RA synovial fibroblasts from passage 1 secreted substantial amounts of both MMP-9 and MMP-2. OA synovial fibroblasts from passage 1 also secreted abundant MMP-2; however, MMP-9 was barely detectable. Normal fibroblasts (passage 1) from neonatal foreskins showed a gelatinase profile similar to that of the OA cells. There was little difference in the secretion of MMP-2 among the 3 types of fibroblasts.

The addition of APC increased in a dose-dependent manner the expression and activation of MMP-2 in passage 1 RA fibroblasts (Figure 1A). Similarly, APC (20 μg/ml) increased MMP-2 activity in culture medium of OA and normal skin fibroblasts (Figure 1A). In contrast, APC decreased in a dose-dependent manner the levels of MMP-9 in culture supernatants from RA synovial fibroblasts (Figures 1A and B). Interestingly, MMP-9 secretion by RA fibroblasts was passage-dependent, decreasing rapidly with passage in culture, and was barely detected in passage 3 cells (Figures 1A and B). There was no significant difference in TIMP-1 levels in response to APC treatment in either RA or OA cells (data not shown).

Figure 2. Increased expression of matrix metalloproteinase 9 (MMP-9) by monocytes from rheumatoid arthritis (RA) patients and inhibition by activated protein C (APC). Expression of A, MMP-9 and B, tissue inhibitor of metalloproteinases 1 (TIMP-1) by monocytes from RA and osteoarthritis (OA) patients was measured by enzyme-linked immunosorbent assay (ELISA). MMP-9 expression by RA monocytes in response to increasing concentrations of APC (0.1, 1, 10, or 20 μg/ml) was measured by C, ELISA and D, zymography. Values in A–C are the mean and SD. * = P < 0.05 versus RA monocytes in A and versus RA monocytes without APC in C; ** = P < 0.01 versus untreated control RA monocytes in B and versus RA monocytes without APC in C.
Attenuated expression of MMP-9 in RA monocytes and Mac6 cells after APC treatment. To help elucidate the mechanism of the differential effects of APC on MMP-2 and MMP-9, we used circulating monocytes from RA and OA patients, which produce abundant MMP-9 regardless of time in culture. Monocytes from RA patients secreted higher levels of MMP-9 than did those from OA patients, as assessed by ELISA (Figure 2A). In contrast, monocytes from OA patients secreted more than 5-fold higher levels of TIMP-1 than did RA monocytes, as measured by ELISA (Figure 2B). There was no significant difference in TIMP-1 levels in either cell type in response to APC treatment (Figure 2B).

APC dose-dependently (0.1–20 µg/ml) inhibited the amount of MMP-9 in the supernatants of RA monocytes (Figures 2C and D). At 20 µg/ml, APC reduced the levels of MMP-9 by ~50% (Figure 2C). Zymographic analysis of culture supernatants confirmed the reduction of MMP-9 by APC and demonstrated that the enzyme was present in its latent form (proMMP-9) (Figure 2D).

Since RA monocytes do not normally produce MMP-2, the Mac6 cell line, which is characteristic of mature monocytes (28) and produces both MMP-2 and MMP-9, was used in some experiments to elucidate the mechanisms of the differential effects of APC on these 2 gelatinases. APC diminished the low basal levels of MMP-9 produced by Mac6 cells, whereas protein C, the inactive precursor, had no effect (Figures 3A and B). The addition of TNF-α to Mac6 cells stimulated MMP-9 secretion by ~5-fold. APC treatment (20 µg/ml) reduced the TNF-α-enhanced levels of MMP-9 by more than 35% (Figure 3A). Similar to its effect on RA fibroblasts (Figure 1), APC consistently increased MMP-2 activity in Mac6 cells (Figure 3B).

To assess whether APC inhibits MMP-9 gene expression, Mac6 cells were treated with 20 µg/ml of
APC for 2 hours and real-time reverse transcription-PCR was used to detect MMP-9 mRNA. APC reduced MMP-9 gene levels by ~20% and ~35% compared with control and TNF-α-stimulated cells, respectively (Figure 3C). APC also inhibited MMP-9 mRNA in early-passage RA fibroblasts (data not shown). To examine whether APC may also directly degrade MMP-9, Mac6 cells were stimulated with TNF-α for 24 hours, and the cell-free medium containing proMMP-9 was collected and incubated with APC in the absence of cells for a further 24 hours. Results of zymography showed that APC activated MMP-2 in a dose-dependent manner but had no direct effect on proMMP-9 at any concentration tested (Figure 3D). Taken together, these results indicate that APC reduces MMP-9 levels by preventing its production at the gene level, and not by degrading the enzyme.

**Involvement of TNF-α, NF-κB, and p38 in the inhibition of MMP-9 by APC.** TNF-α is a predominant mediator of inflammation in RA that is known to stimulate MMP-9 production. A previous study showed that APC inhibits TNF-α production by human monocytes (30), although there have been no reports of studies using activated RA monocytes. We examined the effect of APC on TNF-α production by RA monocytes, as determined by ELISA. Under basal conditions, APC reduced TNF-α in supernatants from Mac6 cells by 25% and by 35% in supernatants from RA monocytes (Figure 4A).

We further examined the involvement of NF-κB in the inhibition of MMP-9 by APC. NF-κB is a major inflammatory signaling molecule in numerous cell types; it is known to be inhibited by APC and activated by TNF-α. In order to achieve this, 2 approaches were taken. First, Western analysis, using an antibody that recognizes the active form of NF-κB, was used to measure the activation of NF-κB in Mac6 cells in response to APC. The results showed that APC suppressed the activation of NF-κB in a dose-dependent
manner (Figure 4B). Second, Mac6 cells were treated with 6-AQ, a specific inhibitor of NF-κB activation, for 24 hours, and culture supernatants were assayed for MMP-9 by ELISA and zymography. Treatment with 6-AQ inhibited TNFα-stimulated MMP-9 production in a dose-dependent manner (Figure 4C). Zymographic analysis showed that this inhibition was specific for MMP-9, since MMP-2 was not affected (Figure 4D). Together, these results suggest that inhibition of MMP-9 by APC may occur via inhibition of TNFα production and inactivation of NF-κB.

Like NF-κB, the MAP kinase family member p38 is one of the major signals for TNFα-mediated inflammatory responses and plays a critical role in the development and progression of arthritis (31,32). We examined whether p38 was involved in APC-induced MMP-9 inhibition. Mac6 cells were treated with TNFα alone or in the presence of APC for 24 hours, and phosphorylated p38 was measured by Western blotting and gelatinases were detected by zymography. APC markedly inhibited TNFα-stimulated p38 activation (Figure 5A). An inhibitor of p38 almost completely suppressed MMP-9 production under basal conditions and in response to TNFα, but had little effect on MMP-2 (Figure 5B). The effect of the p38 inhibitor was similar to that exerted by 10 μg/ml of APC (Figure 5B). Together, these results indicate that APC inhibits MMP-9 via inactivation of p38.

**Figure 5.** Role of p38 in the suppression of matrix metalloproteinase 9 (MMP-9) by activated protein C (APC). A, Mac6 cells were treated with APC and tumor necrosis factor α (TNFα), and levels of phosphorylated p38 (P-p38) were measured by Western blotting. β-actin was used to normalize the equal protein loading. B, Mac6 cells were treated with combinations of APC, TNFα, and p38 inhibitor for 24 hours, and MMP-2 and MMP-9 were detected in cell supernatants by zymography. Images are from 1 representative experiment of 3 experiments performed.

**Figure 6.** Endothelial protein C receptor–dependent inhibition of matrix metalloproteinase 9 (MMP-9) and NF-κB in monocytes after treatment with activated protein C (APC). A, Monocytes from rheumatoid arthritis patients were treated with APC in the presence or absence of RCR252 for 24 hours, and MMP-9 was detected by zymography. B, Mac6 cells were treated with combinations of APC (20 μg/ml), tumor necrosis factor α (TNFα; 100 ng/ml), RCR252 (10 μg/ml), and RCR92 (10 μg/ml) for 24 hours, and MMP-9 was detected by zymography. C, Mac6 cells were treated with combinations of APC (20 μg/ml), TNFα (100 ng/ml), and RCR252 (10 μg/ml) for 24 hours, and the active form of NF-κB in whole cell lysates was detected by Western blotting. Images are from 1 representative experiment of 3 experiments performed.
cells, RCR252 reversed the APC-induced inhibition, whereas RCR92, the nonblocking control antibody to EPCR, had no effect on MMP-9 (Figure 6B). Neither antibody affected the activation of MMP-2 induced by APC. In addition, the inhibitory effect of APC on NF-κB activity in Mac6 cells was completely reversed by RCR252 (Figure 6C), but not RCR92 (data not shown).

DISCUSSION

A major finding from the current study is that APC attenuates gene and protein expression of MMP-9 in circulating monocytes and synovial fibroblasts from RA patients. MMP-9 plays a critical role in the invasion of the synovial tissue, leading to cartilage destruction and bone erosion in RA (7,10,33,34). The importance of this enzyme in arthritis is highlighted by the finding that MMP-9–knockout mice display less severe antibody-induced arthritis than do wild-type mice (10). MMP-9 is implicated in the degradation of basement membrane collagen, synovial membrane neovascularization, transfer of endothelial and hemopoietic stem cells from a quiescent to a proliferative state, and promotion of leukocyte migration from the vascular compartment to sites of inflammation (7,35–37). In the RA synovial joint, levels of leukocyte-derived MMP-9 are directly related to the degree of inflammation. The proinflammatory effects of MMP-9 appear to be due to its ability to process cytokines and chemokines. MMP-9 can cleave IL-8 to potentiate its activity as a neutrophil chemoattractant by at least 10-fold (18), and segment proIL-1β and proTNFα into their active proinflammatory forms (13,16).

TIMPs act as key inhibitors of MMPs in tissue by binding to the active site and forming stable, but inactive, enzyme-inhibitor complexes (38). TIMPs differ in their specificity for gelatinase inhibition, with TIMP-2 having higher affinity for MMP-2 and TIMP-1 preferentially binding to MMP-9. In the current study, higher levels of TIMP-1 were secreted by OA monocytes than by RA monocytes. APC treatment had no significant effect on TIMP-1 expression on either cell type, which suggests that the reduction in MMP-9 gene and protein expression by APC observed in this study represents potential suppression of MMP-9 activity.

Early-passage RA fibroblasts constitutively produced high levels of MMP-9, which was not secreted by normal or OA fibroblasts (Figure 1). Real-time PCR confirmed that MMP-9 was synthesized by these cells and not simply released from cellular storage reservoirs (data not shown). A striking finding of our study was the rapidity of the loss of MMP-9 expression in culture. MMP-9 secretion was restricted to very early-passage cells, decreased rapidly as the cells were passaged, and was barely detectable by passage 3. Rapid loss of MMP-9 secretion has been observed in other cell types, including human endothelial cells (39). In that study, abrogation of MMP-9 secretion by late-passage cells, with no change in MMP-2 secretion, was also observed, similar to the findings of the present study.

The specificity of the inhibitory effect of APC on MMP-9 is evidenced by the opposing effects it exerted on MMP-2. APC stimulated the expression and activation of MMP-2 in RA fibroblasts and Mac6 cells, which is consistent with the previous findings using endothelial cells, normal fibroblasts, and keratinocytes (40,41). Interestingly, in RA monocytes, which do not normally produce MMP-2, APC is unable to induce this enzyme. Although the 2 gelatinases have similar matrix substrate specificity, they vary considerably in their non–matrix-degrading activities. In contrast to the proinflammatory action of MMP-9, MMP-2 exerts antiinflammatory properties by degrading mediators of inflammation (14,15). A number of nonmatrix MMP substrates that potentiate cellular functions, such as cytokines, growth factor receptors, and chemokines, have been identified. For example, MMP-2 efficiently cleaves and inactivates monocyte chemoattractant protein 3, a CC chemokine that promotes monocyte chemotaxis. This action of MMP-2 was shown to result in not only blocking of the initiation of an in vivo inflammatory response, but also complete abrogation of preexisting inflammation in the paws of mice (15). Itoh et al (10) have proposed that although levels of MMP-2 are elevated in the arthritic joint, the matrix-degrading ability of MMP-2 appears to have little effect on the development of arthritis, but instead, it prevents inflammation by cleaving proinflammatory factors. Thus, APC has the potential to exert strong antiinflammatory effects first by inhibiting MMP-9 and second by promoting MMP-2 activity.

Many of the antiinflammatory properties of APC are mediated through EPCR, which itself is antiinflammatory. EPCR is a 46-kd type I transmembrane glycoprotein that is homologous to class I major histocompatibility complex/CD1 family of proteins (19,23). It is predominantly expressed by endothelial cells, mainly those of larger blood vessels, although recent reports have shown it to be present on some leukocytes of the innate immune system (21,42), including RA monocytes (Xue M, et al: unpublished observations). Using a blocking antibody, we have shown that EPCR is required
for APC inhibition of MMP-9 in monocytes. EPCR is not known to be a signaling receptor, so the intracellular signaling mechanism for the inhibition of NF-κB is unknown. It is possible that by binding to EPCR, APC promotes the activation of protease-activated receptors (PARs), in particular PAR-1, which contributes to the anti-inflammatory actions of APC (43).

NF-κB is a transcription factor that regulates the expression of genes involved in immune and inflammatory responses and plays a pivotal role in the regulation of inflammation (44). Direct inhibition of NF-κB by mutated IκB proteins was shown to be sufficient to block generalized symptoms of the inflammatory arthritis and to specifically arrest both bone erosion and inflammatory arthritis in a mouse model (45). The promoter region of MMP-9 possesses several functional enhancer element–binding sites, such as NF-κB and AP-1 sites. These sites make MMP-9 susceptible to induction by proinflammatory cytokines, particularly TNFα, a key mediator in the pathogenesis and maintenance of rheumatoid synovitis. Previous studies have supported the role of NF-κB in the TNFα-stimulated production of MMP-9 in a number of cell types, including normal human fibroblasts (46), human macrophages (47), and rat chondrocytes (48). The current study demonstrates that APC inhibits TNFα production and NF-κB activation in unstimulated and/or stimulated monocytes. Additionally, specific inhibition of NF-κB prevented TNFα-stimulated MMP-9 production. These data suggest that inhibition of MMP-9 by APC is likely to be mediated by suppression of TNFα and inactivation of NF-κB.

Other agents have recently been shown to inhibit MMP-9 via NF-κB in different cell types. The flavonoids wogonin was shown to suppress TNFα-induced MMP-9 expression by blocking NF-κB activation via MAP kinase signaling pathways in human aortic smooth muscle cells (49,50). NF-κB p65–specific small interfering RNA was shown to inhibit the expression of genes, including MMP-9, in IL-1β–induced and TNFα–induced chondrocytes in rats (48). To date, none of these agents have been shown to promote MMP-2 activity. Thus, APC appears to be unique, in that it not only inhibits MMP-9 via NF-κB, but it also stimulates MMP-2 activity.

The MAP kinase family member p38 is a major signaling molecule of inflammation and is considered to be one of the most important signals for TNFα-mediated inflammatory responses (31,32). Inhibitors of p38 have been successfully tested in animal models of arthritis, such as collagen-induced arthritis (32). Inhibition of p38 MAP kinase diminishes clinical signs and all major histopathologic features of chronic arthritis, including synovial inflammation, cartilage damage, and bone loss (31). Effective blockade of p38 MAP kinase might therefore be regarded as a therapeutic option for the protection of joints from inflammatory destruction. In the current study, APC prevented TNFα-stimulated activation of p38, and p38 inhibition abolished TNFα-stimulated MMP-9, thus emphasizing the anti-inflammatory actions of APC.

This study has revealed a novel differential action of APC on the 2 members of the gelatinase family of MMPs. APC appears to be the first physiologic agent that can inhibit MMP-9 while at the same time promoting MMP-2 activity. We provide evidence that the inhibitory effect of APC on MMP-9 occurs through inactivation of 2 mediators of inflammation, NF-κB and p38, after binding to its specific receptor EPCR. These results suggest that APC may be beneficial in the prevention of inflammation in RA.

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AUTHOR CONTRIBUTIONS

Dr. Xue had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Xue, Jackson.

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Development of an Ex Vivo Cellular Model of Rheumatoid Arthritis

Critical Role of CD14-Positive Monocyte/Macrophages in the Development of Pannus Tissue

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Objective. To establish an ex vivo cellular model of pannus, the aberrant overgrowth of human synovial tissue (ST).

Methods. Inflammatory cells that infiltrated pannus tissue from patients with rheumatoid arthritis (RA) were collected without enzyme digestion, and designated as ST-derived inflammatory cells. Single-cell suspensions of ST-derived inflammatory cells were cultured in medium alone. Levels of cytokines produced in culture supernatants were measured using enzyme-linked immunosorbent assay kits. ST-derived inflammatory cells were transferred into the joints of immunodeficient mice to explore whether these cells could develop pannus. CD14 and CD2 cells were depleted by negative selection.

Results. Culture of ST-derived inflammatory cells from 92 of 111 patients with RA resulted in spontaneous reconstruction of inflammatory tissue in vitro within 4 weeks. Ex vivo tissue contained fibroblasts, macrophages, T cells, and tartrate-resistant acid phosphatase-positive multinucleated cells. On calcium phosphate-coated slides, ST-derived inflammatory cell cultures showed numerous resorption pits. ST-derived inflammatory cell cultures continuously produced matrix metalloproteinase 9 and proinflammatory cytokines associated with osteoclastogenesis, such as tumor necrosis factor α, interleukin-8, and macrophage colony-stimulating factor. More importantly, transferring ST-derived inflammatory cells into the joints of immunodeficient mice resulted in the development of pannus tissue and erosive joint lesions. Both in vitro development and in vivo development of pannus tissue by ST-derived inflammatory cells were inhibited by depleting CD14-positive, but not CD2-positive, cells from ST-derived inflammatory cells.

Conclusion. These findings suggest that overgrowth of inflammatory cells from human rheumatoid synovium simulates the development of pannus. This may prove informative in the screening of potential antirheumatic drugs.

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by the overgrowth of synovial tissue (ST), called pannus, which leads to the destruction of articular cartilage and subchondral bone (1,2). Although various inflammatory cell populations and their interactions contribute to the pathogenesis of pannus growth in rheumatoid synovitis, monocyte-derived osteoclasts and fibroblast-like synoviocytes (FLS) are thought to play a critical role in the destruc-
tion of joints by pannus tissue (3–5). Osteoclasts are associated with bone destruction in RA joints (6). Their origin in rheumatoid joints has not yet been identified, but findings of previous studies have indicated that bone marrow-derived CD14-positive cells are responsible for the development of osteoclasts and that both monocytes and dendritic cells can differentiate into functional osteoclasts (7–10). Osteoclast formation requires cell–cell interaction between osteoblasts and osteoclast precursors in bone remodeling.

FLS from rheumatoid synovium have the potential to stimulate macrophages to differentiate into osteoclasts in the absence of any bone or bone marrow-derived cells (4,9). To this end, binding of RANKL expressed on the surface of fibroblasts to the surface of osteoclast precursors promotes the differentiation of these cells to mature cells (11–17). Proinflammatory cytokines produced by rheumatoid synoviocytes, such as tumor necrosis factor α (TNFα), interleukin-6 (IL-6), and macrophage colony-stimulating factor (M-CSF), also promote osteoclastogenesis and bone resorption in inflamed joints (4,12,18–21).

Although there are several experimental models of arthritis, they are far from accurate representations of human disease. Importantly, while numerous drugs for RA have shown great efficacy in animal models, this benefit has not been realized in patients. Adjuvant-induced arthritis (AIA) has been the benchmark for the evaluation of nonsteroidal antiinflammatory drugs (NSAIDs). Cyclooxygenase (COX) inhibitors can control AIA, but in human disease the antiinflammatory and analgesic actions have far less effect on the progression of erosive arthritis. Collagen-induced arthritis (CIA) in rats is weakly responsive to NSAIDs or methotrexate, and is unaffected by gold salts or chloroquine. Cyclosporine effectively inhibits the progression of AIA, pristane-induced arthritis, proteoglycan-induced arthritis, and streptococcal cell wall–induced arthritis (22), but its efficacy in humans is more limited. Thus, important discrepancies between human arthritis and animal models of arthritis impede understanding of the pathogenesis of RA and efforts for reliable screening of potential antirheumatic drugs.

To address this, we developed a method of obtaining ST-derived inflammatory cell populations, without enzyme digestion, and established their mixed cell culture system in vitro to reproduce the morphologic and functional features of pannus tissue seen in RA, such as the development of pannus-like inflammatory tissue, cytokine production, and osteoclastic activity. We believe this system represents an informative model of RA and may turn out to be a useful tool for studying the pathophysiology of RA and for screening potential antirheumatic drugs.

**MATERIALS AND METHODS**

**Reagents.** Methotrexate, gold sodium thiomalate, and a tartrate-resistant acid phosphatase (TRAP) kit were purchased from Sigma (St. Louis, MO).

**Synovial tissue specimens.** ST specimens were obtained from 111 patients who fulfilled the American College of Rheumatology (formerly, the American Rheumatism Association) criteria for RA (23) and from 5 patients with osteoarthritis (OA) undergoing joint replacement surgery. In compliance with institutional policies, informed consent was obtained from all patients. The study was approved by the ethics committee of each institution.

**Isolation of ST-derived inflammatory cells.** ST specimens were cut into small pieces and cultured in 100-mm dishes containing RPMI 1640 (Asahi Technoglass, Chiba, Japan) with 10% fetal calf serum (FCS) and 1,000 units/ml of penicillin G sodium–streptomycin sulfate (Gibco BRL, Grand Island, NY) for 1–3 days in a CO2 incubator (5% CO2, 100% humidity at 37°C). During incubation, the tissue-infiltrating cells migrated and grew out of the tissue and onto the surface of the culture dish. After 1–3 days’ incubation, tissue was removed and single cells were collected by vigorous pipetting. Tissue debris was removed by passing material through a 70-μm cell strainer (Falcon, Oxnard, CA). Cell suspensions were washed once by centrifugation at 1,500 revolutions per minute for 10 minutes at 4°C. Cell suspensions were then layered on 3 ml of Lymphocyte Separation Solution (Nacalai Tesque, Kyoto, Japan) with 10 ml phosphate buffered saline (PBS) and centrifuged at 1,500 rpm for 10 minutes at 4°C. The interface layer was suspended in 50 ml PBS, washed twice by centrifugation at 1,500 rpm for 10 minutes at 4°C, and used as the ST-derived inflammatory cells.

**In vitro reconstruction of inflammatory tissue by ST-derived inflammatory cells.** ST-derived inflammatory cells were seeded at a density of 5 × 105/well in 48-well culture plates and cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco BRL) containing 10% FCS and 1,000 units/ml of penicillin G sodium–streptomycin sulfate. The culture was observed for morphologic changes under an inverted phase-contrast microscope every 3–4 days for 4 weeks. Morphologic changes were scored on a scale of 0–4, as described below. Half of the supernatants were collected every 3–4 days and replaced with fresh medium. Supernatants were frozen at −80°C until assayed. After 4 weeks, the tissue specimens grown in vitro were either fixed in 10% neutral buffered formalin or embedded in TissueTek embedding medium (Sakura, Tokyo, Japan) and snap-frozen.

**FLS culture.** ST-derived inflammatory cells were cultured in 100-mm dishes containing 10 ml of DMEM/10% FCS at a density of 5 × 105/dish. All supernatants were replaced with fresh medium once weekly. Nonadherent cells were removed during the replacement of media. Just before becoming confluent, the adherent cells were collected using 1% trypsin and washed. They were cultured for 1 week in 100-mm dishes containing 10 ml of DMEM/10% FCS at a density of 5 × 105/dish. The adherent cells obtained after 4–6 passages...
with this protocol were used as the FLS. FLS were cultured in 48-well culture plates containing 1 ml of medium/well at a density of 5 × 10^5/well.

**Isolation of subpopulations from ST-derived inflammatory cells.** A mixture of ST-derived inflammatory cells and neuraminidase-treated sheep red blood cells was incubated for 10 minutes at 37°C in a shaking bath and for 1 hour on ice. The cell mixture was layered onto 3 ml of Lymphocyte Separation Solution and centrifuged at 1,500 rpm for 15 minutes at 4°C. The interface layer was resuspended in 50 ml of PBS, washed twice, and used as the non–T cell population. A specific cell surface marker–negative cell population was isolated from the ST-derived inflammatory cells, using specific monoclonal antibodies (mAb) against human CD3, CD4, CD8, and CD14 and Dynabeads, according to the recommendations of the manufacturer (Dynal, New Hyde Park, NY).

**Immunohistochemical staining.** Serial 5-μm sections of paraffin-embedded tissue specimens were cut using a cryostat and air-dried. For immunohistochemical staining, sections were fixed in 4% paraformaldehyde. Monoclonal antibodies against human CD3, CD45RO, CD68 (all from Dako, Hamburg, Germany), vimentin (Genzyme, Cambridge, MA), and human platelet-derived growth factor receptor (PDGFR; Genzyme) were used for staining tissue. Tissue was also stained for TRAP.

**Bone resorption assay.** ST-derived inflammatory cells were incubated for 2 weeks in α-minimum essential medium supplemented with 1% FCS. Mature osteoclasts were identified as TRAP-positive multinucleated cells containing ≥3 nuclei. ST-derived inflammatory cells were seeded (1 × 10^5/well) onto calcium phosphate–coated slides (Osteologic; BD Biosciences, Bedford, MA) and incubated in RPMI 1640 with 1% FCS, 50 μg/ml ascorbic acid (Sigma) and 10 mM β-glycerophosphate (Sigma) for 7–14 days in a CO_2 incubator (5% CO_2, 100% humidity at 37°C). Half of the supernatants were replaced with fresh medium once weekly. The calcium phosphate–coated slides were washed with distilled water and 10% sodium hypochlorite and then air-dried. Resorption pits were counted under a microscope.

**Reproduction of pannus by ST-derived inflammatory cells in vivo.** BALB/cA-bg, SCID (SCID.bg) mice and BALB/cA-rag-2–deficient mice were obtained from the Central Institute for Experimental Animals. ST-derived inflammatory cells were resuspended in normal saline at 2 × 10^7/ml. Fifty microliters of ST-derived inflammatory cells (1 × 10^6 cells/50 μl) was injected, with a 23-gauge needle under sterile laminar flow, into the right knee joints of 6-week-old male mice. Four hundred fifty microliters of ST-derived inflammatory cells was injected subcutaneously into the right dorsal pedis. Mice were housed in sterilized microbarrier units under specific pathogen–free conditions at the Central Institute for Experimental Animals. Mice were killed after 7–8 weeks. Their joints were removed, snap-frozen, and fixed in 10% neutral buffered formalin for paraffin embedding. They were further decalcified.

*Figure 1.* Spontaneous development of pannus-like inflammatory tissue in vitro in synovial tissue (ST)–derived inflammatory cells from rheumatoid arthritis (RA) patients. Cells were cultured in medium alone in 48-well plates at 5 × 10^5 cells/ml/well. Morphologic changes were scored semiquantitatively on a scale of 0–4 according to the degree of tissue development, as described in Materials and Methods. a, ST-derived inflammatory cells at the beginning of culture. b, Aggregation of ST-derived inflammatory cells, resulting in development of foci within a few days (score 1). c, Continuing growth of ST-derived inflammatory cells (score 2). d, Three-dimensional growth of ST-derived inflammatory cells (score 3). e, Higher-magnification view of the boxed area in d, revealing numerous giant cells among small lymphocytes and fibroblast-like synoviocytes. f, Macroscopic tissue resulting from the mixed culture of ST-derived inflammatory cells after 2–4 weeks (score 4). This macroscopic tissue growth (score 4) was reproduced in primary synoviocytes obtained from 37 of 111 patients with RA. (Original magnification × 100 in a–d; × 400 in e; × 40 in f.)
with 0.5M EDTA (pH 7.4), sectioned, and stained with hematoxylin and eosin (H&E).

**Cytokine assay.** ST-derived inflammatory cells were seeded in 24-well culture plates (1 × 10⁶/well) and cultured in DMEM/10% FCS. Half of the supernatants were collected 3 times per week and replaced with fresh medium. Supernatants were frozen at −80°C until assayed, and levels of TNFα, M-CSF, IL-6, and IL-8 (all from R&D Systems, Minneapolis, MN) and matrix metalloproteinase 9 (MMP-9; Amersham Biosciences, Roosendaal, The Netherlands) released into the culture supernatants were measured using enzyme-linked immunosorbent assay kits, according to the manufacturers’ recommendations.

**RESULTS**

**In vitro tissue growth by the mixed culture of ST-derived inflammatory cells.** Microscopic observation of ST-derived inflammatory cells after overnight culture revealed various populations of inflammatory cells, including lymphocytes, FLS, macrophages, dendritic cells, and endothelial cells, as determined by morphologic assessment (Figure 1a). When cultured in either RPMI 1640 or DMEM/10% FCS, ST-derived inflammatory cells started to aggregate, forming foci within a few days (Figure 1b). Further culturing resulted in 3-dimensional (3-D) growth (Figure 1d). Higher magnification revealed the accumulation of large cells among FLS and small lymphocytes (Figure 1e), which ultimately produced macroscopic tissue ≥2 mm in size within 4 weeks (Figure 1f).

Morphologic changes were semiquantitatively scored on a scale of 0–4, according to the degree of tissue development, where 0 = no cellular foci or

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**Figure 2.** Histologic assessment of pannus-like inflammatory tissue that developed spontaneously in vitro upon culture. a, Histologic features of tissue developed in vitro and evaluated after 6 weeks of culture revealed inflammatory cell infiltration within a large number of extracellular matrices. b–d, Immunohistochemical staining with antivimentin (b), anti-CD68 (c), and anti-CD45RO (d) revealed long-term survival of macrophages and T cells within the tissue. (Original magnification × 10 in a; × 100 in b–d.)
aggregates (Figure 1a), 1 = formation of cellular foci or aggregates (Figure 1b), 2 = further growth of cellular aggregates (Figure 1c), 3 = further 3-D growth with a multilayered structure (Figures 1d and e), and 4 = development of macroscopic tissue (Figure 1f). The time course of tissue development and the maximum score reached during the 4-week culture varied among donors. Nevertheless, in vitro tissue development with a score of \( \geq 3 \) was reproduced by ST-derived inflammatory cells from 92 of 111 patients with RA.

The in vitro development of tissue was not reproduced by synovial cells obtained using conventional methods, with the enzymatic treatment of ST using DNase and collagenases. Culture of these cells resulted in a confluent single layer with FLS (results not shown). Synovial cells obtained from 5 patients with OA failed to reproduce tissue development (results not shown).

**Histologic assessment of tissue grown in vitro.**

H&E staining of tissue developed in vitro (Figure 1d) revealed inflammatory tissue with cellular infiltration. Immunohistochemical staining showed the presence of vimentin-positive cells (Figure 2b), CD68-positive macrophages (Figure 2c), and CD45RO-positive T cells (Figure 2d). It appeared that vimentin-positive cells aggregated toward the center, with CD68-positive cells toward the exterior. This arrangement bears some similarity to

Figure 3. Development of tartrate-resistant acid phosphatase (TRAP)-positive cells with osteoclastic activity from in vitro culture of synovial tissue (ST)-derived inflammatory cells. ST-derived inflammatory cells (a and b) and fibroblast-like synoviocytes (c and d) were cultured in medium containing 1% fetal calf serum for 2 weeks and examined for the development of TRAP-positive multinucleated cells (a and c) and resorption pits (b and d), as described in Materials and Methods. Results are from a representative experiment. (Original magnification × 400 in a and c; × 40 in b and d.)
rheumatoid pannus. Of interest, monocyte/macrophages and T cells survived in vitro for >4 weeks without any stimulation. Tissue remained viable in culture for ≥5 months (results not shown).

Development of osteoclastic activity by ST-derived inflammatory cells. Culture of ST-derived inflammatory cells in RPMI/1% FCS resulted in the appearance of giant TRAP-positive multinucleated cells within 2 weeks. (Figure 3a). When incubated on a calcium phosphate–coated slide, ST-derived inflammatory cells developed numerous resorption pits without exogenous addition of M-CSF or soluble RANKL (Figure 3b). However, purified FLS failed to reproduce the development of TRAP-positive cells or any resorption pits without exogenous addition of M-CSF or soluble RANKL (Figure 3b).

Figure 4. Requirement of CD14-positive cells for the in vitro model of human synovitis. a, Tissue growth scores of synovial tissue (ST)–derived inflammatory cells (STICs), CD2-depleted ST-derived inflammatory cells (CD2-depl), CD14-depleted ST-derived inflammatory cells, and fibroblast-like synoviocytes (FLS). Cells were cultured in vitro, and tissue development was scored on a scale of 0–4, as described in Materials and Methods. b–d, Levels of tumor necrosis factor α (TNFα) (b), interleukin-8 (IL-8) (c), and matrix metalloproteinase 9 (MMP-9) (d) in culture supernatants, as measured by enzyme-linked immunosorbent assay. Bars show the mean. P values are versus ST-derived inflammatory cells, by Mann-Whitney test. NS = not significant.
pits (Figures 3c and d). These results suggested that ST-derived inflammatory cells prepared using the technique described above contained osteoclast precursors and that the culture system supported their osteoclastogenesis. To further confirm this hypothesis, we measured the levels of osteoclastogenesis-associated proinflammatory cytokines and metalloproteinase in the supernatants of ST-derived inflammatory cell cultures.

**Production of osteoclastogenesis-associated cytokines and metalloproteinase by ST-derived inflammatory cells.** Excessive and prolonged release of cytokines, such as M-CSF, TNFα, IL-6, and IL-8, seems to play a role in the pathogenesis of inflammatory bone and joint destruction in RA. MMP-9 is expressed in osteoclasts and subchondral bone lesions in RA. Therefore, we measured the levels of production of these cytokines and enzymes during in vitro tissue growth of ST-derived inflammatory cells. When cultured in DMEM/10% FCS, ST-derived inflammatory cells produced the maximum amount of TNFα (mean ± SD 23.6 ± 19.2 pg/ml) and IL-8 (282 ± 473 ng/ml) within 4 days. Further culture resulted in peak production of M-CSF (mean ± SD 306.3 ± 169.6 pg/ml) after 1 week and MMP-9 (2.1 ± 1.1 ng/ml) after 2 weeks. The level of IL-6 production gradually increased during culture (mean ± SD 80.5 ± 40.3 ng/ml at 3 weeks). Cytokine production was not

**Figure 5.** Histologic features of the knee joints of SCID.bg mice injected with synovial tissue (ST)-derived inflammatory cells from patients with rheumatoid arthritis. ST-derived inflammatory cells were injected into the knee joints and subcutaneously into the paws of SCID.bg mice. After 7 weeks, histologic features of the joints were examined, as described in Materials and Methods. **a,** Pannus tissue extending into the joint space and onto the surface of articular cartilage. **b,** Higher-magnification view of **a,** revealing the rich vasculature of pannus tissue. **c,** Invasion of articular cartilage and subchondral bone by pannus tissue. **d,** Multinucleated giant cells (**arrows**) in the leading edge of pannus tissue invading the articular bone. (Original magnification × 20 in **a,** × 100 in **b** and **c,** × 400 in **d.**)
always correlated with tissue growth score, but ST-derived inflammatory cells that were assigned tissue growth scores of 3 or 4 produced significantly higher levels of MMP-9 than those that failed to develop tissue growth (mean ± SD 672.5 ± 77.1 versus 293.4 ± 68.1 ng/ml; P < 0.001).

Requirement of CD14-positive cells, but not CD2-positive T cells, for tissue growth. After passage 4, purified FLS did not reproduce 3-D growth; rather, their culture resulted in a confluent monolayer in the same culture system. In order to study which cell populations contributed to tissue growth, specific cell populations were depleted from ST-derived inflammatory cells by negative selection. ST-derived inflammatory cells depleted of CD14-positive cells were not able to reproduce the tissue growth that occurred in the original ST-derived inflammatory cells. However, tissue growth was not affected by depleting CD2-positive T cells (Figure 4a). The levels of production of TNFα, IL-8, and MMP-9 in culture supernatants were also reduced in CD14-depleted ST-derived inflammatory cells but not in CD2-depleted cells (Figures 4b–d). These results indicated that CD14-positive monocyte/macrophages were essential for tissue growth in ST-derived inflammatory cells.

Pannus formation and joint destruction caused by injecting ST-derived inflammatory cells into the joints of immunodeficient mice. We next studied whether ST-derived inflammatory cells have the potential to develop pannus formation and to lead to joint destruction in vivo. Figure 5 shows the histologic features of the joints of SCID.bg mice 7 weeks after injection with ST-derived inflammatory cells. Typical pannus tissue extended into joint space and invaded articular bone and cartilage. Immunohistochemical analysis using specific anti-human PDGFR mAb showed positive staining of human PDGFR by pannus-forming cells (results not shown), indicating that pannus tissue developed in SCID mice was of human origin.

Pannus formation and joint erosion were reproduced in mice by injection of ST-derived inflammatory cells from all but 1 of 11 donors (Table 1). Pannus formation was not reproduced in either ST-derived inflammatory cells from 3 patients with OA or peripheral blood mononuclear cells from 3 patients with RA. Furthermore, with injection of purified synovial

Table 1. Incidence of arthritis in mice injected with human cells*

<table>
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<th>% with pannus</th>
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*RA = rheumatoid arthritis; ST = synovial tissue; OA = osteoarthritis; PBMCs = peripheral blood mononuclear cells; FLS = fibroblast-like synoviocytes.
fibroblasts or CD14-depleted ST-derived inflammatory cells from 2 RA patients, pannus tissue growth was not reproduced in the joints of SCID/bg mice. Injection of CD2-depleted ST-derived inflammatory cells did, however, reproduce pannus formation. ST-derived inflammatory cells obtained from the same donor (RA 24) developed pannus without CD14 depletion, but not with CD14 depletion (Table 1). These results support the idea that members of the ST-derived inflammatory cell population, particularly CD14-positive cells, have the potential to develop pannus tissue and osteoclastic activity leading to articular bone erosion both in vitro and in vivo.

DISCUSSION

RA is a chronic inflammatory disorder characterized by the aberrant overgrowth of ST, or pannus, leading to the destruction of articular cartilage and subchondral bone. Many experimental models have been developed to study the mechanisms of rheumatoid synovitis both in vitro and in vivo. Animal models include AIA, CIA, and hu-SCID models (22,24). Furthermore, technological developments in molecular biology, such as transgenic mice and gene-knockout mice, have led to new experimental animal models of arthritis and contributed to the understanding of molecular mechanisms of the disease. These experimental models, however, do not always reproduce the pathophysiology of human RA, and they often display significant discrepancies with clinical observations. For example, anti-CD4 mAb and NSAIDs that have been shown to suppress arthritis in animal models have not been efficacious in the treatment of RA (22,25).

In the present study, we established a 2-step in vitro culture system to reproduce morphologic and functional features of pannus tissue seen in RA. The ST-derived inflammatory cells prepared using this method showed potential to develop both pannus-like overgrowth and osteoclastic bone resorption in vitro and in vivo. ST-derived inflammatory cells in an in vitro culture produced high levels of proinflammatory cytokines associated with osteoclastogenesis, and had the ability to develop osteoclastic activity in vitro. Furthermore, in vitro development of pannus-like tissue growth by ST-derived inflammatory cells responded to treatment with antirheumatic drugs, such as methotrexate, gold sodium thiomalate, leflunomide, and infliximab, in doses comparable with those used clinically, while it was resistant to COX inhibitors and anti-CD4 mAb, which have not been efficacious in the treatment of RA in the clinical setting (Nozaki T, Yamada H: unpublished observations).

The novel aspect of the culture system described herein is the method of preparation of synovial cells. Conventional methods used in the past have entailed enzyme digestion of ST by collagenase and other proteinases in order to release synovial cells from the adhered extracellular matrix (4,9,21). In contrast, we collected inflammatory cells infiltrating synovium after a 2-day culture of tissue. This process likely allowed inflammatory cells to migrate from tissue onto the surface of culture plates and may have prevented cellular damage and changes in extracellular matrix caused by enzyme digestion. When isolated by the conventional method using collagenase, synovial cells proliferated in the culture well, reaching a confluent state rather than forming 3-D growth (results not shown).

Another possible explanation for the difference is the cellular composition of the preparation. Our preparation of ST-derived inflammatory cells contained a fair number of monocytes and lymphocytes compared with conventional methods, in which nonadherent cells are discarded, resulting in the enrichment of FLS. Pannus growth by the synovial membrane seems to require multiple cell–cell interactions among FLS and other inflammatory cells, such as monocyte/macrophages and lymphocytes (3,5). Findings of the present study further confirmed the critical role of CD14-positive cells in the development of pannus-like tissue growth both in vitro and in vivo.

Osteoclastic bone resorption is another important feature of pannus tissue in RA. Histologic analysis has demonstrated an increased level of osteoclastic bone resorption at the bone–pannus interface in RA joints (26). Previous studies have shown that both peripheral blood and synovial monocyte/macrophages have the ability to function as osteoclase precursors and develop osteoclastic activity when cocultured with either CD14-negative synovial cells or FLS in the presence of M-CSF (4,7–10). Cultured synovial fibroblasts from RA patients have been shown to express RANKL and to produce M-CSF, an absolute requirement for osteoclast formation (11,27,28).

Rheumatoid ST contains a number of proinflammatory cytokines which influence osteoclast formation and bone resorption. M-CSF is produced constitutively by synovial fibroblasts from RA patients and, in collaboration with RANKL, contributes to the differentiation of synovial macrophages into osteoclasts (9). M-CSF induces monocytes to express CD16 (Fcγ receptor III) and to produce proinflammatory cytokines such as...
TNFα, IL-1β, IL-6, and IL-8 (29). An increased level of plasma M-CSF in patients with RA is correlated with an increased number of CD14+, CD16+ tissue-infiltrating blood monocytes, which in turn contribute to persistent joint inflammation in RA (30). The CD14+, CD16+ monocytes express high levels of Toll-like receptor 2 (TLR-2) and produce proinflammatory cytokines, and levels of expression of CD16 and TLR-2 are increased in RA monocytes and ST macrophages by incubation with M-CSF (31). Therefore, M-CSF might contribute to the expansion of an inflammatory monocyte subset in arthritis patients.

TNFα, IL-6, and IL-8 have been shown to stimulate osteoclast formation by RANKL-independent mechanisms (19,32,33). Furthermore, it has been reported that the α-chemokine IL-8 is a potent and direct activator of osteoclast differentiation and bone resorption (34). The mechanism of action of this chemokine does not require activation of the RANKL pathway (35).

The ST-derived inflammatory cells in the present study contained both macrophages and FLS and produced high levels of TNFα, IL-6, IL-8, and M-CSF. The spontaneous production of these cytokines may explain the spontaneous development of osteoclast formation and bone resorbing activity without exogenous administration of RANKL or M-CSF.

It has been shown that in primary cultures of adherent rheumatoid synovial cells, MMP-9 production is restricted to macrophages (36). The ST-derived inflammatory cells in the present study showed a sustained increase in levels of MMP-9 in the in vitro culture, and the level of MMP-9 correlated with pannus-like tissue growth in vitro. This finding, in addition to the presence of CD68-positive cells in the tissue grown in vitro after 6 weeks of culture (Figure 2c), indicated an important role of monocyte/macrophages in the development of pannus tissue.

MMP-9 plays an active role in cellular diapedesis, augmentation of cellular invasion, and tissue degeneration via inappropriate turnover of the connective tissue matrix. Elevated levels of MMP-9 have been demonstrated in the synovial fluid of patients with various inflammatory diseases; a positive correlation between the level of MMP-9 in the synovial fluid and disease severity has been shown in patients with RA (37). These data further support the notion that in vitro models used in the present study represents the pathophysiology of rheumatoid synovitis.

Similar in vitro and in vivo models of RA using human cells have been developed previously (38,39). (Schultz and colleagues (38) developed a 3-D in vitro model consisting of RA synovial membrane and articular cartilage explants or interactive RA synovial cell-chondrocyte cultures embedded in 3-D fibrin matrices. Geiler et al (39) introduced an in vivo model using SCID mice for studying the invasive properties of RA synovium in the absence of circulating human blood components. These useful models represent the invasive phenotype of RA synovial cells against articular chondrocytes. However, they do not represent the osteoclastogenic activity of rheumatoid pannus that invades articular bone.

In conclusion, we have established a novel model of RA, which can be useful for both analysis of the pathophysiology of RA and screening of potential anti-rheumatic drugs. Additional studies are needed to further document its potential as a surrogate marker of treatment efficacy in humans.

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AUTHOR CONTRIBUTIONS

Dr. Yamada had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Nozaki, Boumpas, Ozaki, Yamada.

Acquisition of data. Nozaki, Takahashi, Ishii, Endo, Hioki, Mori, Kikukawa, Yamada.

Analysis and interpretation of data. Nozaki, Takahashi, Ishii, Endo, Hioki, Mori, Kikukawa, Boumpas, Ozaki, Yamada.


Statistical analysis. Nozaki, Ishii, Yamada.

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Biologic Treatment of Rheumatoid Arthritis and the Risk of Malignancy

Analyses From a Large US Observational Study

Frederick Wolfe¹ and Kaleb Michaud²

Objective. Induction of malignancy is a major concern when rheumatoid arthritis (RA) is treated with biologic therapy. A meta-analysis of RA biologic clinical trials found a general increased risk of malignancy, but this risk was not found in a large observational study. We undertook this study to assess the risk of malignancy among biologic-treated patients in a large US observational database.

Methods. We studied incident cases of cancer among 13,001 patients during ~49,000 patient-years of observation in the years 1998–2005. Cancer rates were compared with population rates using the US National Cancer Institute SEER (Surveillance, Epidemiology, and End-Results) database. Assessment of the risk of biologic therapy utilized conditional logistic regression to calculate odds ratios (ORs) as estimates of the relative risk, further adjusted for 6 confounders: age, sex, education level, smoking history, RA severity, and prednisone use.

Results. Biologic exposure was 49%. There were 623 incident cases of nonmelanotic skin cancer and 537 other cancers. The standardized incidence ratios and 95% confidence intervals (95% CIs) compared with SEER data were as follows: all cancers 1.0 (1.0–1.1), breast 0.8 (0.6–0.9), colon 0.5 (0.4–0.6), lung 1.2 (1.0–1.4), lymphoma 1.7 (1.3–2.2). Biologics were associated with an increased risk of nonmelanotic skin cancer (OR 1.5, 95% CI 1.2–1.8) and melanoma (OR 2.3, 95% CI 0.9–5.4). No other malignancy was associated with biologic use; the OR (overall risk) of any cancer was 1.0 (95% CI 0.8–1.2).

Conclusion. Biologic therapy is associated with increased risk for skin cancers, but not for solid tumors or lymphoproliferative malignancies. These associations were consistent across different biologic therapies.

Although its mechanism of action is substantially different from that of the immunosuppressive drugs
noted above, biologic therapy results in profound immunomodulation, and there are long-term concerns regarding the risk of cancer following treatment with biologic therapies. These concerns were underscored by a recent meta-analysis of the risk of malignancy in patients treated with infliximab or adalimumab in randomized controlled trials (21). That study found that the pooled odds ratio (OR) for malignancy in biologic- versus non–biologic-treated patients in randomized controlled trials was 3.3 (95% confidence interval [95% CI] 1.2–9.1). A followup report indicated an OR of 2.02 (95% CI 0.95–4.29) when additional trial data were added (22).

Observational studies have addressed the risk of malignancy in persons treated with biologics. Askling et al used the Swedish inpatient registry cited above (2,3) to compare 4,160 tumor necrosis factor (TNF) antagonist–treated patients with 53,067 patients in the inpatient registry. They found that cancer risks in treated patients were “largely similar to those of other patients with RA” and that “the pattern of patients treated with TNF antagonists mirrors those of other contemporary as well as historic RA cohorts” (2).

Geborek et al (23) studied 757 patients treated with etanercept or infliximab between February 1999 and December 2002, along with 800 patients who received conventional antirheumatic treatment, as a comparison cohort. Although they found no increase in solid tumors in anti-TNF– versus non–anti-TNF–treated patients, the investigators identified 5 lymphomas among 757 anti-TNF–treated RA patients (1,603 person-years). Compared with a non–anti-TNF–treated cohort, the relative risk of lymphoma in anti-TNF–treated patients was 4.9 (95% CI 0.9–26.2). In an accompanying editorial, Franklin et al (24) considered methodologic problems with this report, including the possibility of confounding by indication, latency, and a low rate of lymphoma in the control population.

In the present report, we first describe the rates and risks of malignancy in RA, and then we try to resolve the issues raised by the meta-analysis. In contrast to the 2 observational studies cited above, we investigated the risk of malignancy conferred by biologic therapy in a large time-matched contemporary cohort of 13,001 patients with RA, 49% of whom were exposed to biologic therapy.

**PATIENTS AND METHODS**

Participants were members of the US National Data Bank for Rheumatic Diseases (NDB) longitudinal study of RA outcomes who completed semiannual questionnaires in the period 1998 through 2005. NDB participants are recruited on an ongoing basis from the practices of US rheumatologists and are followed up prospectively with semiannual, detailed, 28-page questionnaires, as previously described (25–29). The diagnosis of RA was made by the patients’ rheumatologists.

At each semiannual questionnaire assessment, we recorded demographic, disease severity, treatment, and malignancy variables. Patients reported functional status using the Health Assessment Questionnaire (HAQ) (30,31). We determined pain, global severity, and fatigue by visual analog scales (VAS) (32). The VAS measure 21 points from 0 to 10 at 0.5-unit intervals. To assess RA activity, we computed the Patient Activity Scale by multiplying the HAQ score by 3.33 and then dividing the sum of the VAS pain and global scores and rescaled HAQ score by 3. This yields a 0–10 scale with good psychometric properties (33).

At enrollment, participants reported all previous RA medication use. Thereafter, they reported all medication use and timing of use in the previous 6 months. Participants were categorized as having been treated with a biologic agent if they had ever used infliximab, etanercept, adalimumab, or anakinra. For analysis of malignancy risk, only biologic agents used prior to identification of the malignancy were counted as biologic treatments. Approximately 58% of participants who had received infliximab were enrolled in the NDB as part of an infliximab safety registry.

**Case identification.** The determination of malignancy was based on a formal, written protocol and standardized assessment questionnaires. Patients are contacted by specially trained interviewers whose work is periodically reviewed according to written quality control procedures. In the first step of cancer determination we obtain a report of malignancy, and in the second step we validate the report. Initial reports almost always come from the patient, except in cases when the patient is too ill, and in such instances the initial report may come from a family member or friend.

The question on the NDB semiannual questionnaire that addresses malignancy is as follows (using 2006 as an example date): “Between July 1, 2006 and December 31, 2006 were you told that you had any kind of cancer or malignancy? Yes No. (Please list ALL of the types of cancer diagnosed between July and December on the lines below. For example: leukemia, lymphoma, lung, skin, breast, etc.).”

Following the initial patient report, the NDB conducts a detailed telephone interview with each patient (using a standardized form) and immediately thereafter sends for hospital or medical records. If we cannot contact the patient by telephone, we mail a detailed cancer form for him/her to complete. The interview/form requests specific information about cancer type, dates of cancer diagnosis, type of and response to treatment, reoccurrence, other cancers, and name and address of oncologist. Hospital records are requested for all hospitalizations, and physicians are contacted as necessary.

To determine preexisting malignancy, we make use of the above information. In addition, at enrollment into the NDB, all patients are asked the following question from the NDB enrollment questionnaire: “Have you ever been told that you had any kind of cancer or malignancy? If yes, please list all of the types of cancer that you have ever had, and the year each was diagnosed. For example: leukemia, lymphoma, lung, skin,
biologic agents (Table 4) were/was associated with subsequent
are known to be associated with risk of cancer.
level, smoking history, Patient Activity Scale, and prednisone
variables, we included 6 factors as an a priori set of confound-
cancer studied in the RA sample compared with the US
conditioning variable and performed conditional logistic re-
separately, because the number of patients receiving these
were entered into the model simultaneously. Individ-
cancer, we used the summary trend test (P value for trend)
HCQ 25.2
Sulfasalazine 9.4
MTX 56.9
Prednisone 45.6
Anakinra 0.3
* HAQ = Health Assessment Questionnaire; MTX = methotrexate; HCQ = hydroxychloroquine.
cancer. In addition, for each biologic variable, we created a
on the calculation of incidence rate densities. In Table 1, we report on the total
were less likely to enroll in the NDB. In effect, this required
each specific cancer, we excluded patients with that
control for the potential bias associated with delayed discovery of
cancer by the NDB (35), we excluded data from the most
were less likely to enroll in the NDB. In effect, this required
each patient interview.
preexisting cancer from the specific analysis of that cancer. To
cancer (incidence density ratios). Phases represent
were difficult, patient self-report was accepted after a detailed
there was no hospitalization record and medical confirmation
accepted as cases. In cases of nonmelanotic skin cancers where
was difficult, patient self-report was accepted after a detailed
were associated with subsequent
breast, etc.” Incomplete answers are followed up with a
We also searched the National Death Index (34)
annually from 1998 through 2004 and also received reports of
deaths from family and friends. Cancers identified in death
records within 6 months of final patient participation were
excluded at initial assessment. Patient Activity Scale and prednisone

### Table 1. Demographic and clinical characteristics of the study participants (n = 13,001) at first observation*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value ± SD (median)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean ± SD (median) years</td>
<td>58.5 ± 13.1 (58.8)</td>
</tr>
<tr>
<td>Male, %</td>
<td>22.0</td>
</tr>
<tr>
<td>Years of education, %</td>
<td></td>
</tr>
<tr>
<td>0–8</td>
<td>2.8</td>
</tr>
<tr>
<td>&gt;8–11</td>
<td>8.4</td>
</tr>
<tr>
<td>12</td>
<td>37.8</td>
</tr>
<tr>
<td>13–15</td>
<td>25.8</td>
</tr>
<tr>
<td>≥16</td>
<td>25.5</td>
</tr>
<tr>
<td>Ethnic origin, %</td>
<td></td>
</tr>
<tr>
<td>White, not of Hispanic origin</td>
<td>92.5</td>
</tr>
<tr>
<td>Black, not of Hispanic origin</td>
<td>3.9</td>
</tr>
<tr>
<td>Asian or Pacific Islander</td>
<td>1.0</td>
</tr>
<tr>
<td>American Indian or Alaskan Native</td>
<td>0.8</td>
</tr>
<tr>
<td>Hispanic</td>
<td>1.9</td>
</tr>
<tr>
<td>Ever smoked, %</td>
<td>56.4</td>
</tr>
<tr>
<td>Disease duration, mean ± SD (median) years</td>
<td>16.7 ± 12.7 (14.5)</td>
</tr>
<tr>
<td>HAQ score, 0–3</td>
<td>1.1 ± 0.7 (1.1)</td>
</tr>
<tr>
<td>First Patient Activity Scale score, 0–10</td>
<td>3.7 ± 2.2 (3.6)</td>
</tr>
<tr>
<td>Treatment, %</td>
<td></td>
</tr>
<tr>
<td>Prednisone</td>
<td>45.6</td>
</tr>
<tr>
<td>MTX</td>
<td>56.9</td>
</tr>
<tr>
<td>Leflunomide</td>
<td>18.7</td>
</tr>
<tr>
<td>Sulfasalazine</td>
<td>9.4</td>
</tr>
<tr>
<td>HCQ</td>
<td>25.2</td>
</tr>
<tr>
<td>Infliximab</td>
<td>19.9</td>
</tr>
<tr>
<td>Etanercept</td>
<td>7.6</td>
</tr>
<tr>
<td>Adalimumab</td>
<td>0.4</td>
</tr>
<tr>
<td>Anakinra</td>
<td>0.3</td>
</tr>
</tbody>
</table>

* HAQ = Health Assessment Questionnaire; MTX = methotrexate; HCQ = hydroxychloroquine.
We conducted a number of sensitivity analyses with respect to the association of biologics with malignancy. In these analyses we eliminated cancer cases discovered only in death records, allowed all data in 2005 to be used, changed the conditional logistic regression grouping variable from phase of entry/exit to year of entry/exit, and allowed patients with fewer than 2 phases to be analyzed. The results of these analyses were not essentially different from those of the study analyses described below and are therefore not reported.

RESULTS

Of the 13,869 RA patients studied for all cancers, 868 were excluded by the conditional logistic regression requirements. The characteristics of the 13,001 remaining patients are shown in Table 1. At the time of entry to the study, the mean ± SD age of participants was 58.5 ± 13.1 years. Men constituted 22.0% of the sample, non-Hispanic whites 92.5%, and college graduates 25.5%. More than half of the patients had a history of smoking (56.4%), and almost half were receiving prednisone (45.6%). The number and percent of patients using biologic therapy during the study were as follows: any therapy 5,257 (40.7%), infliximab 4,277 (33.1%), etanercept 3,011 (23.3%), adalimumab 763 (5.9%), and anakinra 319 (2.5%). The mean duration (range) in years of each treatment was as follows: any therapy 3.0 (0.5–7.8), infliximab 2.9 (0.5–7.8), etanercept 2.7 (0.5–7.7), adalimumab 1.2 (0.5–7.7), and anakinra 1.6 (0.5–3.9).

The rate of malignancy in RA. As shown in Table 2, there was no increase in the overall rate of cancer in participating RA patients compared with SEER data (SIR 1.0, 95% CI 1.0–1.1). A number of malignancies were more common in the RA patient sample than in the SEER database, including lymphoma (SIR 1.7, 95% CI 1.3–2.2) and melanoma (SIR 1.7, 95% CI 1.3–2.3). The lower limit of the 95% CI crossed 1 for lung cancer, resulting in an SIR of 1.2 (95% CI 1.0–1.4). Rates were reduced for breast cancer (SIR 0.8, 95% CI 0.6–0.9) and

Table 2. Rates of malignancy among 13,869 study participants with rheumatoid arthritis*

<table>
<thead>
<tr>
<th>Cancer</th>
<th>Cases</th>
<th>Exposure, patient-years</th>
<th>Crude rate per 100,000 patient-years (95% CI)</th>
<th>SIR (95% CI)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>All†</td>
<td>543</td>
<td>41,912</td>
<td>1,295.6 (1,188.9–1,409.3)</td>
<td>1.0 (1.0–1.1)</td>
</tr>
<tr>
<td>Bladder</td>
<td>20</td>
<td>49,021</td>
<td>40.8 (24.9–63.0)</td>
<td>0.8 (0.5–1.0)</td>
</tr>
<tr>
<td>Bone</td>
<td>3</td>
<td>49,145</td>
<td>6.1 (1.3–17.8)</td>
<td>5.7 (2.1–14.6)</td>
</tr>
<tr>
<td>Brain</td>
<td>1</td>
<td>49,166</td>
<td>2.0 (0.1–11.1)</td>
<td>0.2 (0.0–0.9)</td>
</tr>
<tr>
<td>Breast</td>
<td>102</td>
<td>47,848</td>
<td>213.2 (173.8–258.8)</td>
<td>0.8 (0.6–0.9)</td>
</tr>
<tr>
<td>Cervix</td>
<td>4</td>
<td>48,582</td>
<td>8.2 (3.1–21.9)</td>
<td>0.8 (0.4–1.9)</td>
</tr>
<tr>
<td>Colon</td>
<td>37</td>
<td>48,870</td>
<td>75.7 (53.3–104.4)</td>
<td>0.5 (0.4–0.6)</td>
</tr>
<tr>
<td>Endocrine</td>
<td>1</td>
<td>49,156</td>
<td>2.0 (0.1–11.3)</td>
<td>0.1 (0.02–0.6)</td>
</tr>
<tr>
<td>Esophagus</td>
<td>10</td>
<td>49,167</td>
<td>20.3 (9.8–37.4)</td>
<td>1.8 (1.1–3.1)</td>
</tr>
<tr>
<td>Gall bladder</td>
<td>1</td>
<td>49,170</td>
<td>2.0 (0.1–11.1)</td>
<td>2.0 (0.3–14.4)</td>
</tr>
<tr>
<td>Head and neck</td>
<td>5</td>
<td>49,171</td>
<td>10.2 (3.3–23.7)</td>
<td>0.3 (0.2–0.7)</td>
</tr>
<tr>
<td>Hodgkin’s</td>
<td>4</td>
<td>49,116</td>
<td>8.1 (2.2–20.8)</td>
<td>3.0 (1.3–6.8)</td>
</tr>
<tr>
<td>Kidney</td>
<td>7</td>
<td>49,101</td>
<td>14.3 (5.7–29.4)</td>
<td>0.5 (0.2–0.9)</td>
</tr>
<tr>
<td>Leukemia</td>
<td>24</td>
<td>49,118</td>
<td>48.9 (31.3–72.7)</td>
<td>1.7 (1.2–2.4)</td>
</tr>
<tr>
<td>Liver</td>
<td>6</td>
<td>49,155</td>
<td>12.2 (4.5–26.0)</td>
<td>0.9 (0.5–1.8)</td>
</tr>
<tr>
<td>Lymphoma</td>
<td>45</td>
<td>49,085</td>
<td>91.7 (66.9–122.7)</td>
<td>1.7 (1.3–2.2)</td>
</tr>
<tr>
<td>Lung</td>
<td>112</td>
<td>49,037</td>
<td>228.8 (188.1–274.8)</td>
<td>1.2 (1.0–1.4)</td>
</tr>
<tr>
<td>Melanoma</td>
<td>32</td>
<td>48,795</td>
<td>65.6 (44.9–92.6)</td>
<td>1.7 (1.3–2.3)</td>
</tr>
<tr>
<td>Non-Hodgkin’s</td>
<td>42</td>
<td>49,103</td>
<td>85.5 (61.6–115.6)</td>
<td>1.7 (1.3–2.2)</td>
</tr>
<tr>
<td>Ovary</td>
<td>7</td>
<td>48,948</td>
<td>14.3 (5.7–29.5)</td>
<td>0.5 (0.3–0.9)</td>
</tr>
<tr>
<td>Pancreas</td>
<td>12</td>
<td>49,175</td>
<td>24.4 (12.6–42.6)</td>
<td>0.7 (0.4–1.1)</td>
</tr>
<tr>
<td>Prostate</td>
<td>56</td>
<td>48,732</td>
<td>114.9 (86.8–149.2)</td>
<td>0.8 (0.6–1.0)</td>
</tr>
<tr>
<td>Skin‡</td>
<td>624</td>
<td>46,494</td>
<td>1,342.1 (1,238.8–1,451.7)</td>
<td>NA</td>
</tr>
<tr>
<td>Soft tissue</td>
<td>4</td>
<td>49,171</td>
<td>8.1 (3.0–21.6)</td>
<td>1.4 (0.6–3.2)</td>
</tr>
<tr>
<td>Solid‡</td>
<td>473</td>
<td>41,763</td>
<td>1,132.6 (1,035.0–1,239.4)</td>
<td>1.0 (0.9–1.0)</td>
</tr>
<tr>
<td>Stomach</td>
<td>4</td>
<td>49,147</td>
<td>8.1 (2.2–20.8)</td>
<td>0.4 (0.2–0.9)</td>
</tr>
<tr>
<td>Testicular</td>
<td>1</td>
<td>49,172</td>
<td>2.0 (0.1–11.3)</td>
<td>2.0 (0.3–14.4)</td>
</tr>
<tr>
<td>Uterus</td>
<td>7</td>
<td>48,520</td>
<td>14.4 (5.8–29.7)</td>
<td>0.3 (0.1–0.4)</td>
</tr>
<tr>
<td>Vagina</td>
<td>2</td>
<td>49,169</td>
<td>4.1 (0.5–14.7)</td>
<td>2.7 (0.8–8.6)</td>
</tr>
</tbody>
</table>

* 95% CI = 95% confidence interval; SIR = standardized incidence ratio; NA = not available.
† Excludes nonmelanoma skin malignancies.
‡ Excludes melanoma.
§ Excludes lymphoma, leukemia, myeloma, and nonmelanoma skin malignancies.
colon cancer (SIR 0.5, 95% CI 0.4–0.6). The upper limit of the 95% CI crossed 1 for bladder cancer (SIR 0.8, 95% CI 0.5–1.0).

The association of biologic therapy and malignancy. In Table 3 the maximum number of patients analyzed was 13,584, and 6,597 of these patients received treatment with biologics. The numbers were slightly smaller when patients with preexisting skin cancer were excluded. In that instance, of 12,916 patients studied, 6,282 had received biologics. When all biologic therapies were considered as a group, the risk of nonmelanotic skin cancer (OR 1.5 [95% CI 1.2–1.8]) and possibly of melanoma (OR 2.3 [95% CI 0.9–5.4], P = 0.070) was increased in patients who received biologics (Table 3). However, no other malignancy was significantly associated with biologic use, and the OR for all cancers overall was 1.0 (95% CI 0.8–1.2). The addition of duration of therapy to the model (the next-to-last column in Table 3) did not strengthen positive associations with biologic therapy.

Table 4 extends the analyses to individual biologics. Infliximab (OR 2.6 [95% CI 1.0–6.7], P = 0.056) and etanercept (OR 2.4 [95% CI 1.0–5.8], P = 0.054) were associated with melanoma. Infliximab (OR 1.7 [95% CI 1.3–2.2], P < 0.001) and etanercept (OR 1.2 [95% CI 1.0–1.5], P = 0.081) were also associated with non-melanotic skin cancer. No association was noted with any other malignancy.

**DISCUSSION**

The main finding of this study is the positive association between biologic therapy and skin cancers and the nonassociation of biologic therapy with all other malignancies. The association of biologic therapy and melanoma (OR 2.3 [95% CI 0.9–5.4]), P = 0.070) was increased in patients who received biologics (Table 3). However, no other malignancy was significantly associated with biologic use, and the OR for all cancers overall was 1.0 (95% CI 0.8–1.2). The addition of duration of therapy to the model (the next-to-last column in Table 3) did not strengthen positive associations with biologic therapy.

**Table 3. Association of biologic therapy and subsequent malignancy**

<table>
<thead>
<tr>
<th>Cancer</th>
<th>Cases</th>
<th>Subjects analyzed</th>
<th>OR (95% CI)†</th>
<th>P</th>
<th>P for trend‡</th>
<th>Users of biologics</th>
</tr>
</thead>
<tbody>
<tr>
<td>All§</td>
<td>537</td>
<td>12,916</td>
<td>1.0 (0.8–1.2)</td>
<td>0.858</td>
<td>0.678</td>
<td>6,282</td>
</tr>
<tr>
<td>Bladder</td>
<td>20</td>
<td>4,687</td>
<td>0.5 (0.1–1.5)</td>
<td>0.197</td>
<td>0.768</td>
<td>1,862</td>
</tr>
<tr>
<td>Bone</td>
<td>3</td>
<td>376</td>
<td>0.0 (0– )</td>
<td>0.999</td>
<td>0.999</td>
<td>102</td>
</tr>
<tr>
<td>Brain</td>
<td>1</td>
<td>111</td>
<td>0.4 (0.2–1.0)</td>
<td>0.44</td>
<td>0.44</td>
<td>44</td>
</tr>
<tr>
<td>Breast</td>
<td>102</td>
<td>10,541</td>
<td>0.9 (0.5–1.3)</td>
<td>0.560</td>
<td>0.539</td>
<td>5,196</td>
</tr>
<tr>
<td>Colon</td>
<td>37</td>
<td>3,795</td>
<td>0.8 (0.3–1.7)</td>
<td>0.506</td>
<td>0.345</td>
<td>2,503</td>
</tr>
<tr>
<td>Endocrine</td>
<td>1</td>
<td>154</td>
<td>0</td>
<td></td>
<td></td>
<td>66</td>
</tr>
<tr>
<td>Esophagus</td>
<td>10</td>
<td>2,374</td>
<td>0.9 (0.2–5.0)</td>
<td>0.907</td>
<td>0.801</td>
<td>1,302</td>
</tr>
<tr>
<td>Gall bladder</td>
<td>1</td>
<td>1,099</td>
<td>0.7 (0.1–5.5)</td>
<td>0.757</td>
<td>0.801</td>
<td>378</td>
</tr>
<tr>
<td>Head and neck</td>
<td>5</td>
<td>926</td>
<td>0.7 (0.1–5.5)</td>
<td>0.757</td>
<td>0.801</td>
<td>378</td>
</tr>
<tr>
<td>Hodgkin’s</td>
<td>4</td>
<td>696</td>
<td>&gt;1,000 (0– )</td>
<td>0.999</td>
<td>0.038</td>
<td>264</td>
</tr>
<tr>
<td>Kidney</td>
<td>7</td>
<td>2,221</td>
<td>1.8 (0.3–9.4)</td>
<td>0.507</td>
<td>0.399</td>
<td>807</td>
</tr>
<tr>
<td>Leukemia</td>
<td>24</td>
<td>3,348</td>
<td>1.2 (0.5–3.1)</td>
<td>0.704</td>
<td>0.696</td>
<td>1,367</td>
</tr>
<tr>
<td>Liver</td>
<td>6</td>
<td>1,762</td>
<td>0.2 (0.0–2.9)</td>
<td>0.256</td>
<td>0.547</td>
<td>675</td>
</tr>
<tr>
<td>Lung</td>
<td>112</td>
<td>8,627</td>
<td>1.1 (0.7–1.8)</td>
<td>0.630</td>
<td>0.737</td>
<td>3,610</td>
</tr>
<tr>
<td>Lymphoma</td>
<td>45</td>
<td>5,901</td>
<td>1.0 (0.5–2.0)</td>
<td>0.967</td>
<td>0.462</td>
<td>2,221</td>
</tr>
<tr>
<td>Melanoma</td>
<td>32</td>
<td>3,260</td>
<td>2.3 (0.9–5.4)</td>
<td>0.070</td>
<td>0.256</td>
<td>1,394</td>
</tr>
<tr>
<td>Non-Hodgkin’s</td>
<td>42</td>
<td>5,589</td>
<td>0.7 (0.3–1.5)</td>
<td>0.335</td>
<td>0.183</td>
<td>2,080</td>
</tr>
<tr>
<td>Ovary</td>
<td>7</td>
<td>1,416</td>
<td>3.6 (0.6–21.1)</td>
<td>0.153</td>
<td>0.076</td>
<td>587</td>
</tr>
<tr>
<td>Pancreas</td>
<td>12</td>
<td>1,857</td>
<td>0.5 (0.1–2.6)</td>
<td>0.440</td>
<td>0.930</td>
<td>481</td>
</tr>
<tr>
<td>Prostate</td>
<td>56</td>
<td>7,511</td>
<td>0.9 (0.4–1.9)</td>
<td>0.734</td>
<td>0.189</td>
<td>2,884</td>
</tr>
<tr>
<td>Skin#</td>
<td>623</td>
<td>13,584</td>
<td>1.5 (1.2–1.8)</td>
<td>&lt;0.001</td>
<td>0.075</td>
<td>6,597</td>
</tr>
<tr>
<td>Soft tissue</td>
<td>4</td>
<td>806</td>
<td>0.3 (0.0–4.8)</td>
<td>0.378</td>
<td>0.998</td>
<td>205</td>
</tr>
<tr>
<td>Solid**</td>
<td>467</td>
<td>12,839</td>
<td>1.0 (0.8–2.2)</td>
<td>0.867</td>
<td>0.851</td>
<td>6,238</td>
</tr>
<tr>
<td>Stomach</td>
<td>4</td>
<td>806</td>
<td>0.8 (0.1–9.3)</td>
<td>0.891</td>
<td>0.675</td>
<td>1,018</td>
</tr>
<tr>
<td>Testicular</td>
<td>1</td>
<td>358</td>
<td>0.0 (0– )</td>
<td>0.999</td>
<td>0.999</td>
<td>63</td>
</tr>
<tr>
<td>Uterus</td>
<td>7</td>
<td>742</td>
<td>0.0 (0– )</td>
<td>0.998</td>
<td>0.993</td>
<td>250</td>
</tr>
<tr>
<td>Vagina</td>
<td>2</td>
<td>960</td>
<td>0.6 (0.0–10.3)</td>
<td>0.735</td>
<td>0.470</td>
<td>706</td>
</tr>
</tbody>
</table>

* OR = odds ratio; 95% CI = 95% confidence interval.
† Adjusted for age, sex, education, smoking history, baseline Patient Activity Scale score, and baseline prednisone use.
‡ “Ever received biologic therapy” was replaced by quartiles of biologic time exposure. Mean values are 0, 1.2, 3.0, and 4.7 years.
§ Excludes nonmelanoma skin malignancies.
¶ Indicates nonconvergence of the statistical algorithm due to an insufficient number of cases.
# Excludes melanoma.
** Excludes lymphoma, leukemia, myeloma, and nonmelanoma skin malignancies.
Table 4. Association of biologic therapies and subsequent malignancy, for malignancies with 20 or more cases in the National Data Bank for Rheumatic Diseases*

<table>
<thead>
<tr>
<th>Cancer, treatment</th>
<th>Treated cases</th>
<th>Total subjects</th>
<th>Treated subjects</th>
<th>OR (95% CI)†</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>All‡</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infliximab</td>
<td>125</td>
<td>12,916</td>
<td>4,277</td>
<td>1.0 (0.8–1.3)</td>
<td>0.820</td>
</tr>
<tr>
<td>Etanercept</td>
<td>93</td>
<td>12,916</td>
<td>3,011</td>
<td>1.0 (0.8–1.3)</td>
<td>0.962</td>
</tr>
<tr>
<td>Adalimumab</td>
<td>7</td>
<td>12,916</td>
<td>763</td>
<td>0.7 (0.3–1.6)</td>
<td>0.393</td>
</tr>
<tr>
<td>Anakinra</td>
<td>6</td>
<td>12,916</td>
<td>319</td>
<td>0.8 (0.3–1.8)</td>
<td>0.515</td>
</tr>
<tr>
<td>Bladder</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infliximab</td>
<td>2</td>
<td>4,687</td>
<td>1,070</td>
<td>0.4 (0.1–1.8)</td>
<td>0.228</td>
</tr>
<tr>
<td>Etanercept</td>
<td>4</td>
<td>4,687</td>
<td>1,037</td>
<td>1.5 (0.4–4.7)</td>
<td>0.513</td>
</tr>
<tr>
<td>Adalimumab</td>
<td>0</td>
<td>4,687</td>
<td>253</td>
<td>0.0 (0.0– )</td>
<td>0.991</td>
</tr>
<tr>
<td>Anakinra</td>
<td>0</td>
<td>4,687</td>
<td>116</td>
<td>0.0 (0.0– )</td>
<td>0.991</td>
</tr>
<tr>
<td>Breast</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infliximab</td>
<td>22</td>
<td>10,541</td>
<td>3,463</td>
<td>0.9 (0.5–1.7)</td>
<td>0.854</td>
</tr>
<tr>
<td>Etanercept</td>
<td>19</td>
<td>10,541</td>
<td>2,571</td>
<td>0.8 (0.5–1.4)</td>
<td>0.505</td>
</tr>
<tr>
<td>Adalimumab</td>
<td>4</td>
<td>10,541</td>
<td>658</td>
<td>1.6 (0.5–4.7)</td>
<td>0.387</td>
</tr>
<tr>
<td>Anakinra</td>
<td>2</td>
<td>10,541</td>
<td>279</td>
<td>1.1 (0.2–4.6)</td>
<td>0.993</td>
</tr>
<tr>
<td>Colon</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infliximab</td>
<td>7</td>
<td>3,795</td>
<td>614</td>
<td>1.1 (0.4–2.9)</td>
<td>0.787</td>
</tr>
<tr>
<td>Etanercept</td>
<td>5</td>
<td>3,795</td>
<td>838</td>
<td>0.7 (0.3–2.0)</td>
<td>0.542</td>
</tr>
<tr>
<td>Adalimumab</td>
<td>0</td>
<td>3,795</td>
<td>114</td>
<td>0.0 (0.0– )</td>
<td>0.993</td>
</tr>
<tr>
<td>Anakinra</td>
<td>0</td>
<td>3,795</td>
<td>66</td>
<td>0.0 (0.0– )</td>
<td>0.990</td>
</tr>
<tr>
<td>Leukemia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infliximab</td>
<td>7</td>
<td>3,438</td>
<td>891</td>
<td>0.9 (0.3–2.7)</td>
<td>0.807</td>
</tr>
<tr>
<td>Etanercept</td>
<td>4</td>
<td>3,438</td>
<td>708</td>
<td>1.0 (0.3–3.1)</td>
<td>1.000</td>
</tr>
<tr>
<td>Adalimumab</td>
<td>0</td>
<td>3,438</td>
<td>95</td>
<td>0.0 (0.0– )</td>
<td>0.993</td>
</tr>
<tr>
<td>Anakinra</td>
<td>0</td>
<td>3,438</td>
<td>59</td>
<td>0.0 (0.0– )</td>
<td>0.993</td>
</tr>
<tr>
<td>Lung</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infliximab</td>
<td>30</td>
<td>8,627</td>
<td>2,412</td>
<td>1.2 (0.7–2.1)</td>
<td>0.465</td>
</tr>
<tr>
<td>Etanercept</td>
<td>19</td>
<td>8,627</td>
<td>1,808</td>
<td>1.0 (0.6–1.8)</td>
<td>0.877</td>
</tr>
<tr>
<td>Adalimumab</td>
<td>0</td>
<td>8,627</td>
<td>335</td>
<td>0.0 (0.0– )</td>
<td>0.990</td>
</tr>
<tr>
<td>Anakinra</td>
<td>0</td>
<td>8,627</td>
<td>183</td>
<td>0.0 (0.0– )</td>
<td>0.989</td>
</tr>
<tr>
<td>Lymphoma</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infliximab</td>
<td>12</td>
<td>5,901</td>
<td>1,182</td>
<td>0.9 (0.4–2.1)</td>
<td>0.898</td>
</tr>
<tr>
<td>Etanercept</td>
<td>10</td>
<td>5,901</td>
<td>1,313</td>
<td>1.3 (0.6–2.8)</td>
<td>0.460</td>
</tr>
<tr>
<td>Adalimumab</td>
<td>1</td>
<td>5,901</td>
<td>251</td>
<td>1.3 (0.2–10.0)</td>
<td>0.826</td>
</tr>
<tr>
<td>Anakinra</td>
<td>0</td>
<td>5,901</td>
<td>95</td>
<td>0.0 (0.0– )</td>
<td>0.992</td>
</tr>
<tr>
<td>Melanoma</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infliximab</td>
<td>11</td>
<td>3,260</td>
<td>790</td>
<td>2.6 (1.0–6.7)</td>
<td>0.056</td>
</tr>
<tr>
<td>Etanercept</td>
<td>9</td>
<td>3,260</td>
<td>754</td>
<td>2.4 (1.0–5.8)</td>
<td>0.054</td>
</tr>
<tr>
<td>Adalimumab</td>
<td>1</td>
<td>3,260</td>
<td>207</td>
<td>0.8 (0.1–6.6)</td>
<td>0.822</td>
</tr>
<tr>
<td>Anakinra</td>
<td>2</td>
<td>3,260</td>
<td>77</td>
<td>4.2 (0.9–20.0)</td>
<td>0.075</td>
</tr>
<tr>
<td>Non-Hodgkin's</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infliximab</td>
<td>11</td>
<td>5,589</td>
<td>1,108</td>
<td>1.0 (0.4–2.3)</td>
<td>0.969</td>
</tr>
<tr>
<td>Etanercept</td>
<td>7</td>
<td>5,589</td>
<td>1,251</td>
<td>1.0 (0.4–2.1)</td>
<td>0.838</td>
</tr>
<tr>
<td>Adalimumab</td>
<td>0</td>
<td>5,589</td>
<td>200</td>
<td>0.0 (0.0– )</td>
<td>0.992</td>
</tr>
<tr>
<td>Anakinra</td>
<td>0</td>
<td>5,589</td>
<td>92</td>
<td>0.0 (0.0– )</td>
<td>0.993</td>
</tr>
<tr>
<td>Prostate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infliximab</td>
<td>6</td>
<td>7,511</td>
<td>1,693</td>
<td>0.5 (0.2–1.5)</td>
<td>0.223</td>
</tr>
<tr>
<td>Etanercept</td>
<td>6</td>
<td>7,511</td>
<td>1,579</td>
<td>0.8 (0.3–2.1)</td>
<td>0.642</td>
</tr>
<tr>
<td>Adalimumab</td>
<td>0</td>
<td>7,511</td>
<td>341</td>
<td>0.0 (0.0– )</td>
<td>0.996</td>
</tr>
<tr>
<td>Anakinra</td>
<td>1</td>
<td>7,511</td>
<td>137</td>
<td>4.0 (0.4–37.4)</td>
<td>0.223</td>
</tr>
<tr>
<td>Skin§</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infliximab</td>
<td>161</td>
<td>13,584</td>
<td>4,430</td>
<td>1.7 (1.3–2.2)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Etanercept</td>
<td>126</td>
<td>13,584</td>
<td>3,163</td>
<td>1.2 (1.0–1.5)</td>
<td>0.081</td>
</tr>
<tr>
<td>Adalimumab</td>
<td>10</td>
<td>13,584</td>
<td>812</td>
<td>0.9 (0.5–1.8)</td>
<td>0.828</td>
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<tr>
<td>Anakinra</td>
<td>11</td>
<td>13,584</td>
<td>317</td>
<td>1.4 (0.7–2.8)</td>
<td>0.289</td>
</tr>
</tbody>
</table>

* OR = odds ratio; 95% CI = 95% confidence interval.
† Adjusted for age, sex, education, smoking history, baseline Patient Activity Scale score, and baseline prednisone use.
‡ Excludes nonmelanoma skin malignancies.
§ Excludes melanoma.
malignancies. Most malignancies have long latency periods in the absence of immunosuppression. For example, the latency period for lung cancer following cigarette smoking and for breast cancer following cosmic radiation exceeds 15 years (37,38). However, immunosuppression shortens the latency period and increases the range of cancers identified.

Immunosuppressant therapy following renal transplantation in Nordic countries between 1964 and 1986 was associated with excess risks for cancers of the colon, larynx, lung, bladder, prostate, and testis, and with particularly high risk for cancers of the lip, skin (nonmelanoma), kidney, endocrine glands, non-Hodgkin’s lymphoma, and cancers of the cervix and vulva-vagina (9). In The Netherlands following renal transplantation, the overall incidence of squamous cell carcinoma was 250 times higher than that in the general Dutch population, and that of basal cell carcinoma was 10 times higher (39). In a study of 35,765 recipients of renal transplants in the US Medicare billing claims database, the above data were confirmed (10). Compared with the incidence of tumors in patients on the waiting list for transplantation in this database, several tumors were more common after transplantation, including nonmelanoma skin cancers (2.6-fold), melanoma (2.2-fold), Hodgkin’s lymphoma (2.6-fold), and non-Hodgkin’s lymphoma (3.3-fold) (10). Not increased in these analyses were cancers of the lung or breast. Colon cancer was reduced (1.3-fold; \( P = 0.086 \)), and prostate cancer was reduced (1.3-fold). However, the risk of malignancy differs according to the degree of immunosuppression used with transplantation, with the strongest associations occurring in cyclosporine-treated patients (11,12).

Although our data do not show associations between malignancy and biologic therapy, except for skin cancers, the mean and median exposure to biologics was only 3.0 years. It is possible that with increasing time of followup or of exposure, the association between malignancy and biologic therapy would become stronger. However, true associations are regularly seen within this time frame, since posttransplantation studies have shown increased risk after the first year of treatment (9,10).

The data in the current report differ substantially from those in the meta-analysis of randomized clinical trials by Bongartz et al (21). Those authors noted that the pooled OR for malignancy in biologic- versus non–biologic-treated patients in randomized controlled trials was 3.3 (95% CI 1.2–9.1).

There are a number of differences between our study and the meta-analysis. In the meta-analysis, there were 3,493 biologic-treated participants and 1,512 non–biologic-treated control subjects. The individual trial durations ranged between 3 months and 1 year. Data regarding malignancies occurring after the trials were available for 3 of the 9 trials and were reported over an indeterminate period of time. Of the malignancies identified, 23 occurred in adalimumab-treated patients and 12 occurred in infliximab-treated patients (some patients had more than 1 malignancy). Patients treated with etanercept were not studied.

In contrast, adalimumab was infrequently used in our cohort, and adalimumab followup was of short duration. The lack of use in our cohort was a function of the recent release of adalimumab for RA treatment and our exclusion requirements. However, our study included participants treated with etanercept. We identified 125 malignancies in 4,277 infliximab-treated patients and 93 malignancies in 3,011 etanercept-treated patients. The mean duration of followup was 4.1 years (median 3.9 years). Among patients exposed to biologics, the mean and median exposure was 3.0 years. The OR for all malignancies was 1.0 (95% CI 0.8–1.3) for patients treated with biologics. This result is substantially different from the OR of 3.3 (95% CI 1.2–9.1) noted by Bongartz et al (21) in their meta-analysis of clinical trials, and also substantially different from the OR of 2.92 (95% CI 0.95–4.29) obtained by Costenbader et al (22) when these investigators used the same methods described by Bongartz et al to update the results of the meta-analysis (21) with additional trial data.

The meta-analysis report has generated commentary and concerns with respect to whether methodologic issues of case identification might have been an important determinant of the observed results (40). The authors of the meta-analysis report replied to the critique and suggested that “treatment registries will provide widely generalizable results about treatment response” (41). We hope that the current study will provide useful data to illuminate this issue.

The primary limitation of observational studies lies in nonrandom assignment to treatment. If severity of arthritis is related to the outcome of interest and persons with severe arthritis are more likely to be treated with biologics, then outcome is confounded by indication and could be more related to arthritis severity than to treatment. With respect to malignancy, such confounding is known to occur with lymphoma (3,42). However, no other malignancy is known to be associated with RA severity. On that basis we might assume that confound-
BIOLOGIC THERAPY AND THE RISK OF MALIGNANCY

A 1996 report on 20,699 persons with RA in Denmark reported statistically significant risk ratios for malignancies, as follows: lung (1.5), hematopoietic malignancies (1.7), nonmelanotic skin (1.3), breast (0.8), colon (0.8), and all cancers (1.08) (1). Similar results using SIRs were obtained from the Swedish RA inpatient registry of 53,067 patients for the years 1964–2004: lymphoma (1.9), lung (1.5), kidney (1.5), nonmelanotic skin (1.7), colon (0.7), breast (0.8), and all cancers (1.05) (2,3). Significant SIR results from a Scottish registry containing 124,143 patients with a rheumatic condition from 1981 to 1996 were as follows: hematopoietic malignancies (males 2.1, females 1.8), lung (males 1.3, females 1.4), prostate (1.3), and colorectal cancer (males 0.9, females 0.7) (4). The results of our study are generally concordant with those of these studies.

With respect to cancer incidence, however, it is possible that we slightly underestimated incidence, although this does not affect biologic/nonbiologic risk estimates. All cancer data represent various degrees of underreporting. The SEER Program, for example, waits several years to capture corrections and additions before publication (35). Even after a 2-year delay, SEER Program reporting accounts for just 88–97% of the estimated final incidence case counts. For the NDB, reporting delay may come in the delayed identification of recent cases or cases in which the patient dies. Death data in some instances depend upon National Death Index (NDI) data, the public release of which is delayed by ~2 years. Therefore, even if NDB data capture was perfect, it would likely underestimate true rates very slightly in the most recent years.

NDB cancer data may also be incomplete if a participant in whom cancer develops withdraws from the NDB study because of that cancer and the accompanying illness. The NDB conducts exit interviews to capture such cancer events. In addition, the NDB may contact the physician to determine cancer status.

Even with the possible underreporting noted above, the SIRs reported in Table 2 are generally concordant with those in other RA cancer incidence studies. In that respect, we noted increased risks for melanoma, nonmelanotic skin cancer, and lymphoma, and decreased risks for breast and colon cancer. The SIR for lung cancer was 1.2 (95% CI 1.0–1.4) compared with 1.5 in the Danish and Swedish studies (1,2). However, this difference might be attributable to the association between smoking and RA (43,44) and the higher rate of smoking in Europe compared with the US (45). In the Nordic studies the overall cancer SIRs were 1.08 and 1.05 compared with 1.0 in the current (NDB) study.

There are other possible limitations to our study. It is possible that a history of malignancy may affect a physician's decision to prescribe an anti-TNF medication, thus allowing for confounding by indication. In the current study we excluded all patients with a previous history of the specific malignancy under study in order to be able to obtain incident data. However, we did not exclude patients with prior malignancies other than those currently under study. To understand whether this made a difference, we conducted sensitivity analyses in which we excluded all patients with preexisting malignancies. The results were essentially unchanged. For lung cancer, for example, the number of cancers and patients analyzed was 111 instead of 112, and the OR was unchanged from that shown in Table 3. For breast cancer, as an example, the number of cancers was 101 instead of 102, and the OR was unchanged.

Another potential limitation comes from our use of the NDI. A small number of cases reported in the current study were identified using only the NDI. It is possible that this could introduce a bias toward more lethal malignancies and away from finding an effect for nonlethal malignancies, such as skin cancers, breast, colon, prostate, etc. To examine this possibility, we conducted sensitivity analyses by excluding all cancer deaths. The ORs for the association of biologic therapy
with major cancers were as follows: lung 1.2 (95% CI 0.6–2.3), \( P = 0.548 \); solid tumors 1.0 (95% CI 0.8–1.3), \( P = 0.991 \); all cancers 1.0 (95% CI 0.8–1.3), \( P = 0.764 \); skin 1.5 (95% CI 1.2–1.8), \( P < 0.001 \); melanoma 2.4 (95% CI 1.0–5.9), \( P = 0.054 \); and breast 0.9 (95% CI 0.6–1.5), \( P = 0.697 \). Therefore, the exclusion of death data did not result in any real changes to the study results presented in Table 3.

In summary, biologic therapy is associated with increased risk for skin cancers, but not for solid tumors or lymphoproliferative malignancies. These associations are consistent across different biologic therapies.

**AUTHOR CONTRIBUTIONS**

Dr. Wolfe had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

**Study design.** Wolfe, Michaud.

**Acquisition of data.** Wolfe, Michaud.

**Analysis and interpretation of data.** Wolfe, Michaud.

**Manuscript preparation.** Wolfe.

**Statistical analysis.** Wolfe, Michaud.

**ROLE OF THE STUDY SPONSOR**

The National Data Bank for Rheumatic Diseases has conducted safety registries for Centocor, Sanofi-Aventis, and Bristol-Myers Squibb. By our safety registry contractual agreement with Centocor, the completed manuscript was reviewed by Centocor. No changes to the manuscript were made after their review.

**REFERENCES**


Serious Infection Following Anti–Tumor Necrosis Factor α Therapy in Patients With Rheumatoid Arthritis

Lessons From Interpreting Data From Observational Studies


Objective. In a recent observational study, we found that the risk of serious infection following anti–tumor necrosis factor α (anti-TNFα) therapy in patients with rheumatoid arthritis (RA) was not importantly increased compared with the background risk in routinely treated RA patients with similar disease severity. Observational data sets are, however, subject to a number of important biases related to selection factors for the timing of starting and stopping therapy. Infection risk is also likely to vary with duration of therapy. This study was undertaken to examine the influences of these biases and of the method of analysis on the risk of infection.

Methods. We compared the risk of serious infection in 8,659 patients treated with anti-TNFα with that in 2,170 patients treated with traditional disease-modifying antirheumatic drugs (DMARDs) recruited to the British Society for Rheumatology Biologics Register. We applied a number of statistical models in which we varied the length of the followup period by using different definitions of the date of discontinuation of treatment and different lag periods of risk following drug cessation.

Results. When the at-risk period was defined as “receiving treatment”, the adjusted incidence rate ratio comparing patients receiving anti-TNFα therapy with patients receiving DMARD therapy was 1.22 (95% confidence interval [95% CI] 0.88–1.69). Limiting followup to the first 90 days, however, revealed an adjusted incidence rate ratio of 4.6 (95% CI 1.8–11.9). Rates of infection were increased in the 90 days immediately following drug discontinuation and beyond, explained by selection factors for drug discontinuation.

Conclusion. These findings show that overall, the way in which UK rheumatologists select patients for starting and discontinuing anti-TNFα therapy explains our previous finding of no increase in risk. However, there may be important increases in true risk, notably early in the course of treatment, that would become more evident depending on the definition of at-risk period.

There are currently 3 anti–tumor necrosis factor α (anti-TNFα) drugs licensed for use in rheumatoid arthritis (RA) in the UK: infliximab and adalimumab, both monoclonal antibodies, and etanercept, a TNFα receptor fusion protein. Since TNFα is involved in host defense and tumor surveillance, there have been concerns that anti-TNFα therapy might lead to adverse...
events, particularly infection and malignancy. These are complex issues, since RA itself increases the risk of serious infection and certain malignancies, acting either via the disease process or secondary to traditional disease-modifying antirheumatic drugs (DMARDs). The question that needs to be addressed is whether anti-TNF\(/\)H9251 therapy further increases that risk.

The long-term safety of treatment with biologic response modifiers cannot, however, be addressed in short-term randomized clinical trials, not only because such trials have a limited duration, but also because they recruit insufficient numbers of patients to detect rare events. Large population-based registers are thus increasingly being used to study drug safety (1–4).

A number of methodologic aspects of register study design are now well-established. There must be a comparison cohort of patients who are as similar as possible to patients in the treatment cohort, aside from taking the drug in question. Adverse events must be reported in a robust manner with avoidance of reporting bias. Information on potential confounders should be collected and adjusted for in the analysis. There are, however, obvious selection factors in determining which patients start, and indeed stop, a particular therapy, which are not necessarily captured even in intensive data sets. Residual confounding is a major concern. In 2 recently published studies from Germany and the UK (2,5), the infection rates observed in the anti-TNF\(/\)H9251 cohorts were very similar, but the 2 groups of investigators drew very different conclusions, the former estimating a doubling of risk and the latter no increased risk. These conclusions highlight important differences between the 2 countries in their methods of selecting comparison cohorts.

There are also a number of issues relating to the method of analysis that are often ignored, but which need to be considered when interpreting the data from individual registers. This study examined the influence

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**Figure 1.** Patterns of constancy of risk of infection while receiving treatment (on drug) and after discontinuation of treatment (off drug). a, Increased risk at start of therapy. b, Constant risk with ongoing drug exposure. c, Increasing risk with cumulative drug exposure. d, Combination of the risk patterns shown in a–c. e, Ongoing constant risk for set lag window after discontinuation of treatment (drug stop). f, Linear decrease in risk back to baseline. g, Nonlinear decrease in risk back to baseline. h, Differing durations of risk windows, based on the half-life of each drug.
of different approaches on the analysis of serious infection rates following anti-TNFα therapy in patients with RA.

The first key question is whether any increase in risk of infection is constant over time, or whether there are specific times when the risk is higher or lower. Plausible models of risk over time include increased risk of an adverse event on initial exposure, constant risk with ongoing drug exposure, or increasing risk with cumulative exposure to the drug (Figures 1a–d). The pattern of risk is likely to differ according to the adverse event considered. An infusion reaction may be more likely to occur early in the course of therapy, whereas a malignancy may be related to cumulative drug exposure. For any given adverse event, the overall risk pattern may be a composite of these patterns.

It is also necessary to define an at-risk window, that is, the period when adverse events should be attributed to a drug. The minimum plausible at-risk window would extend from the beginning of therapy to the therapy discontinuation date (Figure 2a). Defining this, however, is complex in the context of anti-TNFα therapy, given the administration schedule (with, in the case of infliximab, for example, infusions several weeks apart). In addition, depending on the pharmacokinetics and pharmacodynamics of the drug, should the at-risk window extend beyond the drug discontinuation date (Figure 2b)? The third concern is whether a drug confers a long-term risk beyond its period of pharmacologic activity. In this case, an “ever taken drug” model would be applicable (Figure 2c). In combination, these factors can have substantial influences on the measured risk.

Further, the risk once the patient has stopped taking the drug may take one of many patterns (Figures 1e–h). It may remain constant, may decrease back to baseline linearly or, more likely, decrease in a nonlinear manner. It is possible that risk may never return to the pretreatment baseline level.

Finally, the statistical approach to analysis of changing risk pattern over time also needs to be considered. In the analysis of rare events, data may need to be aggregated, or “smoothed,” to obtain a meaningful estimate of risk over time. Interpretation of risk over time may be affected by this smoothing process.

Without carefully considering these sources of variability, the simple description of infection risk as x cases per 1,000 person-years of therapy is impossible to interpret. We used a large national observational study to assess the impact of the various issues addressed above on the estimated risk of infection following anti-TNFα therapy in patients with RA.

PATIENTS AND METHODS

The British Society for Rheumatology Biologics Register (BSRBR). The BSRBR is a national prospective observational study that was established with the primary aim of examining the medium- to long-term safety of biologic response modifiers used in the rheumatic diseases. The methodology has been described in detail elsewhere (5,6). Briefly, it consists of a cohort of patients with RA treated with anti-TNFα, and a comparison cohort of patients with active RA who receive traditional DMARD therapy and have never taken biologic response modifiers. The latter cohort exists in the UK because of budgetary constraints on the prescription of anti-TNFα drugs (7). Members of the BSRBR Control Centre Consortium are shown in Appendix A.

Data on demographic characteristics, disease severity and duration, drug therapy, and comorbidity are collected at baseline from both cohorts. All patients are then followed up using 3 parallel methods. First, consultants are sent a questionnaire every 6 months, requesting details of all changes in therapy and all adverse events that have occurred in that period. Second, patients are sent a 6-month diary in which to document all hospital admissions, new medications, and new hospital referrals. Third, all patients are flagged with the UK General Register Office, which provides the BSRBR with information on deaths. Serious infections, reported from any of these 3 sources, are defined as those that led to death or hospitalization or required intravenous antibiotics.

To be included in the present study, patients had to have been followed up for ≥6 months prior to July 31, 2006. Followup time was censored at the last completed followup prior to July 31, 2006, or the date of switching to a second biologic response modifier (or first biologic response modifier for the comparison cohort), or death, whichever came soonest. In other words, patients who switched to a second or subsequent anti-TNFα drug contributed time and adverse events data to their first drug only.

Statistical analysis. Baseline characteristics of the anti-TNFα cohort were compared with those of the DMARD cohort, using Wilcoxon’s rank sum tests for continuous variables and chi-square tests for categorical outcomes. Between-drug comparisons were made using Kruskal-Wallis rank tests for continuous variables. The incidence of serious infection was then compared both between the anti-TNFα cohort and the comparison cohort and between individual anti-TNFα agents. Rates of serious infection per 1,000 person-years were calculated using a large series of assumptions relating to the issues described above. In each instance, incidence rate ratios were calculated using Poisson regression, comparing the anti-TNFα cohort with the DMARD cohort. All analysis was conducted using Stata, version 8.2 software (StataCorp, College Station, TX).

RESULTS

Baseline characteristics. There were 10,755 patients included in the analysis (8,659 treated with anti-
TNFα and 2,170 treated with DMARDs only). Seventy-four patients switched from the DMARD cohort to the anti-TNFα cohort and were included in both groups. The baseline characteristics of the 2 cohorts are shown in Table 1. The DMARD cohort included proportionally more men, and patients in
this cohort were older and, as expected, had less severe disease.

**Risk of infection while receiving therapy.** There were 1,089 serious infections in total: 114 in the comparison cohort and 975 in the anti-TNFα cohort, 737 occurring while the patients were receiving therapy. Using the “receiving treatment” model of analysis (Figure 2a), the crude rate of serious infection was 39.2 per 1,000 person-years in the DMARD cohort and 55.5 per 1,000 person-years in the anti-TNFα cohort, ranging from 50.4 to 63.0 events per 1,000 person-years in the 3 anti-TNFα drug cohorts (Table 2). For this “receiving treatment” analysis, the at-risk period extended from the start date of anti-TNFα treatment to the first missed dose.

After adjustment for age, sex, disease duration and severity, extraarticular RA, baseline steroid use, diabetes, chronic obstructive pulmonary disease, and smoking history, there was no significant difference in

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**Table 1.** Baseline characteristics of the DMARD-treated and anti-TNFα-treated patients*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>DMARD (n = 2,170)</th>
<th>All anti-TNFα (n = 8,659)</th>
<th>Etanercept (n = 3,844)</th>
<th>Infliximab (n = 2,944)</th>
<th>Adalimumab (n = 1,871)</th>
<th>P†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean ± SD years</td>
<td>60 ± 12</td>
<td>56 ± 12</td>
<td>56 ± 12</td>
<td>56 ± 12</td>
<td>57 ± 12</td>
<td>0.052</td>
</tr>
<tr>
<td>Sex, % female</td>
<td>72</td>
<td>76</td>
<td>77</td>
<td>74</td>
<td>74</td>
<td>0.035</td>
</tr>
<tr>
<td>DAS28 score, mean ± SD</td>
<td>5.0 ± 1.4</td>
<td>6.6 ± 1.0†</td>
<td>6.6 ± 1.0</td>
<td>6.6 ± 1.0</td>
<td>6.5 ± 1.0</td>
<td>0.006</td>
</tr>
<tr>
<td>HAQ score, mean ± SD</td>
<td>1.5 ± 0.8</td>
<td>2.1 ± 0.6‡</td>
<td>2.1 ± 0.6</td>
<td>2.1 ± 0.5</td>
<td>2.0 ± 0.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Disease duration, median (IQR) years</td>
<td>7 (1–15)</td>
<td>12 (6–19)‡</td>
<td>12 (6–19)</td>
<td>12 (6–19)</td>
<td>11 (5–19)</td>
<td>0.009</td>
</tr>
<tr>
<td>Extraarticular RA, no. (%)</td>
<td>415 (19.1)</td>
<td>2,541 (29.3)‡</td>
<td>1,123 (29.2)</td>
<td>896 (30.4)</td>
<td>522 (27.9)</td>
<td>0.131</td>
</tr>
<tr>
<td>Baseline steroid use, no. (%)</td>
<td>418 (19.3)</td>
<td>3,793 (43.8)‡</td>
<td>1,784 (46.4)</td>
<td>1,342 (45.6)</td>
<td>663 (35.4)</td>
<td>0.016</td>
</tr>
<tr>
<td>Diabetes, no. (%)</td>
<td>132 (6.1)</td>
<td>470 (5.4)</td>
<td>230 (6.0)</td>
<td>134 (4.6)</td>
<td>106 (5.7)</td>
<td>0.031</td>
</tr>
<tr>
<td>COPD/asthma, no. (%)</td>
<td>416 (19.2)</td>
<td>1,130 (13.1)‡</td>
<td>536 (13.9)</td>
<td>361 (12.3)</td>
<td>233 (12.5)</td>
<td>0.082</td>
</tr>
<tr>
<td>Smoking history, no. (%)</td>
<td>537 (25)</td>
<td>1,886 (22)§</td>
<td>797 (21)</td>
<td>650 (22)</td>
<td>436 (23)</td>
<td>0.045¶</td>
</tr>
<tr>
<td>Current smoker</td>
<td>849 (39)</td>
<td>3,298 (38)§</td>
<td>1,454 (38)</td>
<td>1,107 (38)</td>
<td>733 (39)</td>
<td>0.001</td>
</tr>
<tr>
<td>Former smoker</td>
<td>767 (35)</td>
<td>3,431 (40)</td>
<td>1,568 (41)</td>
<td>1,169 (40)</td>
<td>690 (37)</td>
<td>0.001 ¶</td>
</tr>
</tbody>
</table>

Infections were counted in both groups. Smoking history was available for 2,153 patients treated with DMARDs and 8,615 patients treated with anti-TNFα drugs. DAS28 = Disease Activity Score in 28 joints; HAQ = Health Assessment Questionnaire; IQR = interquartile range; RA = rheumatoid arthritis; COPD = chronic obstructive pulmonary disease.

* Seventy-four patients switched from the cohort treated with disease-modifying antirheumatic drugs (DMARDs) to the cohort treated with anti-tumor necrosis factor α (anti-TNFα) drugs and were counted in both groups. Smoking history was available for 2,153 patients treated with DMARDs and 8,615 patients treated with anti-TNFα drugs. DAS28 = Disease Activity Score in 28 joints; HAQ = Health Assessment Questionnaire; IQR = interquartile range; RA = rheumatoid arthritis; COPD = chronic obstructive pulmonary disease.

† Adjusted for age, sex, disease duration and severity, extraarticular rheumatoid arthritis, baseline steroid use, diabetes, chronic obstructive pulmonary disease, and smoking history.

‡ P < 0.001 versus DMARD-treated patients.

§ P for trend < 0.001.

¶ P for trend.

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**Table 2.** Rates of serious infections obtained using models that include different definitions of the treatment-attributable at-risk period*

<table>
<thead>
<tr>
<th>Model</th>
<th>Method</th>
<th>DMARD (n = 2,170)</th>
<th>Etanercept (n = 3,844)</th>
<th>Infliximab (n = 2,944)</th>
<th>Adalimumab (n = 1,871)</th>
<th>All anti-TNFα (n = 8,659)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model A, receiving treatment</td>
<td>Person-years</td>
<td>2,908</td>
<td>6,021</td>
<td>5,034</td>
<td>2,221</td>
<td>13,277</td>
</tr>
<tr>
<td></td>
<td>Rate per 1,000 person-years (95% CI)</td>
<td>39.2 (32.3–47.1)</td>
<td>51.2 (45.6–57.2)</td>
<td>63.0 (56.2–70.3)</td>
<td>50.4 (41.5–60.7)</td>
<td>55.5 (51.7–59.5)</td>
</tr>
<tr>
<td></td>
<td>Adjusted incidence rate ratio (95% CI)†</td>
<td>Referent</td>
<td>1.15 (0.82–1.61)</td>
<td>1.28 (0.91–1.81)</td>
<td>1.17 (0.81–1.69)</td>
<td>1.22 (0.88–1.69)</td>
</tr>
<tr>
<td>Model B, duration of treatment plus 90-day lag window</td>
<td>Person-years</td>
<td>2,908</td>
<td>6,274</td>
<td>5,226</td>
<td>2,323</td>
<td>13,823</td>
</tr>
<tr>
<td></td>
<td>Rate per 1,000 person-years (95% CI)</td>
<td>39.2 (32.3–47.1)</td>
<td>57.5 (51.8–63.8)</td>
<td>67.7 (60.9–75.2)</td>
<td>54.7 (45.6–65.0)</td>
<td>60.9 (57.0–65.0)</td>
</tr>
<tr>
<td></td>
<td>Adjusted incidence rate ratio (95% CI)†</td>
<td>Referent</td>
<td>1.26 (0.91–1.74)</td>
<td>1.35 (0.97–1.89)</td>
<td>1.24 (0.87–1.78)</td>
<td>1.30 (0.93–1.78)</td>
</tr>
<tr>
<td>Model C, ever received treatment</td>
<td>Person-years</td>
<td>2,908</td>
<td>6,998</td>
<td>5,874</td>
<td>2,548</td>
<td>15,420</td>
</tr>
<tr>
<td></td>
<td>Rate per 1,000 person-years (95% CI)</td>
<td>39.2 (32.3–47.1)</td>
<td>61.7 (56.0–67.8)</td>
<td>68.9 (62.4–76.0)</td>
<td>54.2 (45.5–64.0)</td>
<td>63.2 (59.4–67.2)</td>
</tr>
<tr>
<td></td>
<td>Adjusted incidence rate ratio (95% CI)†</td>
<td>Referent</td>
<td>1.34 (0.97–1.86)</td>
<td>1.41 (1.02–1.97)</td>
<td>1.25 (0.88–1.77)</td>
<td>1.35 (0.99–1.85)</td>
</tr>
</tbody>
</table>

* 95% CI = 95% confidence interval (see Table 1 for other definitions).

† Adjusted for age, sex, disease duration and severity, extraarticular rheumatoid arthritis, baseline steroid use, diabetes, chronic obstructive pulmonary disease, and smoking history.
risk of infection between any of the anti-TNFα cohorts and the comparison cohort. These results are consistent with the findings published in our previous report on serious infection rates, with followup to September 2005 (5). In this observational study, the treating rheumatologist was aware of the patient’s therapy at the time of infection, possibly leading to a lower threshold for admission into the anti-TNFα cohorts. However, such a bias does not explain these findings.

**Constancy of risk.** A plot of the cumulative incidence of serious infections while receiving treatment (Figure 3a) showed that at any given point in time, it was more likely that patients in the anti-TNFα cohorts had a serious infection compared with patients in the comparison cohort. This does not necessarily mean that the absolute risk was higher at all time points in the anti-TNFα cohorts. In order to explore this, and to evaluate the change in risk over time (as in Figure 1), a plot of the slope of the curves in Figure 3a, or hazard plot, was constructed. This showed a marked increase in hazard in the anti-TNFα cohorts, peaking at ~6 months, but declining over time (Figure 3b).

To further explore a possible early risk of infection (Figure 1a), the rates of serious infections were analyzed with followup censored at 90 days after the start of anti-TNFα therapy (or 90 days after registration date in the DMARD cohort) (Table 3). The adjusted incidence rate ratios for the 3 anti-TNFα drugs compared with the DMARD cohort showed an ~4-fold or greater risk of serious infection in the first 90 days. These ratios were much higher than the incidence rate ratios seen for the entire followup period (Table 2).

This result is biologically plausible, suggesting an increased early risk of infection in the anti-TNFα cohort. However, as shown in Figure 3b, the increased incidence rate ratios in the first 90 days appeared to be driven not only by the elevated crude rates of infection in the anti-TNFα cohorts, but also by the low early crude rates in the DMARD cohort. Both cohorts, but particularly the anti-TNFα group, were screened before initiation of treatment, to avoid inclusion of patients who had an imminent serious infection. However, such a difference between the groups in prescreening would bias against an increased risk in the anti-TNFα group and does not explain this early increased risk.

Selection factors also apply after recruitment. If a patient in the anti-TNFα cohort developed an incident serious infection, the physician made an active treatment decision about whether therapy should be continued once the acute infection had resolved. Some patients were considered to be at high risk of a future infection and did not resume therapy. Therefore, the anti-TNFα cohort in a “receiving treatment” analysis had a progressively lower risk of infection over time because of the selective exclusion of high-risk patients. This may explain the gradual reduction in risk from the 6-month time point onward.

Because of clinical decisions influencing the observed pattern of risk, it is very difficult to ascertain the true pattern of risk, and a possible real increase in cumulative risk may be hidden by the selection factors described above. Thus, over the period of observation, the decisions made in clinical practice in conjunction with the underlying serious infection risk of the drugs...
Influence of choice of date of discontinuation of treatment. Within the BSRBR, stop dates for all 3 anti-TNFα drugs are based on clinical records. An accurate definition of the stop date is particularly important for infliximab, given the prolonged interval between infusions. In ~90% of records, the given stop date for infliximab was the last infusion date. However, an adverse event caused by the drug (apart from an infusion reaction) is unlikely to occur at the time of an infusion, and thus the stop date should be the first missed dose, rather than the last dose given. Indeed, such events are often the reason for discontinuing the drug. If the date of the last dose given were used as the stop date, events which followed the last infusion (event A in Figure 2d) would not be attributable to the drug in the analysis, grossly underestimating the event rate. Defining the stop date as the last dose given for infliximab leads to an infection incidence rate of 49.0 per 1,000 person-years, much lower than the 63.0 per 1,000 person-years shown above. Thus, for all BSRBR analyses, including the “receiving treatment” analysis (Table 2), the stop date was defined as the first missed dose.

Influence of lag window. Once treatment is discontinued, the risk associated with the drug may not immediately return to the predrug baseline. To address this, the at-risk window was extended for an arbitrary period beyond the first missed dose. Thus, in the following analysis, events occurring in the 90 days after the stop date (first missed dose) for each of the 3 drugs were considered attributable to that drug, as in Figure 2b.

The crude rates of serious infection in this analysis ranged from 54.7 to 67.7 per 1,000 person-years for the 3 anti-TNFα drugs (Table 2). These rates were higher than those found when the analysis was restricted to the “receiving treatment” period for all 3 drugs (Table 2). In fact, the rate of serious infection was higher in the 90-day window after drug discontinuation than during the treatment period. The rates per 1,000 person-years (95% confidence intervals [95% CIs]) in the 3 cohorts in this 90-day window after discontinuation of anti-TNFα therapy were 202 (153–256) for etanercept, 193 (139–256) for infliximab, and 147 (85–231) for adalimumab. The incidence rate ratios (95% CIs) in the first 90 days after discontinuation of treatment compared with the rates while receiving treatment were 3.3 (2.4–4.5) for etanercept, 2.8 (1.9–4.1) for infliximab, and 2.5 (1.3–4.8) for adalimumab.

These findings are counterintuitive, since the risk of serious infection would be expected to decrease once anti-TNFα treatment was discontinued. This could be explained by a rebound in disease activity after discontinuation of the drugs. However, the major reason for the increased risk in this 90-day window became clear on reviewing the individual case histories: the infection was causally related to the reason for discontinuing the drug. In the majority of cases, the reason was an adverse event. The subsequent infection may have been either a direct or an indirect consequence of the initial adverse event, e.g., an aspiration pneumonia following a stroke, or an opportunistic infection following chemotherapy for a malignancy. In other cases (for example, prior to surgery), discontinuation of treatment was planned. This event is associated with its own inherent increased risk of infection, elevating the risk in the time period after discontinuation. Further, the drug may have been discontinued because of symptoms of a serious infection, though the infection was only diagnosed some time later. This last scenario would also have resulted in an underestimation of the rate of serious infections in the anti-TNFα cohort in a “receiving treatment” analysis, and

### Table 3. Rates of serious infections obtained using model A: receiving treatment, limited to first 90 days of exposure*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Person-years</th>
<th>No. of infections</th>
<th>Rate per 1,000 person-years (95% CI)†</th>
<th>Adjusted incidence rate ratio (95% CI)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMARD</td>
<td>(n = 2,170)</td>
<td>532</td>
<td>24.4 (13.1–41.4)</td>
<td>Referent 4.1 (1.5–10.8)</td>
</tr>
<tr>
<td>Etanercept</td>
<td>(n = 3,844)</td>
<td>917</td>
<td>60.0 (45.5–77.4)</td>
<td>4.1 (1.5–10.8)</td>
</tr>
<tr>
<td>Infliximab</td>
<td>(n = 2,944)</td>
<td>723</td>
<td>95.4 (75.0–119.2)</td>
<td>5.6 (2.1–15.1)</td>
</tr>
<tr>
<td>Adalimumab</td>
<td>(n = 1,871)</td>
<td>451</td>
<td>59.9 (39.8–85.9)</td>
<td>3.9 (1.3–11.2)</td>
</tr>
<tr>
<td>All anti-TNFα</td>
<td>(n = 8,659)</td>
<td>2,091</td>
<td>72.2 (61.5–84.2)</td>
<td>4.6 (1.8–11.9)</td>
</tr>
</tbody>
</table>

* 95% CI = 95% confidence interval (see Table 1 for other definitions).
† Adjusted for age, sex, disease duration and severity, extraarticular rheumatoid arthritis, baseline steroid use, diabetes, chronic obstructive pulmonary disease, and smoking history.
the analysis of the 90-day lag window might be a better reflection of the true rate.

The influence of these selection variables on serious infection during the lag window makes it very difficult to explore the true pattern of infection risk for the anti-TNFα drugs once discontinued (Figures 1e–h). Such considerations also make it hard to compare the differing patterns between the drugs. For example, is there a longer lag window of risk for infliximab compared with etanercept, given its longer half-life? While it is possible to calculate the rates for lag windows set differently for the 3 drugs based on multiples of their half-lives, these rates are very difficult to interpret given the presence of other factors, such as the reason for drug discontinuation.

**Continued risk after discontinuation of treatment.** An important question to address in relation to anti-TNFα drugs is, assuming there is an increased risk of certain adverse events while taking the drug (and during a lag phase after discontinuation), whether that risk returns to baseline or is persistent. If it is persistent, for how long does the risk continue? Such an ongoing risk would be particularly relevant for adverse events such as malignancy, though less so for serious infections. It would be expected that any effect that anti-TNFα drugs have on susceptibility to infection would subside within months of discontinuing the drug. One way to explore this is to look at the serious infection rate for the entire followup period, regardless of the date of drug discontinuation (Figure 2c). Using this model, the rates of serious infection for the 3 anti-TNFα drugs were 61.7 per 1,000 person-years for etanercept, 68.9 per 1,000 person-years for infliximab, and 54.2 per 1,000 person-years for adalimumab (Table 2). These rates were also higher than those found using either the “receiving treatment” or the “duration of treatment plus 90-day lag window” analyses, as discussed above.

Again, these results seem counterintuitive. As with the lag phase analysis, the reason for drug discontinuation may influence the rate of subsequent infection, but this influence should decline with time. For example, if the treatment is stopped for planned surgery, the postoperative infection risk declines with time, and infection risk should return to presurgical levels.

The reason for the increased risk in this model may relate to the active treatment decisions made in an observational study. For patients who have a serious infection while receiving anti-TNFα therapy, clinicians will decide whether treatment should be resumed, based on their opinion of further infection risk. Thus, there is a “healthy drug continuers” or “depletion of susceptibles” effect, and restricting analysis to those who continue the drug selectively retains those at the lowest risk.

**DISCUSSION**

In a recently published report (5) we showed that the overall risk of severe infection was not increased following anti-TNFα therapy. In this extended analysis it is clear that there are several influences that make it very difficult to generate a robust answer to this superficially simple question. The risk of infection attributable to anti-TNFα therapy measured in an observational study cannot be adequately summarized as a single estimate. Large national registers have the capacity to reveal some of the patterns of adverse events that might otherwise be hidden behind such a point estimate.

It is important to be specific about aspects such as time period of interest during therapy, the choice of stop date, what allowance should be made for continuing pharmacologic action, and how to evaluate risk following these periods. Although standardization of analytical approach will help in comparing the findings of different studies, selection factors for both starting, and as we have shown, discontinuing therapy may seriously compromise interpretation.

In the future, defining stop dates will become even more difficult. Rituximab, an anti-CD20 B cell-depleting drug, is now licensed for the treatment of RA (8). It is given in 2 infusions 2 weeks apart, with its effects lasting for ~6–12 months. When can a patient be considered to be receiving or not receiving this treatment?

Despite these concerns, use of a consistent methodologic approach in all population registers is essential. However, it is equally important to avoid making oversimplistic conclusions about the infection risk conferred by these drugs.

**ACKNOWLEDGMENTS**

The authors acknowledge the enthusiastic collaboration of all consultant rheumatologists and their specialist nurses in the UK in providing the data used in this study. The substantial contribution of Andy Tracey and Katie McGrother in database design and manipulation is acknowledged. We also acknowledge Dr. Ian Griffiths (past Chairman of the BSRBR Management Committee), Professor David Isenberg (present Chairman of the BSRBR Management Committee), Professors Gabriel Panayi, David G. I. Scott, and David Isenberg, and Dr. Andrew Bamji (Presidents of the BSR) for their active role.
in supporting the Register, and Mervyn Hogg, Sam Peters, and the BSR staff for considerable administrative support.

AUTHOR CONTRIBUTIONS

Dr. Symmons had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Dixon, Symmons, Watson, Hyrich, Silman.

Acquisition of data. Dixon, Symmons, Watson, Hyrich, Silman.


ROLE OF THE STUDY SPONSOR

The BSRBR was established primarily to investigate the safety of biologic agents in routine practice. The financial support to the BSRBR comes indirectly from the following UK companies marketing biologic agents in the UK: Schering-Plough, Wyeth Laboratories, Abbott Laboratories, and Amgen, but the independence of the BSRBR and its investigators is assured in the following manner. The resources used to fund the BSRBR are received under contract by the BSR, which then provides a research grant under a separate contract to the University of Manchester, allowing the investigators normal academic freedom in relation to the data, their analysis, and use. Under the terms of the contract between the BSR and the sponsoring pharmaceutical companies, all publications are sent in advance to the companies prior to submission, for the purposes of information. The companies can, if they wish, point out factual errors. Any comments are vetted by 3 members of the steering committee, who decide whether they should be passed on to the authors. All publications are also reviewed by the BSR, but the material presented and the views expressed in all publications from the BSRBR are those of the authors and do not necessarily represent the views of the BSR.

REFERENCES


APPENDIX A: BSRBR CONTROL CENTRE CONSORTIUM

The BSRBR Control Centre Consortium consists of the following institutions (all in the UK): Antrim Area Hospital, Antrim (Nicola Maiden); Cannock Chase Hospital, Cannock Chase (Tom Price); Christchurch Hospital, Christchurch (Neil Hopkinson); Derbyshire Royal Infirmary, Derby (Sheila O’Reilly); Dewsbury and District Hospital, Dewsbury (Lesley Hordon); Freeman Hospital, Newcastle-upon-Tyne (Ian Griffiths); Gartnavel General Hospital, Glasgow (Duncan Porter); Glasgow Royal Infirmary, Glasgow (Hilary Capell); Haywood Hospital, Stoke-on-Trent (Andy Hassell); Hope Hospital, Salford (Romela Benitha); King’s College Hospital, London (Ernest Choy); Kings Mill Centre, Sutton-In-Ashfield (David Walsh); Leeds General Infirmary, Leeds (Paul Emery); Macclesfield District General Hospital, Macclesfield (Susan Knight); Manchester Royal Infirmary, Manchester (Ian Bruce); Musgrave Park Hospital, Belfast (Allister Taggart); Norfolk and Norwich University Hospital, Norwich (David Scott); Poole General Hospital, Poole (Paul Thompson); Queen Alexandra Hospital, Portsmouth (Fiona McCrae); Royal Glamorgan Hospital, Glamorgan (Rhian Goodfellow); Russells Hall Hospital, Dudley (George Kitas); Selly Oak Hospital, Selly Oak (Ronald Jubb); St. Helens Hospital, St. Helens (Rikki Abernethy); and Withington Hospital, Manchester (Paul Sanders).
Reduction in the Incidence of Myocardial Infarction in Patients With Rheumatoid Arthritis Who Respond to Anti–Tumor Necrosis Factor α Therapy

Results From the British Society for Rheumatology Biologics Register


Objective. Rheumatoid arthritis (RA) is associated with an increased risk of coronary artery disease, possibly acting via shared mechanisms of inflammation. This study was undertaken to test the hypothesis that the powerful antiinflammatory effect of anti–tumor necrosis factor (anti-TNFα) therapy might lead to a reduction in the incidence of myocardial infarction (MI) in patients with RA.

Methods. Using data from the British Society for Rheumatology Biologics Register, a national prospective observational study, we compared MI rates in 8,670 patients with RA treated with anti-TNFα and 2,170 patients with active RA treated with traditional disease-modifying antirheumatic drugs (DMARDs).

Results. Through July 2006, 63 MIs occurred in the anti-TNFα cohort during 13,233 person-years of followup and 17 MIs occurred in the DMARD cohort during 2,893 person-years of followup, equivalent to a rate of 4.8 events per 1,000 person-years and 5.9 events per 1,000 person-years, respectively. After adjustment for baseline risk factors, there was no reduction in the rate of MI in the anti-TNFα cohort compared with the DMARD cohort (incidence rate ratio 1.44 [95% confidence interval 0.56–3.67]). In an analysis of anti-TNFα–treated patients who responded to the treatment within 6 months versus those who did not, MI rates were found to be 3.5 events per 1,000 person-years in responders and 9.4 events per 1,000 person-years in nonresponders. The adjusted incidence rate ratio (95% confidence interval) for responders compared with nonresponders was 0.36 (0.19–0.69).

Conclusion. These results indicate that RA patients treated with anti-TNFα do not have a lower incidence of MI compared with RA patients treated with traditional DMARDs. However, the risk of MI is markedly reduced in those who respond to anti-TNFα therapy by 6 months compared with nonresponders. This finding supports the notion that inflammation plays a pivotal role in MI.

It is now well established that rheumatoid arthritis (RA) is associated with increased mortality and morbidity due to accelerated atherosclerosis, including from myocardial infarction (MI) (1–5). This increased risk cannot be attributed to traditional cardiovascular risk factors, such as smoking and hypertension, alone (2,5,6). There is mounting evidence that the increased risk is related to the overall burden of inflammatory disease in RA (7,8). In addition, atherosclerosis itself
is increasingly being viewed as an inflammatory condition (9).

The cytokine tumor necrosis factor α (TNFα) plays a key role in the pathogenesis of RA (10). Introduction of the anti-TNFα therapies infliximab, etanercept, and adalimumab has dramatically improved the outcome of severe RA beyond that achieved with traditional disease-modifying antirheumatic drugs (DMARDs) (11–13). Proinflammatory cytokines, including TNFα, are involved in modification of lipid profile and insulin resistance (14) and the initiation and progression of atherosclerosis (15,16), hemostasis (17), and atherosclerotic plaque rupture, the most common event leading to an acute MI (15). Inhibition of TNFα in patients with RA may therefore lead to a reduction in MI rates by inhibiting one or more of these mechanisms. However, some patients do not respond well to anti-TNFα drugs. Therefore, we hypothesized that any reduction in the incidence of MI would be limited to those patients who displayed a good clinical response to TNFα.

The aims of this study were, first, to determine whether the incidence of MI in RA patients treated with anti-TNFα was lower than that in patients treated with traditional DMARDs and, second, to explore the impact of response to treatment on the rates of MI in the anti-TNFα cohort. To date, 1 study has shown a reduced rate of all cardiovascular events following anti-TNFα therapy (18), but no published studies have so far explored MI incidence or the influence of treatment response.

PATIENTS AND METHODS

Patients. Subjects were participants in a large national prospective observational study, the British Society for Rheumatology Biologics Register (BSRBR). Methods of patient recruitment and followup have been described in detail elsewhere (19). Briefly, the study aims to recruit all UK patients with rheumatic diseases treated with biologic agents, and an appropriate comparison group, in order to examine the long-term safety of these drugs. UK national guidelines recommend that anti-TNFα drugs be reserved for patients with active RA, defined as a Disease Activity Score in 28 joints (DAS28) (20) >5.1 despite previous therapy with at least 2 DMARDs, one of which should be methotrexate (21), and that “any clinician prescribing these medications must (with the patient’s permission) undertake to register the patient with the [BSRBR] and forward information on dosage, outcome and toxicity on a six-monthly basis” (22).

Anti-TNFα cohort. This cohort was restricted to patients registered with the BSRBR who were diagnosed as having RA and were treated with an anti-TNFα drug. Patients who were registered with the BSRBR >6 months after the start of biologic therapy were excluded. Patients had to have been followed up for ≥6 months by July 31, 2006.

Comparison cohort. A cohort of patients with active RA who have never taken biologic agents is being recruited in parallel with the anti-TNFα cohort by the BSRBR Control Centre Consortium (see Appendix A for a list of BSRBR Control Centre Consortium members). The comparison cohort is followed up using the same methodology used to follow up the anti-TNFα cohort (19). The patients in the comparison cohort were diagnosed as having active RA (guideline DAS28 ≥4.2) despite current treatment with a traditional DMARD. These patients also had to have been followed up for ≥6 months by July 31, 2006.

Baseline assessment. Baseline data assessed in both cohorts included demographic characteristics, disease duration, 28-joint counts for swelling and tenderness, erythrocyte sedimentation rate and/or C-reactive protein level, and patient global assessment, and enabled calculation of a DAS28 score (20). Details of all previous and current DMARD therapy and all other current medications were obtained. Patients completed a Health Assessment Questionnaire (HAQ) adapted for British use (23). Townsend scores of multiple deprivation were calculated based on the patient’s area of residence and compared with UK quintiles (24). Data were also collected on other variables that might influence cardiovascular risk, including all baseline drugs, body mass index (BMI), prior cardiovascular comorbidity (including previous MI, angina, and hypertension), diabetes, and smoking history.

Followup. Data on changes in therapy, disease activity, and the occurrence of adverse events were collected in 3 ways. Rheumatologists were sent a questionnaire every 6 months, patients were sent a 6-month diary in which to document all hospital admissions, new medications, and new hospital referrals, and all patients were flagged with the UK General Register Office, which provides the BSRBR with information on deaths and cause of death (coded according to the International Statistical Classification of Diseases and Related Health Problems, Tenth Revision) (25). If an MI was reported from any of the 3 sources, further supporting information, such as a hospital discharge summary, was requested from the rheumatologist.

All available clinical information on the MIs was reviewed by 2 physicians (WGD and KLH) independently to verify the diagnosis according to an adapted European Society of Cardiology (ESC)/American College of Cardiology (ACC) definition (26). ESC/ACC criteria define an acute, evolving, or recent MI as a typical rise and fall of biochemical markers of myocardial necrosis with ≥1 of the following: ischemic symptoms, Q waves, ischemic electrocardiography changes, coronary artery intervention, or pathologic findings of an acute MI. Additional BSRBR verification criteria were thrombolysis and/or MI recorded on death certificate, regardless of autopsy confirmation. Any disagreement was resolved by consensus following discussion. Only verified MIs were included in the analysis.

Statistical analysis. Response to treatment in the anti-TNFα cohort was defined according to European League Against Rheumatism criteria (27). Responders were those patients who achieved either a good or a moderate response, i.e., a reduction in the DAS28 score from baseline to 6 months of >1.2, or a reduction of ≥0.6 in addition to a DAS28 score of ≤5.1 at 6 months. DAS28 scores were not measured at 6
months in the DMARD cohort, and thus response could not be assessed in this group.

For patients in the anti-TNFα cohort, person-years of followup included only the time during which they were actively treated with the first anti-TNFα drug. The date of drug discontinuation was defined as the date of the first missed dose. Person-years were calculated from the first day of anti-TNFα therapy to the date of the most recent completed followup form prior to July 31, 2006, for patients initially registered in the comparison cohort who subsequently received an anti-TNFα drug. Person-years of followup in the comparison cohort included the time up to the date the anti-TNFα drug was started, and person-years of followup in the anti-TNFα cohort included the subsequent time, during which they were actively treated with the anti-TNFα drug.

Incidence rates of MI are presented as events per 1,000 person-years, with 95% confidence intervals (95% CIs). Incidence rate ratios were calculated using Poisson regression, initially comparing the anti-TNFα cohort with the DMARD cohort, and then comparing responders with nonresponders within the anti-TNFα cohort. Stepwise adjustment was performed, first for age and sex, and then additionally for RA disease severity (using baseline DAS28 score, HAQ, and disease duration as continuous variables), BMI, social deprivation (Townsend quintiles), cardiovascular comorbidity (previous MI, angina, hypertension), diabetes, smoking status (current, ever, or never), and baseline use of selected drugs.

### Table 1. Baseline characteristics of the DMARD-treated and anti-TNFα-treated patients*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>DMARD (n = 2,170)</th>
<th>All anti-TNFα (n = 8,659)</th>
<th>Anti-TNFα nonresponders (n = 1,638)</th>
<th>Anti-TNFα responders (n = 5,877)</th>
<th>P†‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean ± SD years</td>
<td>60 ± 12</td>
<td>56 ± 12 ⁺</td>
<td>57 ± 12</td>
<td>56 ± 12</td>
<td>0.002</td>
</tr>
<tr>
<td>Sex, % female</td>
<td>72</td>
<td>76</td>
<td>79</td>
<td>76</td>
<td>0.01</td>
</tr>
<tr>
<td>DAS28 score, mean ± SD</td>
<td>5.0 ± 1.4</td>
<td>6.6 ± 1.0‡</td>
<td>6.4 ± 1.1</td>
<td>6.6 ± 1.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HAQ score, mean ± SD</td>
<td>1.5 ± 0.8</td>
<td>2.1 ± 0.6‡</td>
<td>2.2 ± 0.5</td>
<td>2.0 ± 0.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Disease duration, median (IQR) years</td>
<td>7 (1–15)</td>
<td>12 (6–19)‡</td>
<td>11 (6–19)</td>
<td>11 (6–19)</td>
<td>0.64</td>
</tr>
<tr>
<td>BMI, mean ± SD kg/m²</td>
<td>26.9 ± 5.7</td>
<td>26.7 ± 5.8‡</td>
<td>26.9 ± 6.2</td>
<td>26.7 ± 5.7</td>
<td>0.27</td>
</tr>
<tr>
<td>Smoking history, no. (%)¶</td>
<td>Current smoker</td>
<td>537 (25)</td>
<td>1,886 (22)</td>
<td>382 (23)</td>
<td>1,231 (21)</td>
</tr>
<tr>
<td>Former smoker</td>
<td>849 (39)</td>
<td>3,298 (38)</td>
<td>625 (38)</td>
<td>2,241 (38)</td>
<td></td>
</tr>
<tr>
<td>Never smoked</td>
<td>767 (35)</td>
<td>3,431 (40)</td>
<td>621 (38)</td>
<td>2,369 (40)</td>
<td></td>
</tr>
<tr>
<td>Cardiovascular comorbidity</td>
<td>Prior MI, no. (%)</td>
<td>116 (5.3)</td>
<td>250 (2.9)‡</td>
<td>48 (2.9)</td>
<td>154 (2.6)</td>
</tr>
<tr>
<td>Angina, no. (%)</td>
<td>183 (8.4)</td>
<td>381 (4.4)‡</td>
<td>85 (5.2)</td>
<td>240 (4.1)</td>
<td>0.04</td>
</tr>
<tr>
<td>Hypertension, no. (%)</td>
<td>672 (31.0)</td>
<td>2,581 (29.8)</td>
<td>506 (30.9)</td>
<td>1,731 (29.4)</td>
<td>0.19</td>
</tr>
<tr>
<td>Diabetes, no. (%)</td>
<td>132 (6.1)</td>
<td>470 (5.4)</td>
<td>110 (6.7)</td>
<td>287 (4.9)</td>
<td>0.003</td>
</tr>
<tr>
<td>Corticosteroids, no. (%)</td>
<td>418 (19.3)</td>
<td>3,793 (43.7)‡</td>
<td>743 (45.3)</td>
<td>2,519 (42.9)</td>
<td>0.06</td>
</tr>
<tr>
<td>Lipid-lowering drugs, no. (%)</td>
<td>338 (15.6)</td>
<td>768 (8.9)‡</td>
<td>159 (9.7)</td>
<td>502 (8.5)</td>
<td>0.14</td>
</tr>
<tr>
<td>Antiplatelet drugs, no. (%)</td>
<td>291 (13.4)</td>
<td>648 (7.5)‡</td>
<td>130 (7.9)</td>
<td>418 (7.1)</td>
<td>0.26</td>
</tr>
<tr>
<td>NSAIDs, no. (%)¶</td>
<td>1,344 (61.9)</td>
<td>5,705 (65.9)**</td>
<td>995 (60.8)</td>
<td>3,961 (67.4)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Townsend quintile, no. (%)¶††</td>
<td>1</td>
<td>314 (14.5)</td>
<td>1,388 (16.0)</td>
<td>260 (15.9)</td>
<td>973 (16.6)</td>
</tr>
<tr>
<td>2</td>
<td>301 (13.9)</td>
<td>1,469 (17.0)</td>
<td>250 (15.3)</td>
<td>1,021 (17.4)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>382 (17.6)</td>
<td>1,633 (18.9)</td>
<td>279 (17.0)</td>
<td>1,154 (19.6)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>443 (20.4)</td>
<td>1,881 (21.7)</td>
<td>380 (23.2)</td>
<td>1,263 (21.5)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>655 (30.2)</td>
<td>1,933 (22.3)</td>
<td>402 (24.6)</td>
<td>1,217 (20.7)</td>
<td></td>
</tr>
</tbody>
</table>

* The number of anti–tumor necrosis factor α (anti-TNFα) responders and the number of anti-TNFα nonresponders do not equal the total number of anti-TNFα–treated patients, since information on the change in Disease Activity Score in 28 joints (DAS28) from 0 to 6 months was not available in 1,153 patients. For smoking history, prior myocardial infarction (MI), angina, hypertension, diabetes, corticosteroids, lipid-lowering drugs, antiplatelet drugs, nonsteroidal antiinflammatory drugs (NSAIDs), and Townsend quintiles, data were not available in all patients. HAQ = Health Assessment Questionnaire; IQR = interquartile range; BMI = body mass index.

† Anti-TNFα nonresponders versus anti-TNFα responders.
‡ P < 0.001 versus disease-modifying antirheumatic drug (DMARD)–treated patients.
¶ P = 0.04 versus DMARD–treated patients.
# P for trend < 0.001.
** P = 0.001 versus DMARD-treated patients.
†† Quintile 1 represents the least socially deprived; quintile 5 represents the most socially deprived.
(corticosteroids, nonsteroidal antiinflammatory drugs, lipid-lowering drugs, and antiplatelet drugs). All analyses were performed using Stata, version 8.2 software (StataCorp, College Station, TX).

**RESULTS**

A total of 10,755 patients were included in the analysis: 8,659 in the anti-TNFα/H9251 cohort (3,844 receiving etanercept, 2,944 receiving infliximab, and 1,871 receiving adalimumab) and 2,170 in the comparison cohort. Seventy-four patients switched from the comparison cohort to the anti-TNFα/H9251 cohort and were included in both cohorts. Baseline characteristics are shown in Table 1. The comparison cohort included proportionally more men, and patients in this cohort were older than those in the anti-TNFα group. As anticipated, patients in the comparison cohort had less severe disease of shorter duration and had a lower rate of steroid use at baseline. There were significantly higher rates of previous MI and angina in the DMARD cohort, and significantly higher rates of use of lipid-lowering and antiplatelet drugs. Proportionally more patients in the DMARD cohort were in the most socially deprived quintile. As mentioned above, these differences were adjusted for in subsequent analyses.

The median followup was 1.66 years in the anti-TNFα cohort and 1.34 years in the DMARD cohort. There were 69 reported MIs in the anti-TNFα cohort compared with 20 in the comparison cohort. Of these, 63 and 17, respectively, were verified (Table 2). The crude incidence rate of MI was lower in the anti-TNFα cohort (4.8 events per 1,000 person-years) than in the DMARD cohort (5.9 events per 1,000 person-years), equivalent to an incidence rate ratio (95% CI) of 0.81 (0.47–1.38). However, after adjustment for age and sex, the incidence rate ratio increased to 1.13, and further adjustment for RA disease severity, social deprivation, traditional cardiovascular risk factors, and relevant baseline drug use resulted in an incidence rate ratio (95% CI) of 1.44 (0.56–3.67). Inclusion of the nonverified MIs in the analysis resulted in an adjusted incidence rate ratio.

<table>
<thead>
<tr>
<th>Table 2. Incidence rates of verified first MI in DMARD-treated and anti-TNFα-treated patients*</th>
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<tbody>
<tr>
<td></td>
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<tr>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>DMARD (n = 2,170)</td>
</tr>
<tr>
<td>Person-years</td>
</tr>
<tr>
<td>No. of reported MIs</td>
</tr>
<tr>
<td>Rate of MIs per 1,000 person-years (95% CI)</td>
</tr>
<tr>
<td>Incidence rate ratio</td>
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<tr>
<td>Incidence rate ratio, adjusted for age and sex</td>
</tr>
<tr>
<td>Incidence rate ratio, multivariate analysis†</td>
</tr>
<tr>
<td>DMARD (n = 615)</td>
</tr>
<tr>
<td>Person-years</td>
</tr>
<tr>
<td>No. of reported MIs</td>
</tr>
<tr>
<td>Rate of MIs per 1,000 person-years (95% CI)</td>
</tr>
<tr>
<td>Incidence rate ratio</td>
</tr>
<tr>
<td>Incidence rate ratio, multivariate analysis†</td>
</tr>
<tr>
<td>DMARD (n = 1,555)</td>
</tr>
<tr>
<td>Person-years</td>
</tr>
<tr>
<td>No. of reported MIs</td>
</tr>
<tr>
<td>Rate of MIs per 1,000 person-years (95% CI)</td>
</tr>
<tr>
<td>Incidence rate ratio</td>
</tr>
<tr>
<td>Incidence rate ratio, adjusted for age and sex</td>
</tr>
<tr>
<td>Incidence rate ratio, multivariate analysis†</td>
</tr>
</tbody>
</table>

* 95% CI = 95% confidence interval (see Table 1 for other definitions).
† Adjusted for age, sex, disease severity, body mass index, social deprivation, smoking history, comorbidity, and baseline drug use.

<table>
<thead>
<tr>
<th>Table 3. Incidence rates of verified first MI in nonresponders and responders to anti-TNFα treatment*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<tr>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>Person-years</td>
</tr>
<tr>
<td>No. of reported MIs</td>
</tr>
<tr>
<td>Rate of MIs per 1,000 person-years (95% CI)</td>
</tr>
<tr>
<td>Incidence rate ratio</td>
</tr>
<tr>
<td>Incidence rate ratio, adjusted for age and sex</td>
</tr>
<tr>
<td>Incidence rate ratio, multivariate analysis†</td>
</tr>
<tr>
<td>Incidence rate ratio by sex, multivariate analysis†</td>
</tr>
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<td></td>
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</tbody>
</table>

* 95% CI = 95% confidence interval (see Table 1 for other definitions).
† Adjusted for age, sex, disease severity, body mass index, social deprivation, smoking history, comorbidity, and baseline drug use.
REDUCTION IN MI RATE IN RA PATIENTS WHO RESPOND TO ANTI-TNFα THERAPY

DISCUSSION

We have shown that there is no overall reduction in MI rate in the short term in anti-TNFα–treated RA patients, compared with DMARD-treated patients with active RA who had never taken biologic agents. However, in RA patients who responded to anti-TNFα therapy, the risk of MI was reduced by more than half compared with nonresponders. This is consistent with the hypothesis that suppression of inflammation may reduce cardiovascular risk. Because we did not measure DAS28 scores in the DMARD cohort at 6 months, we cannot determine whether reduction in RA disease activity secondary to any treatment is associated with a reduction in MI incidence, or whether this finding is specific to anti-TNFα therapy.

This early reduction in MI risk in responders cannot be extrapolated to indicate a long-term beneficial cardiac effect. Raised levels of TNFα in chronic heart failure (28), along with the predictive value of high levels of TNFα on adverse outcome in chronic heart failure (29), led to studies to explore the potential benefit of anti-TNFα therapy in severe chronic heart failure. Contrary to expectations, 2 studies of etanercept had to be terminated early when interim analysis showed a lack of efficacy (30), and high-dose infliximab was shown to be detrimental in patients with moderate to severe chronic heart failure (31).

After MI, TNFα inhibition may potentially have both harmful and beneficial effects on myocardial function. TNFα has a protective role in the physiologic adaptive response to injury and limits infarct size (32), although when overexpressed it can lead to maladaptive effects, such as promoting left ventricular dysfunction (33). Evidence of the effect of anti-TNFα therapy on heart failure in RA patients without preexisting heart failure is currently very limited.

Despite the pleiotropic effects of TNFα inhibition on the heart, the focus of the current investigation was solely on MI rates during this early period of exposure. Given the link between plaque inflammation and plaque rupture (34), it is plausible that there might be an early reduction in MI incidence following anti-TNFα treatment, while potential effects on heart failure, if any, may only be noticeable over a longer time period.

There are important methodologic issues which must be considered when interpreting these data. The BSRBR is an observational study aimed at nationwide ascertainment of data related to anti-TNFα treatment, using an appropriate comparison group. As anticipated, patients in the anti-TNFα cohort had more severe disease. This difference should, if anything, have placed the anti-TNFα cohort at increased risk of MI. Conversely, the comparison cohort had higher rates of baseline cardiovascular risk factors, with greater baseline prevalence of ischemic heart disease, a higher percentage of current smokers, and a higher rate of self-reported lipid-lowering and antiplatelet drug use. We adjusted for these differences in the analysis. Nonetheless, better definition of cardiovascular risk, such as knowledge of actual cholesterol levels, may have attenuated the observed protection in our responder group.

Although most traditional cardiovascular risk factors were captured, exercise was not measured in the present study. Responders would be better able to exercise than nonresponders, lowering their cardiovascular risk. However, such a difference would only be apparent following their response to treatment and is not likely to have a large bearing on MI rates within this short followup period.

Anti-TNFα–treated patients were categorized according to the change in DAS28 score from baseline to 6 months. These data were missing at 1 or both time points in 1,153 patients (~15%), and we therefore
explored whether this missing data led to any bias. The MI rate in the patients without a categorized response was 7.7 events per 1,000 person-years. This rate is approximately halfway between the observed rates in the responder and nonresponder groups, suggesting that these data may be missing at random.

The BSRBR has a number of strengths. The size of the cohort far exceeds that of any anti-TNFα clinical trial. The aim is to recruit all RA patients in the UK treated with these agents and thus represent real-life practice. It is a condition of prescribing these agents in the UK National Health Service that the rheumatologist register the patient with the BSRBR (22). Although we cannot accurately assess the completeness of registration, estimates from various sources have suggested a capture rate of \( \geq 70\% \).

It is important to remember that the risk of MI is increased in patients with RA independent of the treatment they receive. The inclusion of a cohort of DMARD-treated patients who had never taken biologic drugs, which would not be possible if MI rates were compared with those in the general population. By using 3 sources of identification of MIs, we increased the chances of identifying all adverse events. Prior to verification, there were 20 MIs reported in the DMARD cohort and 69 in the anti-TNFα cohort. Following collection of available clinical information, 59 (10 in the DMARD cohort and 49 in the anti-TNFα cohort) were verified according to the ESC/ACC criteria. A further 21 (7 in the DMARD cohort and 14 in the anti-TNFα cohort) had either a death certificate diagnosis of MI or received thrombolysis. There was insufficient clinical information available on the remainder (3 in the DMARD cohort and 6 in the anti-TNFα cohort) to allow verification. A secondary analysis including these few nonverified MIs did not noticeably change the results.

It is also appropriate to determine whether the observed MI rate in the comparison group was similar to the expected outcome, which would enhance the external validity of the result. When compared with published data on MI rates in the general population (35) and the risk conferred by RA (5,36), the observed rates in the DMARD cohort of 12.0 and 3.4 events per 1,000 person-years for men and women, respectively, suggest that there was no major under- or overascertainment. Our findings are also consistent with those of a recent study from Sweden (18), which examined the effect of anti-TNFα therapy on all cardiovascular events combined and showed a nonsignificant decrease in the incidence of first-time severe cardiovascular events. Low numbers of events (n = 13) and a short followup time (656 person-years) in the anti-TNFα cohort in that study precluded further analysis (18).

This study showed no protective effect against MI in RA patients treated with anti-TNFα therapy compared with patients treated with traditional DMARDs, after adjustment for baseline risk. However, the suppression of joint disease with anti-TNFα therapy in RA patients may be associated with an early reduced risk of MI. This finding supports the notion that inflammation plays a pivotal role in the pathophysiology of MI.

ACKNOWLEDGMENTS

The authors acknowledge the enthusiastic collaboration of all consultant rheumatologists and their specialist nurses in the UK in providing the data used in this study. The substantial contribution of Andy Tracey and Katie McGrother in database design and manipulation is acknowledged. We also acknowledge Dr. Ian Griffiths (past Chairman of the BSRBR Management Committee), Professor David Isenberg (present Chairman of the BSRBR Management Committee), Professors Gabriel Panayi, David G. I. Scott, and David Isenberg, and Dr. Andrew Bamji (Presidents of the BSR) for their active role in supporting the Register, and Mervyn Hogg, Sam Peters, and the BSR staff for considerable administrative support.

AUTHOR CONTRIBUTIONS

Dr. Symmons had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.


Acquisition of data. Dixon, Watson, Hyrich, Symmons.


ROLE OF THE STUDY SPONSOR

The BSRBR was established primarily to investigate the safety of biologic agents in routine practice. The financial support to the BSRBR comes indirectly from the following UK companies marketing biologic agents in the UK: Schering-Plough, Wyeth Laboratories, Abbott Laboratories, and Amgen, but the independence of the BSRBR and its investigators is assured in the following manner. The resources used to fund the BSRBR are received under contract by the BSR, which then provides a research grant under a separate contract to the University of Manchester, allowing the investigators normal academic freedom in relation to the data, their analysis, and use. Under the terms of the contract between the BSR and the sponsoring pharmaceutical companies, all publications are sent in advance to the companies prior to submission, for the purposes of information. The companies can, if they wish, point out factual errors. Any comments are vetted by 3 members of the steering committee, who decide whether they should be passed on to the authors. All publications are also
reviewed by the BSR, but the material presented and the views expressed in all publications from the BSRBR are those of the authors and do not necessarily represent the views of the BSR.

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APPENDIX A: BSRBR CONTROL CENTRE CONSORTIUM

The BSRBR Control Centre Consortium consists of the following institutions (all in the UK): Antrim Area Hospital, Antrim (Nicola Maiden); Cannock Chase Hospital, Cannock Chase (Tom Price); Christchurch Hospital, Christchurch (Neil Hopkinson); Derbyshire Royal Infirmary, Derby (Sheila O’Reilly); Dewsbury and District Hospital, Dewsbury (Lesley Hordon); Freeman Hospital, Newcastle-upon-Tyne (Ian Griffiths); Gartnavel General Hospital, Glasgow (Duncan Porter); Glasgow Royal Infirmary, Glasgow (Hilary Capell); Haywood Hospital, Stoke-on-Trent (Andy Haswell); Hope Hospital, Salford (Romela Benitha); King’s College Hospital, London (Ernest Choy); Kings Mill Centre, Sutton-in-Ashfield (David Walsh); Leeds General Infirmary, Leeds (Paul Emery); Maclesfield District General Hospital, Macclesfield (Susan Knight); Manchester Royal Infirmary, Manchester (Ian Bruce); Musgrave Park Hospital, Belfast (Allister Taggart); Norfolk and Norwich University Hospital, Norwich (David Scott); Poole General Hospital, Poole (Paul Thompson); Queen Alexandra Hospital, Portsmouth (Fiona McCrae); Royal Glamorgan Hospital, Glamorgan (Rhian Goodfellow); Russells Hall Hospital, Dudley (George Kitas); Selly Oak Hospital, Selly Oak (Ronald Jubb); St. Helens Hospital, St. Helens (Rikki Abernethy); and Withington Hospital, Manchester (Paul Sanders).
Association of Smoking With the Constitution of the Anti–Cyclic Citrullinated Peptide Response in the Absence of HLA–DRB1 Shared Epitope Alleles


Objective. Smoking is a risk factor for anti–cyclic citrullinated peptide (anti-CCP) antibody–positive rheumatoid arthritis (RA) in patients with HLA–DRB1 shared epitope (SE) alleles. It is unknown whether smoking influences not only the presence of these antibodies, but also other characteristics of the anti-CCP response, such as isotype usage. The aim of this study was to determine the influence of smoking on anti-CCP isotypes in RA patients, and to determine whether this influence is observed in the presence and/or absence of SE alleles.

Methods. IgA, IgM, and IgG subclasses of anti-CCP antibodies were measured by enzyme-linked immunosorbent assay in serum obtained at the first visit to the Leiden Early Arthritis Clinic from 216 patients with anti-CCP–positive RA whose smoking habits were also assessed. HLA genotyping data were available for 202 of these patients.

Results. IgA and IgM anti-CCP were more frequent in RA patients who were smokers than in those who were nonsmokers (odds ratio 2.8 and 1.8, respectively). In addition, levels of all isotypes of anti-CCP, except IgG3, were significantly higher ($P < 0.05$) in smokers. The number of anti-CCP isotypes was higher in smokers compared with nonsmokers, both in SE-negative RA ($P = 0.04$) and in SE-positive RA ($P = 0.07$).

Conclusion. Patients with anti-CCP–positive RA who have a current or former tobacco exposure display a more extensive anti-CCP isotype usage in general, and IgA and IgM in particular, compared with patients with anti-CCP–positive RA who have never smoked. In contrast to its influence on the incidence of anti-CCP positivity, the influence of tobacco exposure on the constitution of the anti-CCP response is significant in SE-negative RA. These findings suggest a differential effect of tobacco exposure on the induction as compared with the propagation of the anti-CCP antibody response.

Antibodies against citrullinated proteins are thought to play a pivotal role in the progression of rheumatoid arthritis (RA) because they are highly specific and predictive of RA (1), are associated with the extent of joint destruction (2), and have been shown to enhance disease severity in mice with experimental arthritis (3). The most prominent genetic risk factors for RA, the HLA–DRB1 shared epitope (SE) alleles, encode for a common amino acid sequence in the peptide presenting part of the HLA class II molecule. These SE alleles have been described recently to be a risk factor for the development of anti–cyclic citrullinated peptide (anti-CCP) antibodies, rather than for anti-CCP–positive RA per se (4).
disease expression, disease activity, and radiologic joint damage (6,7). However, tobacco exposure has been associated with anti-CCP–positive RA only, as opposed to RA in general. This association was only observed in the context of SE alleles, and not with SE-negative RA, thus demonstrating a gene–environment interaction between the HLA–SE and smoking (8,9). Together, these observations were the basis for the hypothesis, first postulated by Klareskog et al (9), that smoking may trigger RA-specific immune reactions to citrullinated proteins, possibly by inducing citrullination of damaged, dying cells in the bronchoalveolar tract.

The observation that the gene–environment interaction between HLA–SE alleles and smoking was only present in anti-CCP–positive disease (9) makes it attractive to speculate that smoking may affect not only the presence, but also the “nature” of the anti-CCP response. For example, it is conceivable that the contribution of HLA–SE alleles to the association between smoking and anti-CCP–positive disease is routed through CD4+ T helper cells, which influence the magnitude and/or quality of the citrullinated protein–directed B cell responses and thereby the overall anti-CCP response.

We have recently shown that the levels of anti-CCP antibodies in patients with SE-positive, anti-CCP–positive arthritis who smoked were higher compared with those in patients with SE-positive, anti-CCP–positive arthritis who never smoked (10). However, no information is available on the constitution of the anti-CCP response with respect to, for example, isotype usage as a characteristic of the anti-CCP response. This information could be of relevance because it might provide novel details on the relationship between anti-CCP antibodies, the HLA–SE, and smoking in patients with RA, and subsequently may increase the understanding of how tobacco exposure contributes to the development and progression of RA.

Smoking is associated with a higher prevalence of citrullinated proteins in cells obtained by bronchoalveolar lavage (9), presumably caused by abundant protein citrullination in damaged cells. Therefore, the effect of smoking on the anti-CCP response could be mediated through modulation of citrulline-directed immune responses in the bronchus-associated lymphoid tissue (BALT). We hypothesized that the prevalence of IgA anti-CCP, and possibly other isotypes, would differ between RA patients with and those without tobacco exposure, since IgA is an isotype that is typically, although not exclusively, produced in mucosa-associated lymphoid tissue such as BALT.

Levels of total IgG anti-CCP are commonly measured in studies and in daily clinical practice. However, little information on the IgA, IgM, and subclasses of IgG anti-CCP antibodies is available, in contrast to the extensive study findings on the isotypes used by rheumatoid factor (RF)–producing B cells. IgA-RF has been reported to be associated with a more severe disease outcome, and smokers have been reported to produce more IgM-RF and IgA-RF as compared with the levels of these isotypes in nonsmokers (7).

In this study we first addressed whether the isotype usage in patients with anti-CCP–positive RA who were smokers differed from the isotype usage in patients with anti-CCP–positive RA who were nonsmokers, focusing especially on the participation of IgA in the anti-CCP response. We then analyzed whether this influence of tobacco exposure on isotype usage depended on the presence of HLA–DRB1 SE alleles, as was recently described with respect to the influence of tobacco exposure on the presence of IgG anti-CCP antibodies in RA patients. Our data demonstrate that smoking influences the pattern of isotype usage in the anti-CCP response, and that this effect is not limited to SE-positive RA.

PATIENTS AND METHODS

Study population and serum samples. Patients having received a diagnosis of RA within the first year after their initial clinic visit were selected from the Leiden Early Arthritis Clinic (EAC), which provides an inception cohort of patients with recent-onset arthritis (duration of symptoms <2 years). The EAC was started at the Department of Rheumatology of the Leiden University Medical Center in 1993 and is described in detail by van Aken et al (11). RA was diagnosed according to the American College of Rheumatology (ACR; formerly, the American Rheumatism Association) 1987 revised criteria for the classification of RA (12).

At the first EAC visit, serum samples were obtained and smoking history (all sorts of active tobacco exposure) was assessed by means of patient questionnaires. Patients who were current smokers and those with a history of smoking were classified as smokers, while those who had never smoked were classified as nonsmokers. Patients for whom baseline serum samples and smoking history were available were selected for the present study (n = 416). Anti-CCP antibody isotypes were assessed in IgG anti-CCP–positive patients only, resulting in a cohort of 216 patients for inclusion in the present study. Among the 216 patients with anti-CCP–positive RA, 202 had data available on the HLA genotype. Patients provided their informed consent, and the study was approved by the local review board of medical ethics.

Isotypes of anti-CCP antibodies. Total IgG anti-CCP antibodies were assessed by an enzyme-linked immunosorbent assay (ELISA) (Immunoscan RA Mark 2; Euro-Diagnostica,
Arnhem, The Netherlands). The cutoff for IgG anti-CCP positivity was set at a level of 25 units/ml, according to the manufacturer's instructions.

Levels of the IgG subclasses of anti-CCP as well as levels of IgA and IgM anti-CCP were determined by a sandwich ELISA technique as described previously (13). Briefly, microtiter plates coated with CCPs (Immunoscan RA Mark 2; Euro-Diagnostica) were incubated with the patients' serum. The next incubation step was performed with conjugated polyclonal antibodies for the detection of IgM and IgA (AHI 0605 and AHI 0105; BioSource International, Camarillo, CA), and unconjugated mouse monoclonal antibodies followed by conjugated rabbit anti-mouse Ig for the detection of the IgG subclasses. A series of successive dilutions of pooled patient sera was used as a reference standard in all plates. Microtiter plates coated with uncitrullinated control peptide (Euro-Diagnostica) were used as a control for citrulline specificity of the antibodies.

Cutoff values for the presence of the different isotypes of anti-CCP antibodies were defined as the mean plus 2 SD in serum samples of a group of 50 IgG anti-CCP–negative control subjects, and were corrected for a high background level of response against the control peptide, as described previously (13). The cutoff values for positivity were as follows: 25 units/ml for IgA anti-CCP, 32 units/ml for IgM anti-CCP, 2 units/ml for IgG1 anti-CCP, 20 units/ml for IgG2 anti-CCP, 52 units/ml for IgG3 anti-CCP, and 0.1 units/ml for IgG4 anti-CCP.

HLA genotyping. The HLA–DRB1 alleles were determined in 202 patients with anti-CCP–positive RA. HLA–DRB1 (sub)typing was performed by polymerase chain reaction using specific primers and hybridization with sequence-specific oligonucleotides as previously described (14). The SE alleles were DRB1*0101, *0102, *0104, *0401, *0404, *0405, *0408, *1001, and *1402.

Statistical analysis. Odds ratios (ORs) were calculated by comparing patients whose serum was positive and patients whose serum was negative for the different anti-CCP isotypes. Differences in levels of anti-CCP isotypes and differences in the number of anti-CCP isotypes were analyzed using the Mann-Whitney U test. SPSS software, version 12.0 (SPSS, Chicago, IL) was used for all statistical analyses. In all tests, P values less than 0.05 were considered significant.

RESULTS

Different classes and subclasses of anti-CCP antibodies were determined in 216 patients with anti-CCP–positive RA to determine whether tobacco exposure influences the usage of the different isotypes, and in particular the presence of IgA anti-CCP. We found that IgA anti-CCP was more frequently present in smokers than in nonsmokers, with an OR of 2.8 (95% confidence interval [95% CI] 1.60–5.04). IgM anti-CCP was also more frequent in smokers than in nonsmokers (OR 1.8, 95% CI 1.03–3.15), whereas the subclasses of IgG anti-CCP were not significantly more frequent among smokers (Table 1).

A trend toward longer disease duration at the time of inclusion was observed for the patients classified as smokers compared with those classified as nonsmokers (P = 0.08 by Mann-Whitney U test), and therefore additional logistic regression analyses were performed to correct for disease duration. Smoking was still found to

Table 1. Distribution of isotypes of anti-CCP in 117 smokers versus 99 nonsmokers*

<table>
<thead>
<tr>
<th>Anti-CCP isotype</th>
<th>Nonsmokers, no. (%)</th>
<th>Smokers, no. (%)</th>
<th>Odds ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgA</td>
<td>50 (51)</td>
<td>87 (74)</td>
<td>2.8 (1.60–5.04)</td>
</tr>
<tr>
<td>IgM</td>
<td>55 (56)</td>
<td>81 (69)</td>
<td>1.8 (1.03–3.15)</td>
</tr>
<tr>
<td>IgG1</td>
<td>99 (100)</td>
<td>116 (99)</td>
<td>–</td>
</tr>
<tr>
<td>IgG2</td>
<td>76 (77)</td>
<td>99 (85)</td>
<td>1.7 (0.84–3.30)</td>
</tr>
<tr>
<td>IgG3</td>
<td>50 (51)</td>
<td>70 (60)</td>
<td>1.5 (0.85–2.51)</td>
</tr>
<tr>
<td>IgG4</td>
<td>97 (98)</td>
<td>113 (97)</td>
<td>0.6 (0.10–3.25)</td>
</tr>
</tbody>
</table>

* Anti-CCP = anti–cyclic citrullinated peptide; 95% CI = 95% confidence interval.

Table 2. Levels of anti-CCP isotypes in smokers versus nonsmokers*

<table>
<thead>
<tr>
<th>Anti-CCP isotype</th>
<th>Nonsmokers, units/ml</th>
<th>Smokers, units/ml</th>
<th>P†</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgA</td>
<td>67 (42–141)</td>
<td>109 (47–352)</td>
<td>0.012</td>
</tr>
<tr>
<td>IgM</td>
<td>57 (42–98)</td>
<td>94 (52–166)</td>
<td>0.001</td>
</tr>
<tr>
<td>IgG1</td>
<td>145 (79–208)</td>
<td>201 (108–312)</td>
<td>0.003</td>
</tr>
<tr>
<td>IgG2</td>
<td>71 (40–167)</td>
<td>125 (57–328)</td>
<td>0.016</td>
</tr>
<tr>
<td>IgG3</td>
<td>145 (72–266)</td>
<td>186 (86–870)</td>
<td>0.102</td>
</tr>
<tr>
<td>IgG4</td>
<td>17 (5–63)</td>
<td>75 (15–363)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* Values are the median (interquartile range). Anti-CCP = anti–cyclic citrullinated peptide.
† Calculated by Mann-Whitney U test.
be a significant predictor of both the presence of IgM ($P = 0.022$) and the presence of IgA ($P = 0.001$) after correction for disease duration.

To summarize the extensiveness of the isotype usage, the number of different isotypes participating in the anti-CCP response in individual patients was calculated. Although the median number of isotypes used was equal between patients who were smokers and those who were nonsmokers (median 5 isotypes, range 1–6), the number of isotypes detected per patient was higher in smokers compared with nonsmokers ($P = 0.013$ by Mann-Whitney U test) (Figure 1), indicating that tobacco exposure influences the extensiveness of anti-CCP antibody isotype usage in general, and of IgM anti-CCP and IgA anti-CCP in particular.

To determine whether tobacco exposure influences not only the presence or absence of the different isotypes of anti-CCP antibodies, but also the level of each isotype, the different anti-CCP isotypes were measured by ELISA in the serum, and levels were compared

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**Figure 2.** Levels of IgA anti–cyclic citrullinated peptide (anti-CCP) (A), IgM anti-CCP (B), IgG1 anti-CCP (C), IgG2 anti-CCP (D), IgG3 anti-CCP (E), and IgG4 anti-CCP (F) in patients with anti-CCP–positive rheumatoid arthritis who were positive for the respective isotypes and classified as nonsmokers or smokers. Circles indicate individual data points. Broken lines indicate the cutoff level for positivity. Bars show the geometric mean with 95% confidence interval.
according to tobacco exposure in RA patients who were positive for the respective anti-CCP isotypes. Levels of all isotypes of anti-CCP antibodies, except those of IgG3, were significantly higher in the patients classified as smokers than in the patients who had never smoked (Table 2 and Figure 2), which is consistent with previous results with regard to total levels of IgG anti-CCP antibodies (10).

We then assessed whether the influence of tobacco exposure on isotype usage can be observed in both SE-positive and SE-negative RA, and whether the influence of smoking is dependent on the presence of SE alleles in RA, as was recently described with respect to the influence of tobacco exposure on the presence of anti-CCP antibodies. In the present analysis, patients were stratified according to tobacco exposure and the presence or absence of SE alleles.

IgA anti-CCP, irrespective of SE status, was significantly more frequent among smokers. Similarly, IgM anti-CCP was more often detected in smokers as compared with nonsmokers regardless of whether these patients had SE-positive or SE-negative disease, although the differences were not statistically significant (data not shown). A trend toward a higher number of different isotypes of anti-CCP antibodies in smokers compared with nonsmokers was observed in those with SE-positive RA \( (P = 0.07) \).

More intriguingly, however, we observed that in the patients with SE-negative RA, tobacco exposure was associated with a more extensive isotype usage within the anti-CCP response \( (P = 0.04) \) (Figure 3). No interaction between SE status and smoking status in relation to usage of the anti-CCP antibody isotypes could be detected (data not shown). However, the data obtained indicated that the influence of smoking on isotype usage in patients with anti-CCP–positive RA does not depend on the presence of SE alleles.

**DISCUSSION**

B cells activated in the bronchoalveolar tract are prominent producers of IgA antibodies, and the organized BALT that is involved in the generation of IgA-producing cells can be detected more frequently in smokers than in nonsmokers (15). This finding, together with the observation that individuals who are smokers display higher citrullination in cells obtained by bronchoalveolar lavage (9), fueled the hypothesis that IgA anti-CCP would be present more frequently and detected at higher levels in smokers than in nonsmokers.

Indeed, not only were IgA anti-CCP antibodies more frequently present in smokers, but also the levels of IgA anti-CCP antibodies were higher in smokers than in nonsmokers. However, in addition to the findings regarding IgA anti-CCP antibodies, IgM anti-CCP antibodies were also more frequently detected in smokers, and the levels of all isotypes, except IgG3, as well as the number of isotypes used in the anti-CCP response were higher in smokers than in nonsmokers. These data indicate a more diverse anti-CCP response in general in patients with anti-CCP–positive RA who have been exposed to tobacco compared with patients who are nonsmokers.

Smoking not only is associated with anti-CCP–positive RA, but also has been identified as a risk factor for the development of RA among patients with anti-CCP–positive undifferentiated arthritis (UA) (10) and as a factor that influences the extent of joint damage in RA (6). The differences in isotype usage and/or the differences in levels of anti-CCP antibodies between patients with anti-CCP–positive RA who are smokers and those who are nonsmokers possibly contribute to a more severe progression of RA and a faster fulfillment of the ACR criteria within patients with UA. This is a subject of interest that should be explored further, but
was not included in the present study due to insufficient power to detect differences in disease progression.

Tobacco exposure was recently described as a contributor to the risk of anti-CCP–positive RA only among patients with SE-positive disease (8). In this study, we addressed whether the effect of smoking on the constitution of the anti-CCP response, in terms of isotype usage, was dependent on the presence of the SE as well. We observed a higher number of anti-CCP isotypes in anti-CCP–positive smokers compared with anti-CCP–positive nonsmokers, both in patients with SE-positive RA (P not significant, possibly as a result of a ceiling effect) and in patients with SE-negative RA (P = 0.04) (Figure 3). These data indicate that, at least in SE-negative RA, tobacco exposure influences the extensiveness of isotype usage in the anti-CCP response. Moreover, the results suggest that tobacco exposure is involved in the development of anti-CCP only in patients with SE-positive RA, whereas once the tolerance for citrullinated antigens is broken, the effect of tobacco exposure on the response becomes independent of T cell help via SE-bearing HLA molecules. This could, for example, be mediated by exerting a direct effect on the B cell response and/or a diversification of the underlying T cell response that now recognizes the antigen in the context of other HLA molecules.

In conclusion, patients with anti-CCP–positive RA who are current or former smokers display a more extensive anti-CCP isotype usage and a higher percentage of IgA and IgM anti-CCP antibodies than do patients with anti-CCP–positive RA who are nonsmokers. Additionally, in contrast to the influence of smoking on the presence of anti-CCP antibodies, the influence of smoking on the constitution of the anti-CCP response is not observed exclusively in patients with SE-positive RA, but also in patients with SE-negative RA, possibly reflecting the differential effects of tobacco exposure on the induction as compared with propagation of the anti-CCP response.

**AUTHOR CONTRIBUTIONS**

Dr. Verpoort had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. **Study design.** Breedveld, Huizinga, Toes. **Acquisition of data.** Verpoort, Papendrecht-van der Voort, Jol-van der Zijde, Drijfhout, Breedveld, de Vries. **Analysis and interpretation of data.** Verpoort, Papendrecht-van der Voort, van der Helm-van Mil, Jol-van der Zijde, van Tol, Drijfhout, Huizinga, Toes. **Manuscript preparation.** Verpoort, van der Helm-van Mil, Jol-van der Zijde, de Vries, Toes. **Statistical analysis.** Verpoort.

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14. Verduyn W, Doxiadis II, Anholts J, Drabbels JJ, Naipal A, Meyer O, et al. Tobacco exposure was recently described as a contributor to the risk of anti-CCP–positive RA only among patients with SE-positive disease (8). In this study, we addressed whether the effect of smoking on the constitution of the anti-CCP response, in terms of isotype usage, was dependent on the presence of the SE as well. We observed a higher number of anti-CCP isotypes in anti-CCP–positive smokers compared with anti-CCP–positive nonsmokers, both in patients with SE-positive RA (P not significant, possibly as a result of a ceiling effect) and in patients with SE-negative RA (P = 0.04) (Figure 3). These data indicate that, at least in SE-negative RA, tobacco exposure influences the extensiveness of isotype usage in the anti-CCP response. Moreover, the results suggest that tobacco exposure is involved in the development of anti-CCP only in patients with SE-positive RA, whereas once the tolerance for citrullinated antigens is broken, the effect of tobacco exposure on the response becomes independent of T cell help via SE-bearing HLA molecules. This could, for example, be mediated by exerting a direct effect on the B cell response and/or a diversification of the underlying T cell response that now recognizes the antigen in the context of other HLA molecules.

In conclusion, patients with anti-CCP–positive RA who are current or former smokers display a more extensive anti-CCP isotype usage and a higher percentage of IgA and IgM anti-CCP antibodies than do patients with anti-CCP–positive RA who are nonsmokers. Additionally, in contrast to the influence of smoking on the presence of anti-CCP antibodies, the influence of smoking on the constitution of the anti-CCP response is not observed exclusively in patients with SE-positive RA, but also in patients with SE-negative RA, possibly reflecting the differential effects of tobacco exposure on the induction as compared with propagation of the anti-CCP response.

**AUTHOR CONTRIBUTIONS**

Dr. Verpoort had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. **Study design.** Breedveld, Huizinga, Toes. **Acquisition of data.** Verpoort, Papendrecht-van der Voort, Jol-van der Zijde, Drijfhout, Breedveld, de Vries. **Analysis and interpretation of data.** Verpoort, Papendrecht-van der Voort, van der Helm-van Mil, Jol-van der Zijde, van Tol, Drijfhout, Huizinga, Toes. **Manuscript preparation.** Verpoort, van der Helm-van Mil, Jol-van der Zijde, de Vries, Toes. **Statistical analysis.** Verpoort.
Early Changes in Serum Type II Collagen Biomarkers Predict Radiographic Progression at One Year in Inflammatory Arthritis Patients After Biologic Therapy

Ronan H. Mullan,1 Clare Matthews,1 Barry Bresnihan,1 Oliver FitzGerald,1 Lindsay King,2 A. Robin Poole,3 Ursula Fearon,1 and Douglas J. Veale1

Objective. To investigate whether short-term changes in serum biomarkers of type II collagen degradation (C2C) and types I and II collagen degradation (C1,2C), as well as the biomarker for the synthesis of type II procollagen (CPII) can predict radiographic progression at 1 year following initiation of biologic therapy in patients with inflammatory arthritis.

Methods. Serum levels of biomarkers were measured at baseline and at 1, 3, 6, 9, and 12 months after initiation of biologic therapy. A composite score reflecting changes from baseline in all 3 biomarkers (ΔCOL) was calculated. Associations with clinical responses according to the 28-joint count Disease Activity Score and with radiographic progression according to the modified Sharp/van der Heijde score (SHS) were assessed.

Results. The 1-year increase in the SHS correlated with the 1-month change in C2C results (r = 0.311, P = 0.028) and the ΔCOL score (r = 0.342, P = 0.015). Radiographic progression was predicted by increases in serum C2C at 1 month (P = 0.031). The ΔCOL score was significantly associated with 1-year radiographic progression after 1 (P = 0.022), 3 (P = 0.015), 6 (P = 0.048), and 9 (P = 0.019) months of therapy. Clinical remission was predicted by 1-month decreases in serum levels of C2C (P = 0.031) and C1,2C (P = 0.036). By regression analysis, 1-month changes in C2C, C1,2C, and CPII levels were independently associated with, and correctly predicted radiographic outcome in, 88% of the patients.

Conclusion. Short-term changes in serum levels of collagen biomarkers following initiation of biologic therapy may better predict long-term clinical and radiographic outcomes. These collagen biomarkers may therefore be valuable new early indicators of short-term biologic treatment efficacy in clinical trials and in individual patients with inflammatory erosive arthritis.

Rheumatoid arthritis (RA) and psoriatic arthritis (PsA) are forms of progressive inflammatory arthritis characterized by pain and progressive destruction of the joints. Joint destruction occurs when inflamed synovial tissue erodes and degrades adjacent cartilage and bone through the action of locally produced cytokines and metalloproteinases (1). This process can be measured by quantifying the changes in joint space narrowing and erosions that are visible on serial plain radiographs (2). Radiographic joint damage is associated with long-term functional disability (3) and is the accepted assessment tool for monitoring clinical progression and long-term response to therapy (4). Radiographic progression develops over many months and years, however, and is not suitable for assessment of treatment efficacy over short periods.
The treatment of RA and PsA with tumor necrosis factor α (TNFα) inhibitors has transformed clinical practice by inducing remission and preventing radiographic progression in a larger proportion of patients than with previously available therapies (5,6). In current clinical practice, treatment response is measured through suppression of inflammation, either in isolation, as with the systemic acute-phase response indicators C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR), or in combination with clinical information, as in the American College of Rheumatology response criteria (4) or the European League Against Rheumatism (EULAR) improvement criteria (7). Despite the considerable efficacy of biologic therapies, a significant proportion of patients receiving them either fail to respond, or have a suboptimal response, to treatment (8). Patients who fail to enter clinical remission can have significant levels of ongoing inflammation, despite meeting currently accepted treatment response criteria (4,7).

Other prognostic factors, including the presence of rheumatoid factor, rheumatoid nodules, HLA–DR4/ shared epitope, and anti–cyclic citrullinated peptide antibodies (9–12), are associated with worse outcomes when using grouped data, but they are not useful in predicting individual outcomes or responses to therapy. New biomarkers are therefore needed that can predict and be used to help prevent long-term radiographic progression in individual patients.

The destruction and remodeling of articular cartilage in arthritis involves increased degradation and changes in the synthesis of cartilage matrix (13). Cartilage metabolism can be monitored by measurement of the synthesis and the degradation products of cartilage-specific collagens and proteoglycans, which are released into synovial fluid, serum, and urine as byproducts (14). Studies of cartilage turnover using these biomarkers have indicated significant changes that relate to the erosion of articular cartilage in arthritis (15–18). Recently, the use of collagen biomarkers as a possible tool for monitoring disease activity and predicting radiographic outcome in RA has been highlighted but requires further study (18,19).

Type II collagen (CII) is the main collagen of articular cartilage, and CII is excessively degraded in RA, PsA, and osteoarthritis (OA) (20–25), an effect believed to result from its cleavage by collagenases. Cleavage of the triple helix of CII by collagenase exposes a helical neocollagen Col2-3/4Cshort (C1,2C), which can be detected by immunoassay (26–28). Changes in type II procollagen synthesis also occur in inflammatory arthritis, and this is measurable by immunoassay of the C-propeptide of this molecule (CPII) (16,17,29–31). A third epitope, Col2-3/4Cshort (C1,2C), indicates the cleavage by collagenases of both type I and type II collagen (18,24,32). We have previously shown increases in serum C2C levels in RA (26) and correlations between C2C and C1,2C neoepitope levels with measures of disease activity in early RA and PsA (18,26), coupled with a reduction in CPII levels in the synovial fluid of these patients (20), in contrast to increases in the serum (17,31). These findings indicate a possible role of these biomarkers of cartilage destruction in arthritis.

In the present study, we investigated the changes in cartilage biomarkers in a cohort of patients following initiation of biologic therapy for active RA and PsA that was resistant to treatment with standard disease-modifying antirheumatic drugs (DMARDs). Specifically, we assessed the relationship of biomarkers of CII and type I collagen cleavage as well as CII synthesis to treatment responses and to radiographic outcome at 12 months. We demonstrated that serum biomarkers of collagen turnover and the relationships between these markers of synthesis and degradation may more accurately assess the presence or absence of early skeletal protection following treatment with biologic therapies in patients with RA and PsA.

**PATIENTS AND METHODS**

**Patient recruitment.** A total of 62 patients were recruited from rheumatology outpatient clinics at St. Vincent’s University Hospital and were followed up prospectively for 1 year. Forty-five patients fulfilled diagnostic criteria for RA according to the American College of Rheumatology (formerly, the American Rheumatism Association) criteria (33), and 17 patients had a diagnosis of PsA that met previously defined criteria (34). All patients had clinically active disease, with 28-joint count Disease Activity Scores (DAS28) >3.2 points despite conventional DMARD therapy, and were offered treatment with biologic agents. Patients who had previously received biologic therapy were excluded from the study.

Changes in conventional therapy were permitted during biologic therapy at the discretion of the patient’s treating rheumatologist; however, no changes in the DMARD dosage were made during the study.

Following approval by the institutional ethics committee at St. Vincent’s University Hospital, all patients gave their informed written consent prior to inclusion in the study. All 62 patients began biologic therapy after their baseline assessment of disease activity. Fifty-eight patients received TNF-targeted therapies: infliximab in 11 (3 RA and 8 PsA), adalimumab in 44 (36 RA and 8 PsA), and etanercept in 3 (2 RA and 1 PsA). The remaining 4 RA patients received treatment with the interleukin-1 (IL-1) receptor antagonist anakinra. Patients were evaluated before and at 1, 3, 6, 9, and 12 months after initiation of biologic therapy.
At each evaluation, blood samples were obtained and sera were separated and stored at −80°C until used for biomarker analyses. Clinical evaluation at each assessment was performed using the DAS28 (35), which has now been validated for use in PsA patients as well as RA patients (36), and the modified Health Assessment Questionnaire (HAQ) (37). The DAS28 response was analyzed both by changes in scores from baseline and by response categories according to the EULAR criteria (7). A DAS28 response at 3 months was defined as a reduction in the DAS28 score of ≥0.6 points and a final DAS28 score of ≤5.1 points. A DAS28 nonresponse was defined as an improvement of <0.6 points or a final DAS28 score of >5.1 points. Patients achieving clinical remission at 6 months were identified according to EULAR criteria (DAS28 <2.6 points) (38). In addition, the patient’s global assessment of his or her overall health was recorded at each visit, using a visual analog scale of 0–100 mm, where 0 = best and 100 = worst.

Radiographic evaluation. Anteroposterior radiographs of the hands and feet were obtained at baseline and after 1 year. The radiographs were analyzed by 2 independent blinded observers (RHM and CM) who were aware of the chronologic sequence of the radiographs. Radiographs were scored according to the modified Sharp/van der Heijde scoring (SHS) method (2). Both baseline radiographic damage and radiographic progression were defined as binomial variables on the basis of the smallest detectable change calculated from the scoring of radiographs between the 2 observers (SHS 1.02). Calculations for the smallest interobserver detectable change were performed as previously described (39). Patients were therefore defined as having baseline radiographic damage when their initial SHS was ≥3.5. One-year radiographic progression was defined as an increase in the SHS of ≥1.5.

Assays for cartilage matrix molecules and markers of inflammation. Serum biomarkers were measured using 2-step competitive immunomasys (Ibex, Montreal, Quebec, Canada). The C2C and C1,2C assays, also known as the Col2-3/4Clong mono and Col2-3/4Cshort assays, respectively, measure the cleavage by collagenases of CII (C2C) and types I and II collagens (C1,2C), respectively, as previously described (24,26). The CPII enzyme-linked immunosorbent assay is based on a previously described radioimmunomasay (31,32). These commercially available assays have been carefully standardized for use with human sera, and full details of their reproducibility and accuracy have recently been published. The interassay reproducibility of measurements of C2C, CPII, and C1,2C concentrations in 30 masked pairs of sera was 9.7%, 6.4%, and 10%, respectively (18,40). The ESR was determined using the Westergren technique. CRP levels were measured by nephelometry.

Statistical analysis. Clinical data are expressed as the mean ± SEM except where stated otherwise. Statistically significant correlations found during Spearman’s correlation testing were verified and confirmed by using alternative statistical approaches (one-way analysis of variance [ANOVA] and logistic regression analysis) to ensure that our initial results were not achieved through multiple testing. No significant difference in the effect of treatment on levels of biomarkers between RA and PsA patients was observed (data not shown). To evaluate the overall utility of biomarkers in assessing the response to biologic therapy in clinical practice, the effects of treatment on levels of biomarkers were calculated using combined data from both RA and PsA patients. The effect of treatment on biomarkers was assessed by analysis of serum levels at each assessment point and by the change in serum levels (ng/ml) relative to pretreatment levels, with a negative value ascribed to a fall in serum level. Comparisons of paired continuous data were performed using Wilcoxon’s signed rank test. Comparisons between 2 sets of categorical data were performed using the chi-square test. Correlations of biomarkers, markers of inflammation, and clinical scores with radiographic progression, both as a continuous variable using Spearman’s rank correlation and as a categorical variable (<1.5 SHS changes [no progression] versus ≥1.5 SHS changes [progression]) using one-way ANOVA, were also performed.

To determine independent predictors of radiographic progression, multivariate analysis was performed using binary logistic regression. Regression analysis included baseline SHS and ESR (mm/hour) values, which were both significantly associated with radiographic damage on univariate analysis, as well as the change from baseline in serum levels of C2C (ΔC2C), C1,2C (ΔC1,2C), and CPII (ΔCPII) from their pretreatment levels was calculated at each followup time point, with a negative value ascribed to a fall in the level. The ΔCOL score for each time point (in arbitrary units [AU]) was calculated by subtracting the percentage change in the collagen synthesis marker CPII from the sum of the changes in the collagen cleavage markers, as follows:

\[ ΔCOL = ΔC2C + ΔC1,2C - ΔCPII \]

The ΔCOL score therefore reflects the effects of therapeutic intervention on collagen metabolism by taking account of the effects on both collagen synthesis and collagen degradation.

To determine independent predictors of radiographic progression, multivariate analysis was performed using binary logistic regression. Regression analysis included baseline SHS and ESR (mm/hour) values, which were both significantly associated with radiographic damage on univariate analysis, as well as the change from baseline in serum levels of C2C (ng/ml), C1,2C (ng/ml), and CPII (ng/ml). All multivariate models were controlled for sex (male versus female), age (years), and diagnosis (RA versus PsA). CRP was not independently associated with radiographic progression either before \( P = 0.112 \) or after \( P = 0.935 \) correction for the ESR and was not included in the model. The regression model was also tested to assess its accuracy in predicting radiographic progression at 1 year. Receiver operating characteristic (ROC) analysis was used to determine the best discriminative cutoff value for changes in biomarker levels using the radiographic progression grouping as the dependent variable. All statistical analyses were performed using SPSS version 12 software for Windows (SPSS, Chicago, IL).

RESULTS

Patients. Baseline clinical, laboratory, and radiologic disease characteristics of the 62 study patients are shown in Table 1. Patients had high levels of disease activity at baseline, as measured by the DAS28 (mean ± SD 5.4 ± 1.4), and significant functional impairment,
with elevated baseline scores on the modified HAQ (mean ± SD 1.4 ± 0.7). Sixty-eight percent of patients were receiving a DMARD and 63% were receiving corticosteroids at the time biologic therapy was started. There were no significant differences between the RA and PsA patients in terms of serum levels of collagen biomarkers, serum markers of inflammation, disease duration, sex ratio, or frequency of DMARD use. The proportion of patients with radiographic damage at baseline was equal in the RA and PsA groups (83%), although patients with RA were significantly older, had higher baseline SHS and DAS28 scores, and were more likely to be receiving corticosteroid therapy (Table 1).

**Radiographic progression after 1 year.** Seven patients (3 with RA and 4 with PsA) did not have radiographs taken at 1 year and were therefore excluded from followup radiographic analysis. No differences in disease activity measures at baseline or at followup were observed between these patients and the remaining 55 patients with followup radiographic data. Of the remaining 55 patients, 47 (85%) had evidence of radiographic damage at baseline, with a mean ± SEM SHS of 52 ± 7.5 units. Radiographic progression occurred in 24 patients (44%; median increase in SHS 2.75 [interquartile range 2.0–6.125]), all of whom had evidence of radiographic damage at baseline. The presence of radiographic damage at baseline was strongly associated with radiographic progression following 1 year of treatment with biologic therapy ($P = 0.007$). No patient with normal radiographic findings at their baseline assessment developed radiographic damage while receiving biologic therapy.

**Biomarkers correlate with and predict radiographic progression.** The results of an explorative analysis of associations of biomarkers and clinical responses with radiographic progression at 1 year are shown in Table 2. Changes at 1 month in C2C levels (r = 0.311, $P = 0.028$) and the ΔCOL score (r = 0.342, $P = 0.015$) correlated significantly with the increase in total SHS, as determined by Spearman’s rank correlation. In contrast, there was no association between radiographic progression and the change at 1 month in the CRP level, ESR, or DAS28, although significant associations with their baseline values were seen (Table 2).

To confirm the associations of the biomarkers with joint damage, patients were divided into radiographic progressors (change in SHS of ≥1.5) and non-progressors (change in SHS of <1.5). Changes in the levels of C2C, C1,2C, and CPII at each time point were determined by Spearman’s rank correlation. In contrast, there was no association between radiographic progression and the change at 1 month in the CRP level, ESR, or DAS28, although significant associations with their baseline values were seen (Table 2).
90% of the patients (area under the curve [AUC] predicted the absence of radiographic progression in Table 2.

Correlations with increased SHS at 1 year

<table>
<thead>
<tr>
<th></th>
<th>r for baseline level</th>
<th>r for change at 1 month</th>
</tr>
</thead>
<tbody>
<tr>
<td>C2C</td>
<td>0.23</td>
<td>0.311†</td>
</tr>
<tr>
<td>∆COL</td>
<td>NA</td>
<td>0.342†</td>
</tr>
<tr>
<td>Baseline SHS</td>
<td>0.411‡</td>
<td>NA</td>
</tr>
<tr>
<td>ESR</td>
<td>0.512‡</td>
<td>0.28</td>
</tr>
<tr>
<td>CRP</td>
<td>0.41†</td>
<td>0.025</td>
</tr>
<tr>
<td>DAS28</td>
<td>0.28†</td>
<td>0.036</td>
</tr>
<tr>
<td>Modified HAQ</td>
<td>0.37†</td>
<td>0.29</td>
</tr>
</tbody>
</table>

* Baseline levels of biomarkers and clinical measures of disease activity and changes in these levels at 1 month were correlated with radiographic progression at 1 year, as measured by increases in the modified Sharp/van der Heijde score (SHS), by Spearman’s rank correlation. C2C = Col2-3/4Clong mono; ∆COL = composite score for changes in collagen biomarkers from baseline (see Patients and Methods for details); NA = not applicable; ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; DAS28 = 28-joint Disease Activity Score; HAQ = Health Assessment Questionnaire.

† P < 0.05.
‡ P < 0.005.

progressors at 1 month, with a significant difference in the change from baseline (P = 0.031). This association was maintained at 3 months (144 ± 19 ng/ml in progressors versus 111 ± 8 ng/ml in nonprogressors; P = 0.049).

Baseline levels of C1,2C were similar in the 2 groups (mean ± SEM 384 ± 17 ng/ml in progressors versus 379 ± 30 ng/ml in nonprogressors). C1,2C levels fell significantly from 382 ± 17 ng/ml to 360 ± 13 ng/ml after 1 month (P = 0.04). This trend was maintained after 3 months, with a significant fall in C1,2C levels in radiographic nonprogressors (to 357 ± 15 ng/ml; P = 0.02).

No significant differences were seen in levels of the collagen synthesis marker CPII at baseline (mean ± SEM 1,105 ± 122 ng/ml in progressors versus 954 ± 56 ng/ml in nonprogressors) or in the change during followup.

The associations between ∆COL scores (in AU) at 1, 3, 6, 9, and 12 months and radiographic progression are shown in Figure 1. When radiographic progressors and nonprogressors were compared, significant differences in the ∆COL score were found at 1 month (decrease of 7 AU in nonprogressors versus rise of 11 AU in progressors; P = 0.022). This statistical association was maintained at 3 (P = 0.015), 6 (P = 0.048), and 9 (P = 0.019) months of therapy. In order to calculate the best discriminative cutoff for the ∆COL score at 1 month, ROC analysis was performed. A ∆COL score cutoff point of <20 AU (range −80 to +74) correctly predicted the absence of radiographic progression in 90% of the patients (area under the curve [AUC] = 0.69; P = 0.02), which was superior to the sensitivity of changes in single biomarkers alone, as shown in Table 3.

Radiographic progressors had significantly higher baseline values for the ESR, CRP, DAS28, and modified HAQ, and they tended to have higher disease activity throughout the 12 months of followup (Figure 2). The change in DAS28 scores at 3 months did not discriminate between radiographic progressors and nonprogressors, whether using its absolute reduction from baseline (mean ± SEM 2.0 ± 0.4 units in radiographic progressors versus 2.1 ± 0.2 units in nonprogressors; P = 0.8) or using the DAS28 response rates (64% in radiographic progressors versus 86% in nonprogressors; P = 0.06). When baseline biomarker levels were compared with baseline levels of markers of inflammation, a significant correlation was found between the C1,2C value and the ESR (r = 0.278, P = 0.03). There were no correlations between the ESR and either the C2C or the CPII level, and no correlations of any biomarker with the CRP level.

**Clinical responses to biologic therapy.** After 3 months of therapy, a clinical response in the DAS28 was seen in 76% of patients. To investigate the relationship between very early changes in biomarkers with the subsequent clinical response, the changes in C2C, C1,2C, CPII, and ∆COL values at 1 month were analyzed according to the DAS28 response at 3 months. The C1,2C levels fell from 385 ± 18 ng/ml to 365 ± 18 ng/ml in the DAS28 responders, but rose from 376 ± 43 ng/ml to 425 ± 36 ng/ml in the nonresponders, a statistically significant difference between groups (P = 0.01). A

![Figure 1](image-url)
significant difference was also seen with the ΔCOL score, which fell by 6 AU in DAS28 responders but increased by 16 AU in nonresponders ($P = 0.014$). No statistically significant relationship was seen between serum levels of C2C or CPII and the clinical response at 3 months.

Clinical remission (DAS28 of $<2.6$) was seen in 35% of patients after 6 months of followup. The very early changes in biomarker levels significantly correlated with clinical remission at 6 months. C2C levels determined at 1 month after starting biologic therapy were significantly lower in patients who experienced clinical remission at 6 months ($141 \pm 14$ ng/ml in the nonremission group versus $92 \pm 6.6$ ng/ml in the remission group; $P = 0.008$). A statistical association was also seen at 1 month for the C1,2C value ($389 \pm 17$ ng/ml in the nonremission group versus $336 \pm 15$ ng/ml in the remission group; $P = 0.036$) and the change in the ΔCOL score at 1 month (increase of 6 AU in the nonremission group versus decrease of 10 AU in the remission group; $P = 0.026$). Baseline levels of C2C, C1,2C, and CPII were not associated with remission at 6 months.

**Composite model for predicting radiographic progression.** Predictive factors for radiographic progression that were identified in the univariate analysis were further investigated using binary logistic regression analysis. C2C and C1,2C levels were independently associated with radiographic progression at 1 month, with CPII tending to show a negative independent association (Table 4). These data suggest that 1-month changes in all 3 collagen biomarker levels are independent determinants of radiographic progression at 1 year. Using the 1-month changes in biomarkers, the model increased the percentage of patients that were correctly predicted from 80% to 88% (Table 5). At later time points, biomarker changes were less predictive and were

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**Table 3. Changes in levels of biomarkers at 1 month and prediction of radiographic progression**

<table>
<thead>
<tr>
<th>Cutoff for radiographic progression</th>
<th>AUC</th>
<th>No progression if ≤</th>
<th>Range</th>
<th>Sensitivity, %</th>
<th>1 – specificity, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>C2C, ng/ml</td>
<td>0.68</td>
<td>16</td>
<td>−63 to 47</td>
<td>87</td>
<td>55</td>
</tr>
<tr>
<td>C1,2C, ng/ml</td>
<td>0.65</td>
<td>1</td>
<td>−125 to 111</td>
<td>67</td>
<td>35</td>
</tr>
<tr>
<td>CPII, ng/ml</td>
<td>0.55</td>
<td>−93</td>
<td>−857 to 514</td>
<td>50</td>
<td>25</td>
</tr>
<tr>
<td>ΔCOL, AU</td>
<td>0.69</td>
<td>20</td>
<td>−80 to 74</td>
<td>90</td>
<td>55</td>
</tr>
</tbody>
</table>

*Receiver operating characteristic analysis of changes in biomarker levels at 1 month using radiographic progression as the dependent variable. Cutoff points that best predicted both radiographic nonprogression and progression are shown. AUC = area under the curve; C2C = Col2-3/4Clong mono; C1,2C = Col2-3/4Cshort; CPII = C-propeptide of type II collagen; ΔCOL = composite score for changes in collagen biomarkers from baseline (see Patients and Methods for details).

**Table 4. Binary logistic regression analysis for radiographic progression**

<table>
<thead>
<tr>
<th>Input covariate</th>
<th>OR</th>
<th>95% CI</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline SHS (per unit)</td>
<td>1.04</td>
<td>1.01–1.07</td>
<td>0.004</td>
</tr>
<tr>
<td>Baseline ESR, mm/hour (per unit)</td>
<td>1.1</td>
<td>1.02–1.18</td>
<td>0.01</td>
</tr>
<tr>
<td>1-month C2C change, ng/ml (per unit)</td>
<td>1.08</td>
<td>1.01–1.2</td>
<td>0.022</td>
</tr>
<tr>
<td>1-month C1,2C change, ng/ml (per unit)</td>
<td>1.03</td>
<td>1.001–1.06</td>
<td>0.046</td>
</tr>
<tr>
<td>1-month CPII change, ng/ml (per unit)</td>
<td>0.99</td>
<td>0.987–0.999</td>
<td>0.046</td>
</tr>
</tbody>
</table>

*Analyses were controlled for age, sex, and diagnosis. $P$ values less than 0.05 indicate that the variable was independently associated with radiographic progression. OR = odds ratio; 95% CI = 95% confidence interval; SHS = modified Sharp/van der Heijde score; ESR = erythrocyte sedimentation rate; C2C = Col2-3/4Clong mono; C1,2C = Col2-3/4Cshort; CPII = C-propeptide of type II collagen.
no longer independently associated with progression (data not shown).

To confirm that the associations of collagen biomarkers with radiographic progression following biologic therapy were independent of changes in the inflammatory and clinical response measures used in current clinical practice, further regression models were generated that included 1-month changes from baseline for the ESR (mm/hour), the CRP level (mg/liter), or the DAS28 (Table 6). All 3 biomarkers remained independently associated ($P < 0.05$) with radiographic outcome when corrected for the change in ESR or CRP value. Both the 1-month changes in C2C and CPII levels remained independently associated with the 1-year radiographic progression after correction for the change in the DAS28.

**Analysis of responses by biologic agent or by diagnosis.** When disease activity was examined in relation to the different biologic agents used in this study, no difference was observed in baseline levels of markers of inflammation, DAS28 scores, modified HAQ scores, or baseline radiographic damage between treatment groups, indicating a lack of prescribing bias on the basis of disease activity. No difference was seen in clinical responses to any of the 3 anti-TNF therapies throughout the 12 months of this study. No difference in radiographic progression rates were observed between any of the biologic agents used in this study (data not shown).

Comparison of clinical responses between RA and PsA patients was performed excluding the RA patients who were treated with the IL-1 receptor antagonist anakinra. No significant difference was seen between the disease groups with respect to the DAS28 response at 3 months (71% of RA patients versus 87% of PsA patients; $P = 0.2$) or radiographic progression at 12 months (43% of RA patients versus 46% of PsA patients; $P = 0.8$). Furthermore, there was no significant difference in the increase in the SHS between the disease groups (mean ± SEM 2.7 ± 1 units in RA patients versus 2.2 ± 0.8 units in PsA patients; $P = 0.8$).

**Table 5.** Predictive power of serum biomarkers*

<table>
<thead>
<tr>
<th>Input covariate</th>
<th>Nonprogressors</th>
<th>Progressors</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline ESR, mm/hour, and SHS</td>
<td>84</td>
<td>75</td>
<td>80</td>
</tr>
<tr>
<td>Plus 1-month change in</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C2C, ng/ml</td>
<td>86</td>
<td>80</td>
<td>84</td>
</tr>
<tr>
<td>C1,2C, ng/ml</td>
<td>86</td>
<td>75</td>
<td>82</td>
</tr>
<tr>
<td>CPII, ng/ml</td>
<td>83</td>
<td>65</td>
<td>76</td>
</tr>
<tr>
<td>C2C and C1,2C, ng/ml</td>
<td>87</td>
<td>75</td>
<td>82</td>
</tr>
<tr>
<td>C2C, C1,2C, and CPII, ng/ml</td>
<td>90</td>
<td>85</td>
<td>88</td>
</tr>
</tbody>
</table>

* Binary regression models corrected for age, sex, and diagnosis were used to determine the power of the biomarkers to predict which patients would be radiographic nonprogressors and radiographic progressors. ESR = erythrocyte sedimentation rate; SHS = modified Sharp/van der Heijde score; C2C = Col2-3/4Clong mono; C1,2C = Col2-3/4Cshort; CPII = C-propeptide of type II collagen.

**Table 6.** Regression analysis of changes in collagen biomarkers from baseline to 1 month against radiographic progression at 1 year*

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Model 1</th>
<th>Model 2</th>
<th>Model 3</th>
<th>Model 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>C2C, ng/ml (per unit)</td>
<td>1.04 (0.022)</td>
<td>1.08 (0.029)</td>
<td>1.1 (0.041)</td>
<td>1.08 (0.033)</td>
</tr>
<tr>
<td>CPII, ng/ml (per unit)</td>
<td>0.99 (0.046)</td>
<td>0.99 (0.036)</td>
<td>0.99 (0.048)</td>
<td>0.99 (0.049)</td>
</tr>
<tr>
<td>C1,2C, ng/ml (per unit)</td>
<td>1.03 (0.046)</td>
<td>1.04 (0.036)</td>
<td>1.05 (0.049)</td>
<td>1.03 (0.067)</td>
</tr>
</tbody>
</table>

* Analyses were corrected for changes in the erythrocyte sedimentation rate (ESR), C-reactive protein level, and 28-joint count Disease Activity Score. Model 1 variables were the changes at 1 month in the levels of Col2-3/4Clong mono ($C_{2C}$), Col2-3/4Cshort ($C_{1,2C}$), and C-propeptide of type II collagen (CPII), controlling for the baseline modified Sharp/van der Heijde score, the baseline ESR, age, sex, and diagnosis. Model 2 variables were the same as those for model 1 plus the change at 1 month in the C-reactive protein level. Model 3 variables were the same as those for model 1 plus the change at 1 month in the ESR. Model 4 variables were the same as those for model 1 plus the change at 1 month in the 28-joint count Disease Activity Score. $P$ values less than 0.05 indicate that the variable was independently associated with radiographic progression. OR = odds ratio.
With regard to collagen biomarker levels, there were no statistically significant differences in serum levels of any of the collagen biomarkers between the RA patients and the PsA patients either at baseline or during followup (data not shown).

**DISCUSSION**

This study is the first to show that early changes in serum biomarkers of type I collagen and cartilage type II collagen turnover during biologic therapy are significantly associated with subsequent radiographic progression in a prospective cohort of patients with erosive inflammatory arthritis. Increased levels of the cartilage marker C2C and increased ΔCOL scores at 1 month correlated with increased radiographic progression at 12 months. In contrast, response to therapy in nonprogressors was associated with an equally early fall in these markers. Serum cartilage C2C levels have previously been shown to be reduced by etanercept therapy in patients with ankylosing spondylitis (41). In a previous longitudinal study of RA patients, elevated levels of C2C and C1,2C measured at 1 year after diagnosis predicted increased radiographic progression rates for the next 3 years (18).

In our study of the response to treatment, serum levels of the collagen synthesis biomarker CPII did not change, nor did they add to the prediction of radiographic progression when examined in isolation. This finding confirms previous studies demonstrating no difference in serum CPII levels between RA patients with slow or fast rates of radiographic progression (18). Furthermore, ROC analysis showed that the predictive potential of the composite ΔCOL score was explained to a greater extent by the effect of treatment on levels of collagen cleavage markers. Despite this, a slight additional advantage was consistently conferred by including both the synthesis and degradation markers together when analyzed using a number of different statistical techniques.

ROC analysis demonstrated that in this study, the power of these collagen biomarkers was chiefly their ability to identify clinical study patients who were most likely to have stable disease. The discriminatory capacities of these combined assays to predict progression with 88% accuracy is encouraging, although their utility in clinical practice may be limited. In addition, since our cohort was relatively small and the duration of followup short, we would be cautious in interpreting the significance of early changes in the CPII biomarker levels during treatment. Further studies with larger cohorts evaluated over a more prolonged period are required to further assess the robustness of these findings in wider clinical practice.

Findings of our study are important, in that we have demonstrated that collagen biomarker levels have the potential to provide an indication of a treatment response as well as a treatment failure early after a therapeutic intervention. An early and accurate indication of treatment failure would alert clinicians of the need to further optimize treatment before significant and permanent joint damage occurs.

Previous candidates for a readily measurable biomarker that could accurately predict radiographic progression in patients with erosive arthritis have included key matrix-degrading enzymes, such as matrix metalloproteinase 1 (MMP-1) and MMP-3 (42), and other byproducts of cartilage and bone metabolism that are elevated in serum, such as cartilage oligomeric matrix protein (12), the cartilage aggrecan epitope chondroitin sulfate 846 (17), and the CII biomarker C-telopeptide of type II collagen (CTX-II), which are elevated in the serum of arthritis patients. To date, however, no single biomarker has been found to reflect joint destruction with sufficient accuracy to use in the clinic, and it has been suggested that use of a panel of biomarkers could provide the specificity required to inform clinical decision-making for individual patients. In this study, we provide evidence that a triad of markers specific for different aspects of CII metabolism can predict radiographic progression at 1 month of biologic therapy with somewhat greater accuracy than a single biomarker alone.

We have also shown that the very early effect of biologic therapy on levels of collagen biomarkers is significantly associated with subsequent clinical remission, in addition to radiographic response, but the relationship of these biomarkers to radiographic progression occurs independently of changes in systemic inflammation. At present, clinicians rely heavily on clinical and laboratory measures of inflammatory disease activity to assess the efficacy of anti-TNF agents in controlling disease activity. However, there is increasing evidence to support a decoupling of the inflammatory and destructive processes of arthritis, both experimentally and in the clinical setting (6,43). Biomarkers of cartilage turnover may therefore provide the additional information needed to rapidly identify patients who are at risk of disease progression despite ongoing therapy.

In this study, we examined the effect of a common treatment strategy across 2 disease categories, RA and PsA, and as such, this is a direct reflection of current
clinical practice. Rates of radiographic progression were low during the 1-year followup study and are likely to reflect the ameliorating effects of biologic therapy on disease progression. Despite this and the relatively low number of study patients and the heterogeneity of this cohort, consistent significant associations between serum biomarker levels and radiographic progression were found. The data also demonstrate that these collagen biomarkers may be of value in predicting disease progression and associated radiologically defined skeletal changes, as was observed for C2C in a recent study (18). We have previously shown that there are increased levels of C2C in synovial fluid from patients with RA and patients with PsA, and that the C2C levels correlated with systemic markers of inflammation and with levels of TNFα and MMP-1 in synovial fluid (20). The findings from the present study extend the evidence that these biomarkers reflect pathologic destruction of cartilage and bone in both diseases. Because of the relatively small sample size in this study, however, further studies of these biomarkers in larger groups of patients are required.

In summary, this is the first study in a prospective cohort of patients to show that biomarkers specific for type II and type I collagen turnover can be used to monitor short-term treatment response to biologic agents and can accurately predict radiographic outcome at 1 year in 88% of patients. Use of biomarkers to optimize treatment strategies for these diseases may prevent joint damage and lead to better functional outcomes for patients. Opportunities are also identified for the use of biomarkers in shortening clinical trials in patients with inflammatory erosive arthritis.

ACKNOWLEDGMENTS

The authors thank Professor Leslie Daly, PhD, and Yaw Bimpeh, BSc (Department of Biostatistics, University College Dublin, Dublin, Ireland), for their assistance with the data analysis.

AUTHOR CONTRIBUTIONS

Dr. Veale had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Mullan, Bresnihan, FitzGerald, Poole, Fearon, Veale.

Acquisition of data. Mullan, Matthews, Bresnihan, FitzGerald, Poole, Fearon, Veale.

Analysis and interpretation of data. Mullan, Matthews, Bresnihan, FitzGerald, Poole, Fearon, Veale.

Manuscript preparation. Mullan, Bresnihan, FitzGerald, Poole, Fearon, Veale.


Development of some of the biomarker assays used. King.

REFERENCES


The Performance of Anti–Cyclic Citrullinated Peptide Antibodies in Predicting the Severity of Radiologic Damage in Inflammatory Polyarthritis

Results from the Norfolk Arthritis Register

M. Bukhari,1 W. Thomson,1 H. Naseem,1 D. Bunn,2 A. Silman,1 D. Symmons,1 and A. Barton1

Objective. Anti–cyclic citrullinated peptide (anti-CCP) antibodies are a stronger predictor of the severity of rheumatoid arthritis than is rheumatoid factor (RF). Their role in predicting outcome in unselected patients with new-onset inflammatory polyarthritis (IP) has not been examined. The aims of this study were to examine the role of baseline RF and anti-CCP antibodies in determining the likelihood of patients having erosions at presentation or in predicting future radiologic damage, and to determine whether anti-CCP antibodies or RF is sufficiently robust to be clinically useful in guiding treatment decisions in early IP.

Methods. Patients were recruited from the Norfolk Arthritis Register. Logistic regression models were fitted to test the ability of anti-CCP antibodies and RF to predict erosions. Further models were investigated to examine the role of anti-CCP antibodies in patients stratified by RF status.

Results. The presence of anti-CCP antibodies at baseline was strongly associated with both prevalent erosions (odds ratio [OR] 2.53 [95% confidence interval (95% CI) 1.48–4.30]) and developing erosions at 5 years (OR 10.2 [95% CI 6.2–16.9]). These ORs were higher than those for RF (OR 1.63 [95% CI 0.94–2.82] and OR 3.4 [95% CI 2.2–5.2], respectively). The likelihood ratio (LR) for the prediction of prevalent erosions and erosions at 5 years was highest in the RF−subgroup (LR 2.2 and 5.8, respectively). However, 27% of anti-CCP−patients had developed erosions by 5 years.

Conclusion. Despite their strong association with the presence, development, and extent of erosions, anti-CCP antibodies alone are not a sufficiently accurate measure upon which to base clinical treatment decisions. Knowledge of anti-CCP antibody status is most informative in RF−negative patients.

There is considerable evidence of the benefit of early treatment with disease-modifying antirheumatic drugs (DMARDs) in patients with rheumatoid arthritis (RA). These studies have shown that there is a window of opportunity early in the disease course during which DMARDs have the greatest effect in altering disease progression, as measured by the development of radiologic erosions (1,2). The identification of a marker at the onset of disease that could reliably predict which patients will or, perhaps more importantly, will not develop erosions would be a major clinical advance because the latter group could be spared potentially toxic therapies, while the former group may be targeted for combination or biologic therapy. There have been several prospective studies that have examined the relative role of different clinical and laboratory predictors. The presence of rheumatoid factor (RF) and of shared epitope (SE) alleles of the HLA–DRB1 gene has been consistently associated with an adverse outcome (3–5).

More recently, studies have focused on the role of antibodies that recognize cyclic citrullinated
peptides (anti–citrullinated protein antibodies [ACPAs]) (6). ACPAs, as measured by anti–CCP-2 enzyme-linked immunosorbent assays (ELISAs), are highly specific and reasonably sensitive for diagnosing RA (7), although RF may still be present in persons with RA who are negative for anti-CCP antibodies (8). Cross-sectional surveys of prevalent RA cases have also shown that both RF and anti-CCP antibodies are associated with radiographic severity (6,9–11), but recent studies suggest that their effects are not completely overlapping (10). Prospective studies have confirmed the association of anti-CCP antibodies with worsening radiographic outcome in patients with RA at baseline (6,12–18). It has also been shown that the presence of these antibodies in patients presenting with undifferentiated inflammatory arthritis is associated with an increased likelihood of being classified as having RA (13).

A limitation of many previous studies has been the restriction of the investigation to patients with definite RA. In this group, it is difficult to evaluate erosions as an outcome since erosions are one of the criteria used for classification of RA. We have argued previously that an unselected series of patients with inflammatory polyarthritis (IP) would provide a more representative cohort in which to develop prognostic models because the American College of Rheumatology (ACR; formerly, the American Rheumatism Association) classification criteria for RA (19) do not perform well in early disease (20). Furthermore, rheumatologists increasingly want to make therapeutic decisions before patients satisfy the ACR criteria, because there is considerable evidence to suggest that it is in the early stages of the disease that treatment is most likely to affect outcome (21).

In our previous study of primary care–based unselected series of patients with IP presenting between 1990 and 1994, we reported that RF was the most important baseline predictor of erosive disease at 5 years (22), but we have not examined the role of anti-CCP antibodies. The aim of this study was to compare the roles of RF and anti-CCP antibody status in determining the likelihood of having erosions at presentation and in predicting future radiologic damage. We also determined whether anti-CCP antibody status, either alone or in combination with RF, is sufficiently robust to be useful in guiding clinical treatment decisions.

PATIENTS AND METHODS

Study protocol. Subjects were recruited from the Norfolk Arthritis Register (NOAR), a primary care–based inception cohort of patients with IP. Details of NOAR have been published previously (23). Briefly, patients with swelling in 2 or more joints that lasted 4 weeks or longer were referred to NOAR and were assessed by a trained metrologist within 2 weeks of referral using a standardized approach. Data gathered included joint counts for swelling and tenderness and responses on the Health Assessment Questionnaire (24). Blood was obtained for serum analysis (initially for RF, but C-reactive protein and anti-CCP testing have been introduced more recently) and DNA extraction.

Radiographs of the hands and feet were requested for each consenting patient and were scored using the Larsen scale (25). All radiographs were scored by 2 observers, with a third observer arbitrating in case of disagreement (MB, DS, and AB). Details of the radiographic scoring process used in NOAR have been published elsewhere (22). Briefly, a Larsen score of ≥2 in any joint indicated the presence of erosions. Joints assessed included all proximal interphalangeal joints, the interphalangeal joint of the thumbs, all metacarpophalangeal joints, both wrists, and the second through fifth metatarsophalangeal joints in both feet. A weighting factor of 5 was applied to each wrist. The total possible score was 190.

Criteria for ascertainment of radiographs have changed over the time period that patients have been recruited to NOAR. Two cohorts of patients were included in the current study. The prospective cohort was composed of 427 consecutive unselected patients recruited between 1990 and 1994 who had both a baseline serum sample and a radiograph at 5 years available for analysis. These patients did not have a baseline radiograph performed. A more recently recruited cross-sectional cohort was studied based on 254 consecutive patients recruited after January 1, 2000 who had a baseline radiograph performed.

Serum testing. RF was measured using a latex method, and a titer of ≥1:40 was regarded as positive. Anti-CCP testing was performed using the Axis-Shield DIASTAT kit according to the manufacturer’s instructions (Axis-Shield, Dundee, UK), using the recommended cutoff of >5 units/ml as positive.

Statistical analysis. The baseline characteristics of the cohorts were compared, stratified according to their anti-CCP and RF status. For the prospective cohort, erosion status and Larsen score at 5 years were also analyzed in this way. Categorical values were compared using the chi-square test, and continuous variables were compared using the Mann-Whitney U test.

We constructed 2 × 2 tables examining the prevalence of erosions in the various groups, depending on their anti-CCP and RF status alone and in combination. Odds ratios (ORs) and 95% confidence intervals (95% CIs) were calculated using Stata software (StataCorp, College Station, TX). The absolute values of ELISA-measured anti-CCP antibody levels were also used as a continuous variable to determine their association with the development of erosions at 5 years using a receiver operating curve (ROC) analysis.

Four groups were identified within each cohort, depending on their autoantibody profile: patients who were both anti-CCP+ and RF+, patients who were RF+ and anti-CCP+, patients who were RF– and anti-CCP+, and those who were both anti-CCP– and RF–. Larsen scores for subjects with erosions in the 4 groups were ascertained and compared using the Mann-Whitney U test. Sensitivity,
specificity, and likelihood ratios (LRs) were calculated for each of these groups using the “diagt” command in Stata. All analyses were repeated, adjusting for the use of DMARDs or steroids at 5 years. P values less than 0.05 were considered significant.

RESULTS

Clinical characteristics at baseline. The baseline characteristics of each cohort are shown in Table 1. At 5 years, 311 subjects in the prospective cohort (72.8%) had satisfied ACR criteria for RA by year 5, no. (%) – 311 (72.8)

Table 1. Patient characteristics at baseline and at 5 years, in patients with available data*

<table>
<thead>
<tr>
<th></th>
<th>Cross-sectional cohort (n = 254)</th>
<th>Prospective cohort (n = 427)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female, no. (%)</td>
<td>173 (68.1)</td>
<td>283 (66.3)</td>
</tr>
<tr>
<td>Age at symptom onset, median (IQR) years</td>
<td>59.2 (48.4–70.6)</td>
<td>53.3 (42.6–70.5)</td>
</tr>
<tr>
<td>HAQ score, median (IQR)</td>
<td>0.88 (0.25–1.50)</td>
<td>0.75 (0.25–1.38)</td>
</tr>
<tr>
<td>RF+, no. (%)</td>
<td>71 (28.0)</td>
<td>113 (26.5)</td>
</tr>
<tr>
<td>Anti-CCP+, no. (%)</td>
<td>88 (34.6)</td>
<td>125 (29.3)</td>
</tr>
<tr>
<td>No. of tender joints, median (IQR)</td>
<td>3 (1–8)</td>
<td>8 (3–16)</td>
</tr>
<tr>
<td>No. of swollen joints, median (IQR)</td>
<td>3 (1–8)</td>
<td>6 (2–14)</td>
</tr>
<tr>
<td>Shared epitope alleles, no. (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>79 (48.5)</td>
<td>155 (39.4)</td>
</tr>
<tr>
<td>1</td>
<td>63 (38.6)</td>
<td>181 (46.1)</td>
</tr>
<tr>
<td>2</td>
<td>21 (12.9)</td>
<td>57 (14.5)</td>
</tr>
<tr>
<td>5 years</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of tender joints, median (IQR)</td>
<td>–</td>
<td>0 (0–4)</td>
</tr>
<tr>
<td>No. of swollen joints, median (IQR)</td>
<td>–</td>
<td>0 (0–2)</td>
</tr>
<tr>
<td>HAQ score, median (IQR)</td>
<td>–</td>
<td>0.75 (0.25–1.50)</td>
</tr>
<tr>
<td>Treated with DMARD or steroid by 5 years, no. (%)</td>
<td>–</td>
<td>257 (60.2)</td>
</tr>
<tr>
<td>Satisfied ACR criteria for RA by year 5, no. (%)</td>
<td>–</td>
<td>311 (72.8)</td>
</tr>
<tr>
<td>Symptom duration at baseline, median (IQR) months</td>
<td>5 (3–10)</td>
<td>5 (2–12)</td>
</tr>
</tbody>
</table>

* IQR = interquartile range; HAQ = Health Assessment Questionnaire; RF = rheumatoid factor; anti-CCP = anti–cyclic citrullinated peptide antibody; DMARD = disease-modifying antirheumatic drug; ACR = American College of Rheumatology; RA = rheumatoid arthritis.

Table 2. Clinical characteristics of the patients at baseline, according to the presence and absence of anti-CCP and RF at baseline*

<table>
<thead>
<tr>
<th></th>
<th>Anti-CCP+</th>
<th>Anti-CCP−</th>
<th>RF+</th>
<th>RF−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cross-sectional cohort</td>
<td>88</td>
<td>166</td>
<td>71</td>
<td>183</td>
</tr>
<tr>
<td>No. of subjects</td>
<td>64 (72.7)</td>
<td>109 (65.7)</td>
<td>50 (70.4)</td>
<td>90 (67.2)</td>
</tr>
<tr>
<td>Age at symptom onset, median (IQR) years</td>
<td>60.7 (52.2–71.4)</td>
<td>58.5 (45.0–71.1)</td>
<td>62.7 (48.9–72.0)</td>
<td>58.5 (47.7–71.1)</td>
</tr>
<tr>
<td>HAQ score at baseline, median (IQR)</td>
<td>1.0 (0.38–1.75)</td>
<td>0.75 (0.25–1.50)</td>
<td>1.0 (0.38–1.5)</td>
<td>0.88 (0.25–1.50)</td>
</tr>
<tr>
<td>Erosions at baseline, no. (%)</td>
<td>55 (62.5)</td>
<td>66 (39.8)§</td>
<td>40 (56.3)</td>
<td>81 (44.3)</td>
</tr>
<tr>
<td>Larsen score at baseline, median (IQR)</td>
<td>6.5 (1–14.5)</td>
<td>2 (0–10)§§</td>
<td>5 (0–15)</td>
<td>3 (0–12)</td>
</tr>
<tr>
<td>Prospective cohort</td>
<td>125</td>
<td>302</td>
<td>112</td>
<td>308</td>
</tr>
<tr>
<td>Female, no. (%)</td>
<td>77 (61.6)</td>
<td>206 (68.2)</td>
<td>71 (62.8)</td>
<td>212 (67.5)</td>
</tr>
<tr>
<td>Age at symptom onset, median (IQR) years</td>
<td>55.7 (48.4–64.0)</td>
<td>51.7 (40.8–62.7)</td>
<td>55.7 (46.8–64.0)</td>
<td>52.4 (42.1–62.4)</td>
</tr>
<tr>
<td>HAQ score, median (IQR)</td>
<td>0.88 (0.38–1.62)</td>
<td>0.63 (0.25–1.25)</td>
<td>0.75 (0.38–1.50)</td>
<td>0.75 (0.25–1.25)</td>
</tr>
<tr>
<td>Erosions at 5 years, no. (%)</td>
<td>99 (79.2)</td>
<td>82 (27.2)**</td>
<td>74 (65.5)</td>
<td>107 (34.1)††</td>
</tr>
<tr>
<td>Larsen score at 5 years, median (IQR)</td>
<td>29 (12–44)</td>
<td>2 (0–10)¶</td>
<td>17 (3–41)</td>
<td>4 (0–14)¶</td>
</tr>
<tr>
<td>Treated with DMARD or steroid by 5 years, no. (%)</td>
<td>119 (95.2)</td>
<td>138 (45.7)¶¶</td>
<td>91 (80.5)</td>
<td>166 (52.9)§§</td>
</tr>
</tbody>
</table>

* See Table 1 for definitions.
† P = 0.04 versus anti-CCP+ group.
‡ P = 0.001 versus anti-CCP+ group.
§ P < 0.02 versus anti-CCP+ group.
¶ P < 1 × 10−5 versus anti-CCP+ group.
# P = 0.01 versus RF+ group.
** P = 4 × 10−23 versus anti-CCP+ group.
†† P = 6.7 × 10−9 versus RF+ group.
¶¶ P = 1.9 × 10−21 versus anti-CCP+ group.
§§ P = 2.6 × 10−7 versus RF+ group.
satisfied the ACR criteria for RA, modified for genetics studies. In the cross-sectional cohort, 88 (34.6%) were anti-CCP+, 71 (28.0%) were RF+, and 50 (19.7%) were anti-CCP+ and RF+, while in the prospective cohort, 125 (29.3%) were anti-CCP+, 113 (26.5%) were RF+, and 80 (18.7%) were anti-CCP+ and RF+. The presence of anti-CCP antibodies was highly, but not perfectly, correlated with the presence of RF. For example, the kappa statistic was 0.47 in the cross-sectional cohort ($P < 0.001$) and 0.59 in the prospective cohort ($P < 0.001$). The presence of anti-CCP antibodies, but not RF, at baseline was associated with prevalent erosions, and both were associated with erosions and was the presence of Larsen score by 5 years (Table 2).

The presence of anti-CCP antibodies at baseline was a more powerful predictor of both prevalent erosions (OR 2.53 [95% CI 1.48–4.30]) and developing erosions by 5 years (OR 10.2 [95% CI 6.2–16.9]) than was the presence of RF (OR 1.63 [95% CI 0.94–2.82] and 3.4 [2.2–5.2], respectively) (Table 3). Modeling the role of anti-CCP antibodies stratified by RF status showed that anti-CCP antibodies perform equally well in predicting erosions in RF+ and RF− patients, and the presence of both autoantibodies did not yield significantly higher odds of erosions (either prevalent or at 5 years) than did anti-CCP alone (OR for erosions at 5 years in anti−CCP+ patients 10.2 [95% CI 6.2–16.9]; OR for erosions at 5 years in anti-CCP+, RF+ patients 11.6 [95% CI 4.5–29.9]).

Larsen scores were higher in anti-CCP+ patients than in anti-CCP− patients, both at baseline and at 5 years (Table 2). Patients with erosions who were anti-CCP+ at baseline had higher Larsen scores at 5 years (median 36, interquartile range [IQR] 20–48) than did anti-CCP− patients (median 16 [IQR 10–27]), although no difference in their baseline demographic features were noted. The median Larsen score at 5 years, but not at baseline, was higher in the anti-CCP+ patients, regardless of their RF status (Figures 1 and 2).

### Development of erosions.

At 5 years, erosions had developed in 81% of RF+, anti-CCP+ patients and 27% of RF−, anti-CCP− patients. The titers of anti-CCP antibodies were higher in RF+ (median 25.9 [95% CI 1.0–74.3]) than in RF− (median 0.74 [95% CI 0.48–1.26]) patients. The sensitivity and specificity of the models derived from Table 3 showed that both RF and anti-CCP antibodies were stronger predictors of erosions at 5 years than at presentation (Table 4). The specificities for predicting erosions at 5 years were similar for RF and anti-CCP antibodies, but the latter were more sensitive. Interestingly, the presence of anti-CCP antibodies had the highest LR for erosions, whether at baseline or at 5 years, in RF− patients. However, the area under the curve in the ROC analysis was higher in RF+, anti-CCP+ subjects than in RF−, anti-CCP+ subjects (0.78 [95% CI 0.69–0.87] and 0.66 [95% CI 0.59–0.73], respectively), suggesting that anti-CCP is a better predictor of erosions at 5 years in the presence of RF positivity.

The ROC analysis was also used to determine whether there is a cutoff of anti-CCP antibody values at which erosions at 5 years can be accurately predicted. The peak of the ROC curve occurred at an anti-CCP antibody value of 2.4 when the whole data set was

### Table 3. Prevalence and odds of erosions in patients at presentation and at 5 years, by autoantibody status at baseline*

<table>
<thead>
<tr>
<th>Antibody status at baseline</th>
<th>No. (%) with erosions</th>
<th>No. (%) without erosions</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cross-sectional cohort (at presentation)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All anti-CCP+</td>
<td>55 (63)</td>
<td>33 (37)</td>
<td>2.53 (1.48–4.30)</td>
</tr>
<tr>
<td>Anti-CCP+, RF−</td>
<td>24 (63)</td>
<td>14 (37)</td>
<td>1.63 (0.94–2.82)</td>
</tr>
<tr>
<td>Anti-CCP+, RF+</td>
<td>31 (62)</td>
<td>19 (38)</td>
<td>3.4 (2.2–5.2)</td>
</tr>
<tr>
<td>All RF+</td>
<td>40 (56)</td>
<td>31 (44)</td>
<td>2.53 (1.48–4.30)</td>
</tr>
<tr>
<td>RF+, anti-CCP−</td>
<td>9 (43)</td>
<td>12 (57)</td>
<td>1.16 (0.46–2.92)</td>
</tr>
<tr>
<td>RF+, anti-CCP+</td>
<td>31 (62)</td>
<td>19 (38)</td>
<td>0.95 (0.40–2.28)</td>
</tr>
<tr>
<td><strong>Prospective cohort (at 5 years)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All anti-CCP+</td>
<td>99 (79)</td>
<td>26 (21)</td>
<td>10.2 (6.2–16.9)</td>
</tr>
<tr>
<td>Anti-CCP+, RF−</td>
<td>34 (76)</td>
<td>11 (24)</td>
<td>8.3 (4.0–17.2)</td>
</tr>
<tr>
<td>Anti-CCP+, RF+</td>
<td>65 (81)</td>
<td>15 (19)</td>
<td>11.6 (4.5–29.9)</td>
</tr>
<tr>
<td>All RF+</td>
<td>83 (63)</td>
<td>48 (37)</td>
<td>3.4 (2.2–5.2)</td>
</tr>
<tr>
<td>RF+, anti-CCP−</td>
<td>9 (27)</td>
<td>24 (73)</td>
<td>1.01 (0.45–2.27)</td>
</tr>
<tr>
<td>RF+, anti-CCP+</td>
<td>65 (81)</td>
<td>15 (19)</td>
<td>1.40 (0.58–3.39)</td>
</tr>
</tbody>
</table>

* OR = odds ratio; 95% CI = 95% confidence interval (see Table 1 for other definitions).
included and at 0.65 when analysis was restricted to RF− subjects, indicating that, even at low titers, the presence of the antibody is associated with erosive change.

Logistic regression analyses were repeated using DMARDs or steroids at 5 years as a cofactor, but in no situation did this correction alter the conclusions, although it did attenuate the effects seen. For example, the OR of developing erosions at 5 years in the presence of anti-CCP antibodies at baseline was reduced to 7.1

Figure 1. Distribution of Larsen scores at baseline in all subjects (A) and in subjects with prevalent erosions (B), according to baseline antibody status in the cross-sectional cohort. Values are presented as box and whisker plots, where the boxes represent the interquartile range, the lines within the boxes represent the median Larsen score, the whiskers represent the range from the smallest to the largest score, and the circles represent outliers. No significant differences between the groups were noted. 1 represents subjects who were RF+ and anti-CCP+ (P = 0.07 versus subjects who were RF− and anti-CCP− in the total group and P = 0.70 versus subjects who were RF− and anti-CCP− in the group with erosions). 2 represents subjects who were RF− and anti-CCP+ (P = 0.06 versus subjects who were RF− and anti-CCP− in the total group and P = 0.49 versus subjects who were RF− and anti-CCP− in the group with erosions). 3 represents subjects who were RF+ and anti-CCP− (P = 0.90 versus subjects who were RF− and anti-CCP− in the total group and P = 0.34 versus subjects who were RF− and anti-CCP− in the group with erosions). 4 represents subjects who were RF− and anti-CCP− (referent).

Figure 2. Distribution of Larsen scores at 5 years in all subjects (A) and in subjects with erosions (B), according to baseline antibody status in the prospective cohort. Values are presented as box and whisker plots, where the boxes represent the interquartile range, the lines within the boxes represent the median Larsen score, the whiskers represent the range from the smallest to the largest score, and the circles represent outliers. 1 represents subjects who were RF+ and anti-CCP+ (P < 0.0001 versus subjects who were RF− and anti-CCP−, both in the total group and in the group with erosions). 2 represents subjects who were RF− and anti-CCP− (P < versus subjects who were RF− and anti-CCP− in the total group and P = 0.0001 versus subjects who were RF− and anti-CCP− in the group with erosions). 3 represents subjects who were RF+ and anti-CCP− (P = 0.90 versus subjects who were RF− and anti-CCP− in the total group and P = 0.85 versus subjects who were RF− and anti-CCP− in the group with erosions). 4 represents subjects who were RF− and anti-CCP− (referent).
Table 4. Sensitivity, specificity, and likelihood ratios for predicting erosions at baseline or at 5 years using baseline RF and anti-CCP status either alone or in combination

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity, %</th>
<th>Specificity, %</th>
<th>Likelihood ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cross-sectional cohort</strong></td>
<td></td>
<td></td>
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<tr>
<td>All anti-CCP+</td>
<td>45.5</td>
<td>75.2</td>
<td>1.8</td>
</tr>
<tr>
<td>Anti-CCP+, RF−</td>
<td>29.6</td>
<td>86.3</td>
<td>2.2</td>
</tr>
<tr>
<td>Anti-CCP+, RF+</td>
<td>77.5</td>
<td>38.7</td>
<td>1.3</td>
</tr>
<tr>
<td>All RF+</td>
<td>33.1</td>
<td>76.7</td>
<td>1.4</td>
</tr>
<tr>
<td>RF+, anti-CCP−</td>
<td>13.6</td>
<td>88.0</td>
<td>1.1</td>
</tr>
<tr>
<td>RF+, anti-CCP+</td>
<td>56.4</td>
<td>42.4</td>
<td>1.0</td>
</tr>
<tr>
<td><strong>Prospective cohort</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All anti-CCP+</td>
<td>54.7</td>
<td>89.4</td>
<td>5.2</td>
</tr>
<tr>
<td>Anti-CCP+, in RF−</td>
<td>31.8</td>
<td>94.5</td>
<td>6.0</td>
</tr>
<tr>
<td>Anti-CCP+, in RF+</td>
<td>87.8</td>
<td>61.5</td>
<td>2.3</td>
</tr>
<tr>
<td>All RF+</td>
<td>40.9</td>
<td>85.2</td>
<td>2.4</td>
</tr>
<tr>
<td>RF+, anti-CCP−</td>
<td>11.0</td>
<td>89.1</td>
<td>1.0</td>
</tr>
<tr>
<td>RF+, anti-CCP+</td>
<td>65.7</td>
<td>42.3</td>
<td>1.1</td>
</tr>
</tbody>
</table>

* See Table 1 for definitions.

(95% CI 4.1–12.1) after accounting for this potential confounder.

**DISCUSSION**

We have shown that anti-CCP antibody status, measured at presentation of IP, predicts both prevalent erosions and development of erosions at 5 years. Furthermore, anti-CCP antibody status is a better predictor of future erosions than is RF, despite the fact that both are correlated.

Anti-CCP antibody status has been proposed as a new biomarker of disease severity, since it has been found to be more sensitive than RF by all who have published studies on this area. These antibodies have the advantage that the status is stable over time as compared with RF, which is known to vary (26). Questions remain, however, regarding their usefulness in clinical practice, particularly because the assay cost is higher than that of RF, and it is unclear how much better than RF they are at determining outcome. It is unclear whether both RF and anti-CCP antibodies should be tested routinely in patients or whether anti-CCP antibody testing should be reserved for those who are RF negative.

Our study aimed to inform the debate and has several advantages over previous investigations. First, we used a primary care–based cohort of patients with unselected IP, thus reflecting the mix of patients attending early arthritis clinics. Second, the study design removes possible biases introduced when analysis is restricted to RA patients, because erosions and RF (with which anti-CCP antibodies are correlated) are both criteria used to classify RA. Finally, the radiographs were read blinded to anti-CCP status, removing the possibility of observer bias. We tested radiologic erosions as the primary outcome measure because they are an objective, reliable, and standardized method of measuring arthritis severity (27).

The results show that both RF and anti-CCP antibody status are useful as predictors of adverse outcome but that anti-CCP antibody status is stronger. The group of patients who were anti-CCP− but RF+ had similar Larsen scores as the group negative for both antibodies and significantly lower scores than the anti-CCP+, RF− group, although the small numbers in some of the groups may have limited the robustness of these conclusions. The LR for predicting erosions was higher in the anti-CCP+, RF− group, suggesting that one possible strategy would be to test for anti-CCP antibodies only in patients seronegative for RF.

It should be noted, however, that a significant number of anti-CCP− patients developed erosions at 5 years (27% of the prospective cohort). Larsen scores were significantly lower in these patients compared with anti-CCP+ subjects with erosions (median 16 [IQR 10–27] and 36 [IQR 20–48], respectively), suggesting that anti-CCP antibodies may affect the extent or severity of radiologic damage as well as susceptibility to it. However, the absence of anti-CCP antibodies cannot be used to identify subjects who do not require treatment, since even very low titers can be associated with the development of erosions, particularly in RF− subjects.

Conversely, 21% of patients positive for the presence of anti-CCP antibodies had not developed erosions at 5 years. This may reflect the benefit of treatment if treatment was more likely to be provided to anti-CCP+ patients, and indeed, this was found to be the case. Thus, although the treating physician was unaware of the antibody status, the presence of anti-CCP antibodies was strongly associated with the likelihood of receiving DMARDs or steroid therapy (OR 16.6 [95% CI 8.9–30.7]), presumably because of other markers of disease severity. The presence of RF was also associated with receiving treatment, but to a lesser degree (OR 5.2 [95% CI 3.4–7.8]). To address the issue of possible confounding by treatment, we adjusted for ever use of DMARDs or steroids at 5 years in the analysis. Such an adjustment is inevitably quite crude and almost certainly will not have accounted for all the treatment effects. The results, after this adjustment, showed that although the effects were attenuated, the presence of either antibody remained strongly predictive of the development of erosions.

In summary, ACPAs, as measured by anti-CCP ELISAs, are strongly associated with both prevalent erosions and the development of erosions at 5 years. In this respect, it is a stronger predictor than RF, but
because its detection contributes no additional value in RF+ patients, testing could be restricted to seronegative patients. Finally, despite the strong association of anti-CCP antibodies with the presence, development, and extent of erosions, knowledge of anti-CCP status alone is still not a sufficiently accurate measure upon which to base clinical treatment decisions, since a significant proportion of anti-CCP+ patients develop erosions.

**AUTHOR CONTRIBUTIONS**

Dr. Bukhari had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.  

**Study design.** Silman, Symmons, Barton.  
**Acquisition of data.** Thomson, Naseem, Bunn, Symmons.  
**Analysis and interpretation of data.** Bukhari, Naseem, Silman, Symmons, Barton.  
**Statistical analysis.** Bukhari, Naseem, Barton.  

**REFERENCES**


Distinct Regulation of Interleukin-17 in Human T Helper Lymphocytes

Zhi Chen, Cristina M. Tato, Linda Muul, Arian Laurence, and John J. O'Shea

Objective. Interleukin-17 (IL-17)–producing T helper cells have been proposed to represent a separate lineage of CD4+ cells, designated Th17 cells, which are regulated by the transcription factor retinoic acid–related orphan receptor γt (RORγt). However, despite advances in understanding murine Th17 differentiation, a systematic assessment of factors that promote the differentiation of naive human T cells to Th17 cells has not been reported. The present study was undertaken to assess the effects on naive human CD4+ T cells of cytokines known to promote murine Th17 cells.

Methods. Human naive and memory CD4+ T cells isolated from peripheral blood were activated and cultured with various cytokines. Cytokine production was measured by enzyme-linked immunosorbent assay and flow cytometry. Messenger RNA was measured by quantitative polymerase chain reaction.

Results. In response to anti-CD3/anti-CD28 stimulation alone, human memory T cells rapidly produced IL-17, whereas naive T cells expressed low levels. Transforming growth factor β1 and IL-6 up-regulated RORγt expression but did not induce Th17 differentiation of naive CD4+ T cells. However, IL-23 up-regulated its own receptor and was an important inducer of IL-17 and IL-22.

Conclusion. The present data demonstrate the differential regulation of IL-17 and RORγt expression in human CD4+ T cells compared with murine cells. Optimal conditions for the development of IL-17–producing T cells from murine naive precursors are ineffective in human T cells. Conversely, IL-23 promoted the generation of human Th17 cells but was also a very potent inducer of other proinflammatory cytokines. These findings may have important implications in the pathogenesis of human autoimmunity as compared with mouse models.

Classically, naive CD4+ T cells have been thought to differentiate into 2 possible helper lineages, Th1 or Th2 cells. Th1 cells produce the signature cytokine interferon-γ (IFNγ), a critical factor that promotes cellular immunity. Interleukin-12 (IL-12), acting via the transcription factor STAT-4 in concert with T-box expressed in T cells/T-box 21 (T-BET), is critical for Th1 differentiation. In contrast, Th2 development is initiated by IL-4 signaling with the participation of the transcription factors STAT-6 and GATA-3. The hallmark cytokine secreted by Th2 cells is IL-4, which is crucial for host defense against helminths and the pathogenesis of asthma and allergy. Th1 and Th2 lineage decision appears to be made at a very early stage of T helper cell differentiation, with the respective Th1/Th2 cytokines enforcing their own expression and inhibiting alternative commitment. This occurs by regulation of receptor levels, expression of transcription factors, and epigenetic changes (1–3). Another important aspect of Th1/Th2 counterregulation is interchromosomal interaction between Th1- or Th2-specific cytokine genes (4). As a result of these mechanisms, Th1 and Th2 cells develop into mature effectors with stable phenotypes and important roles in host defense, as documented in a number of murine models.

While the simple dichotomous model of T helper cell differentiation fits well with many models of infection, fitting autoimmune disease into such models has been problematic. CD4+ T cells can also differentiate to
become Treg cells, and it is clear that dysregulation of this subset has major consequences with respect to the pathogenesis of autoimmunity (5). An additional complication relates to the discovery of another cytokine, IL-23. IL-23 is a dimer that shares a subunit with IL-12, IL-12p40, and both utilize a receptor subunit designated IL-12 receptor β1 (IL-12Rβ1) (6). The complex biology of IL-12 and IL-23 is relevant to the pathogenesis of autoimmunity, in that gene targeting of IL-12p40 attenuates the development of disease in many models of autoimmunity. Similarly, anti-p40 antibody is efficacious in the treatment of Crohn’s disease (7). Although these effects were initially misattributed to interference with the actions of IL-12, using specific deletion of IL-23 (p19−/− mice), it is now recognized that IL-23 (and not IL-12) is the culprit, at least in many of the animal models (8–13).

One way that IL-23 is thought to promote autoimmune disease is through the regulation of IL-17A and IL-17F. IL-17A is a proinflammatory cytokine initially identified in mouse cytotoxic T cells more than 10 years ago. This family now includes 6 members (IL-17 [IL-17A], IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F) (14–17) that share 16–50% amino acid identity and have different tissue expression patterns. IL-17 acts on epithelial cells, endothelial cells, fibroblasts, synoviocytes, and myeloid cells to induce secretion of a variety of mediators, including IL-8, CXCL1, CXCL6, IL-6, granulocyte–macrophage colony-stimulating factor, granulocyte colony-stimulating factor, tumor necrosis factor α (TNFα), and IL-1β. IL-17 family cytokines thus induce cellular infiltration and production of inflammatory cytokines. Dysregulated production of IL-17 is associated with human autoimmune diseases, including multiple sclerosis, inflammatory bowel disease, and psoriasis (13,18–20). Importantly, studies in a murine experimental arthritis model showed that IL-17 was involved in both the initiation and the progression of the disease. Furthermore, elevated levels of IL-17 were detected in synovial fluid from patients with rheumatoid arthritis, and osteoclast formation was inhibited by anti–IL-17 antibody, suggesting an effect on bone resorption (21–25).

IL-17A was originally reported to be produced by activated CD4+ and CD8+ T cells. More recently, it has been proposed that IL-17–producing CD4+ T cells represent a distinct lineage (Th17), a lineage that does not produce IL-4 or IFNγ (26–28). In fact, these products of Th1 and Th2 cells antagonize the differentiation of Th17 cells. Additionally, cytokines that promote Th1 differentiation are distinct from those that promote Th1 and Th2 differentiation. The current model is that whereas transforming growth factor β1 (TGFβ1) promotes Treg cell differentiation, the combination of TGFβ1 and IL-6 promotes Th17 lineage commitment (29–31). This subset is expanded and maintained by IL-23. Both IL-6 and IL-23 activate the transcription factor STAT-3, which directly binds to the IL-17 promoter to regulate IL-17 expression (32). Conversely, suppressor of cytokine signaling 3 negatively regulates Th17 differentiation by inhibiting STAT-3 phosphorylation (32,33). In addition to STAT-3, retinoic acid–related orphan receptor γt (RORγt) is an important transcription factor for initiation of Th17 differentiation (34).

The current models of Th17 differentiation are all derived from studies using murine cells. Given the pathogenic relevance of IL-17 family members, it is obviously important to understand how this family of cytokines is controlled in human T cells and to define the conditions under which human naive CD4+ T cells might become Th17 cells. Previously, it was reported that T cell receptor (TCR) crosslinking leads to IL-17 production (35). It has also been reported that IL-23 induces IL-17 expression (26). However, these studies did not separate effects on memory cells versus naive cells. The ability of naive human CD4+ T cells to differentiate into IL-17 producers has not been examined, nor has a comprehensive assessment been undertaken to define the relative importance of various conditions in promoting the differentiation of this putative human lineage. Additionally, the extent to which human T cell subsets fully commit to (or extinguish) IL-17 production has not been addressed. This is important not only with respect to the pathogenesis of human autoimmune disease, but also because human T helper cell differentiation appears to exhibit much more plasticity than murine T helper cell differentiation (36).

In the present report, we show that human memory T cells up-regulate RORγt and IL-17 in response to TCR occupancy. Importantly, in sharp contrast to murine naive CD4+ T cells, in which Th17 lineage commitment is initiated by TGFβ1 and IL-6, human Th17 differentiation does not occur in response to this cytokine combination, even though these cytokines promote RORγt expression. Instead, IL-23 up-regulates its own receptor on T cells and increases RORγt and IL-17 expression. IL-23 also induces other proinflammatory cytokines, including IL-22. These findings may have important implications in the pathogenesis of human autoimmunity as compared with mouse models.
MATERIALS AND METHODS

Cell cultures. Peripheral blood mononuclear cells were isolated from peripheral blood of healthy donors (Department of Transfusion Medicine, National Institutes of Health, Bethesda, MD) by Ficoll-Paque Plus (Amersham Pharmacia Biotech, Uppsala, Sweden). Naive or memory CD4 T cells were further purified using a human naive or memory CD4 T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of CD45RA, CD45RO cells was 90%. Naive CD4 T cells were activated with plate-bound anti-CD3 and soluble anti-CD28 (BD PharMingen, San Diego, CA) and cultured under neutral (Th0), Th1 (IL-12, anti–IL-4), Th2 (IL-4, anti–IL-12, anti-IFNγ), or Th17 (TGFβ1 and IL-6, anti-IFNγ and anti–IL-4) polarizing conditions with IL-2 or stimulated with IL-23 (10 ng/ml; R&D Systems, Minneapolis, MN). On day 7, cells were restimulated with plate-bound anti-CD3 and anti-CD28 and continuously cultured until day 14 under Th0, Th1, Th2, or Th17 polarizing conditions.

Cytokine detection. Quantitation of cytokine production in cell culture supernatants was determined by enzyme-linked immunosorbent assay (R&D Systems) according to the manufacturer’s instructions. Cytokine-producing cells were determined by intracellular staining using phycoerythrin-conjugated anti-IFNγ (BD PharMingen), Alexa Fluor 647-conjugated anti-human IL-17 (eBioscience, San Diego, CA), and Alexa Fluor 488-conjugated anti-human forkhead box P3 (FoxP3; BioLegend, San Diego, CA). Briefly, cells were stimulated with phorbol myristate acetate and ionomycin for 4 hours, and Golgiplug (BD Biosciences, San Jose, CA) was added after 2 hours. Cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% saponin, stained with fluorescent antibodies, and analyzed on a FACSCalibur flow cytometer (BD Biosciences). CellQuest software (BD Biosciences) was used for data acquisition, and Flow Jo software (Tree Star, Ashland, OR) was used for analysis.

Real-time quantitative polymerase chain reaction (TaqMan) analysis. Total RNA was isolated with an RNeasy kit (Qiagen, Valencia, CA). Complementary DNA was synthesized with the use of a TaqMan Reverse Transcription kit (Applied Biosystems, Foster City, CA) using random hexamers as primers according to the manufacturer’s instructions. Hypoxanthine guanine phosphoribosyltransferase (HPRT) was used as an endogenous control. TaqMan primers and probes for human IL-17A (IL-17), IL-17B, IL-17C, IL-17D, IL-17E, IL-17F, IL-22, IFNγ, IL-4, T-BET, GATA-3, IL-23R, RORγt, and HPRT were purchased from Applied Biosystems, and samples were analyzed using the ABI Prism 7700 Sequence Detection System (Applied Biosystems).

Statistical analysis. Statistical analyses were performed using Small Stata 9.2 software (StataCorp, College Station, TX). Geometric means were computed separately for each stimulation. The log difference (geometric mean) in IL-17 or IL-22 secretion between the 2 stimulations for each donor was used to conduct a paired t-test and compute a 95% confidence interval.

RESULTS

High levels of IL-17 produced by human memory T helper cells. We first assessed whether IL-17 production is detected in normal human donors. To this end, we fractionated CD4+ cells into memory (CD45RO+) and naive (CD45RA+) cells. As shown in Figure 1a, freshly isolated memory CD4+ T cells secreted large quantities of IL-17 in response to anti-CD3 and anti-CD28. While there was considerable individual variation in the propensity to produce IL-17, memory CD4+ T cells made significantly more IL-17 than did naive CD4+ T cells.

Figure 1. Significantly increased levels of interleukin-17 (IL-17) produced by human memory T helper cells. Isolated human naive and memory CD4+ T cells were stimulated with plate-bound anti-CD3 and anti-CD28 for 24 hours. IL-17 production was detected using enzyme-linked immunosorbent assay kits. a, A representative result. Values are the mean and SD. unstim = unstimulated. b, Data from 5 healthy donors. Horizontal lines indicate the geometric mean. Statistical significance was calculated based on the geometric mean, using a paired t-test.
(Figure 1b), although modest levels of IL-17 were clearly produced by naive CD4+ T cells (~15–20% of the levels produced by memory cells). In this setting, plate-bound antibodies and antibody-coated microbeads gave similar results (data not shown).

**Human Th17 cells are not generated under optimal conditions for murine Th17 cells.** Since IL-17-producing T cells were readily detectable in human memory CD4+ T cells, we next sought to determine what conditions might favor the generation of such cells from naive CD4+ T cells. Recent studies have shown that TGFβ1 and IL-6, combined with blockade of IL-4 and IFNγ, quickly generate large numbers of IL-17-producing T cells from isolated naive murine CD4+ T cells within a few days (see supplementary Figure 1, available online at www.niams.nih.gov/rtbc/labs_branches/miib/lcbs/publications.htm) (29–31,37). Therefore, we cultured naive human CD4+ T cells under these conditions (termed “Th17 conditions”) to assess whether this regimen would generate human IL-17-producing T cells.

Surprisingly, the combination of TGFβ1 and IL-6 with anti–IL-4 and anti-IFNγ was no better than anti-CD3 and anti-CD28 alone in terms of IL-17 production ($P = 0.67$; $n = 5$) (Figure 2a). This was the case regardless of the concentration of anti-CD3 used, with or without anti-CD28 (see supplementary Figure 2, available online at www.niams.nih.gov/rtbc/labs_branches/miib/lcbs/publications.htm). Kinetic analysis of IL-17 production during 2-week culture further sup-
ported this contention (Figure 2b). The effect of TGFβ1 was evidenced by the up-regulation of FoxP3, although, surprisingly, the combination of TGFβ1 and IL-6 enhanced FoxP3 expression, rather than inhibiting expression as is the case in murine cells (31) (Figure 2c). Some protocols for in vitro generation of murine Th17 cells use mononuclear cells along with soluble anti-CD3/anti-CD28 (29) and exogenous TGFβ1 and IL-6. However, this also failed to induce human cells to produce IL-17 (data not shown); in fact, activated monocyte-derived dendritic cells enhanced IFNγ production and inhibited Th17 differentiation (data not shown).

In murine CD4+ T cells, the combination of TGFβ1 and IL-6 with anti–IL-4 and anti-IFNγ not only efficiently generates IL-17–producing T cells, but also extinguishes IFNγ production. This is the basis of the idea that Th17 cells are a distinct functional lineage of mouse CD4+ effector cells (30,31). Culture of human naive CD4+ T cells with or without exogenous TGFβ1 and IL-6 generated similar numbers of IL-17–producing cells, and notably, half of the IL-17–producing cells expressed both IFNγ and IL-17 (Figure 2d). Therefore, we next polarized naive human CD4+ T cells under Th1 (IL-12 and anti–IL-4) and Th2 (IL-4 and anti–IL-12 and anti-IFNγ) polarizing conditions and assessed their ability to produce IL-17. Surprisingly, IL-17 production was not extinguished under these conditions (see supplementary Figure 3, available online at www.niams.nih.gov/rtbc/labs_branches/miib/lcbs/publications.htm). Despite the absence of IL-17 messenger RNA (mRNA) in cells polarized under Th2 conditions for 7 days, re-stimulation via TCR occupancy (anti-CD3/anti-CD28 stimulation) permitted considerable IL-17 production, indicating the plasticity of human CD4+ T cells with respect to IL-17 production (Figure 3a). IFNγ and IL-17 dual producers were also observed in freshly isolated human memory CD4+ T cells activated with anti-CD3/anti-CD28, indicating that the incomplete differentiation of human Th17 cells was not simply an in vitro phenomenon (Figure 3b).

**Figure 3.** Plasticity of human IL-17–producing T cells. a, Isolated naive CD4+ T cells were stimulated with anti-CD3 and anti-CD28 under Th0, Th1 (IL-12, anti–IL-4), Th2 (IL-4, anti–IL-12, anti-IFNγ), or Th17 polarizing conditions for 7 days, and IL-17 protein production was measured by ELISA directly or after restimulation with anti-CD3 and anti-CD28 for an additional 24 hours. Values are the mean and SD. b, Isolated memory CD4+ T cells from 2 different donors were activated with plate-bound anti-CD3 and anti-CD28 for the indicated periods of time. IFNγ and IL-17–producing cells were analyzed by intracellular cytokine staining using flow cytometry. See Figure 2 for other definitions.

IL-23 up-regulation of IL-23R and enhancement of IL-17 production by human CD4+ T cells. IL-23 is a heterodimeric cytokine that shares a ligand subunit (p40) and a receptor subunit (IL-12Rβ1) with IL-12 (38). IL-23 has been reported to be essential for the development of autoimmunity in mouse models, and current data suggest that this is due to the production of IL-17 (6,8–10,26,39). We found that naive CD4+ T cells expressed low levels of IL-23R mRNA compared with memory CD4+ T cells (Figure 4a). Under Th0, Th1, and Th2 conditions, the expression of this receptor remained low; however, IL-23R mRNA expression was strongly
up-regulated in anti-CD3/anti-CD28–activated T cells cultured with IL-23 (Figure 4b). Furthermore, IL-23R was inhibited by “Th17 conditions.” In mice, IL-23 was initially thought to promote Th17 polarization, but was later viewed as a regulator of Th17 expansion/maintenance (29). Therefore, we next studied the effect of inclusion of IL-23 in cultures on the polarization of naive human precursors compared with other conditions. As shown in Figure 4c, anti-CD3/anti-CD28–induced IL-17 alone had little additional effect at early time points. Addition of IL-23 in Th17 polarizing conditions was also ineffectual. However, after 2 weeks of culture, cells cultured with IL-23 and restimulated with anti-CD3/anti-CD28 produced significantly higher amounts of IL-17 compared with cells cultured under other conditions. Cells cultured under Th1 and Th17 conditions had poor IL-17 production, and addition of IL-23 did not rescue cells cultured under Th17 conditions. IL-23 had relatively little effect on memory (CD45RO+) T cells to produce IL-17 (Figure 4d); this contrasted greatly with its effect on IL-22 (see below). Again, IFNγ and IL-17 dual producers were observed under all these conditions (data not shown).

**Figure 4.** IL-23 up-regulation of its receptor (IL-23R) and enhancement of IL-17 production in human CD4+ T cells. **a,** IL-23R mRNA expression in isolated naive or memory CD4+ T cells was analyzed by real-time quantitative polymerase chain reaction (PCR). Values are the mean and SD of 3 measurements. **b,** Naive CD4+ T cells were activated with plate-bound anti-CD3 and anti-CD28 and cultured under Th0, Th1 (IL-12, anti–IL-4), Th2 (IL-4, anti–IL-12, anti–IFNγ), Th17, Th1+IL-23 (10 ng/ml), or Th0+IL-23 (IL-23, anti–IL-4, anti–IFNγ; 10 ng/ml) conditions for 2 days. IL-23R mRNA expression was detected by real-time quantitative PCR. Values are the mean and SD of duplicate experiments. **c,** Naive CD4+ T cells were cultured under Th0, Th1, Th17, Th17+IL-23, or Th0+IL-23 conditions for 14 days. On day 14, cells were restimulated with anti-CD3 and anti-CD28 for 24 hours. IL-17 in cell culture supernatants was detected by ELISA. Values are the mean and SD of 3 separate donors analyzed in duplicate. Statistical significance was calculated with Student’s paired t-test (n = 6). **d,** Naive or memory CD4+ T cells were activated with plate-bound anti-CD3 and anti-CD28 with either no polarizing cytokines or under Th0+IL-23 conditions for 24 hours. IL-17 was measured by ELISA. Values are the mean and SD. See Figure 2 for other definitions.
TGFβ1 and IL-6, and increased even more by the combination of TGFβ1, IL-6, and IL-23 (Figure 5a), despite the fact that IL-17 mRNA levels were not affected (Figure 5b). The effects of cytokines on RORγt mRNA expression were also evident when starting with naive CD4+ T cells, but were more pronounced at 2 weeks of culture, especially after anti-CD3/anti-CD28 restimulation (Figure 5c).

**Distinct regulation of IL-17 and IL-22.** IL-22 is a member of the IL-10 family, and its gene is located on chromosome 12q15 between the IFNγ and IL-26 loci (40,41). Recently, IL-22 has been reported to be produced by murine Th17 cells in vitro and in vivo (12,42). We sought to determine whether the expression of IL-22 in human CD4+ T cells was regulated in the same manner as that of IL-17A and IL-17F. Again, we found that freshly isolated naive CD4+ T cells did not express detectable levels of IL-22 mRNA. After TCR stimulation, IL-22 production was significantly up-regulated, and the induction was much greater in memory than in naive CD4+ T cells (Figure 6a). Interestingly, IL-23 greatly enhanced IL-22 production, especially in memory CD4+ T cells, contrasting sharply with its minimal effects on IL-17 production (Figure 4d). Addition of IL-23 to cultures of naive CD4+ T cells activated with anti-CD3 and anti-CD28 also resulted in enhanced IL-22 production (Figures 6c and d). In contrast, “Th17 conditions” inhibited IL-22 production (Figure 6b). Conversely, previous studies have shown that IL-22 is produced by Th1 cells (43), and we confirmed the effects of addition of exogenous IL-12 in the experiment shown in Figure 6c. Compared with anti-CD3/anti-CD28 alone, IL-12 enhanced IL-22 production, whereas optimal “Th17 conditions” attenuated IL-22 production (Figures 6b and c). However, IL-23 was clearly a more potent inducer of IL-22 (Figure 6e).

**DISCUSSION**

Although the cytokine IL-17 was discovered more than 10 years ago, the existence of a new lineage of
T helper cells (Th17 cells) that selectively produce IL-17 was only recently recognized (26–28). More recently, it has been argued that Th17 cells are also major producers of IL-22 (12,42). The current view of mouse Th17 development is that TGF\(_{\beta}\) and IL-6 are the key cytokines that initiate the differentiation process and that IL-23 plays an essential role in the expansion and maintenance of this lineage. Support for the notion of an IL-23/IL-17 axis has been provided by a number of models of autoimmunity (6,8–11,26,39,44–46), but despite these compelling data in mice, a systematic analysis of human Th17 differentiation from naive cells has not yet been undertaken.

In the present study, we showed that regulation of IL-17 and IL-22 in human CD4\(^+\) T cells is surprisingly different from that in mouse T cells. In the mouse, TCR stimulation with a costimulatory signal alone is not sufficient to induce IL-17, especially in naive CD4\(^+\) T cells. In contrast, in human CD4\(^+\) T cells, TCR stimulation of memory cells, and to a lesser extent naive cells, is sufficient. Although IL-17–producing T cells are readily detectable among human memory CD4\(^+\) T cells, the cytokine cocktail that effectively promotes murine Th17 differentiation (TGF\(_{\beta}\) and IL-6) was completely ineffective in driving Th17 differentiation of naive human CD4\(^+\) T cells. Moreover, IL-23, a cytokine that does not effectively induce murine Th17 differentiation, was effective in promoting IL-17 production in human T cells. One aspect of this regulation is the ability of IL-23 to induce its own receptor, which is analogous to other cytokines, such as IL-2, IL-4, and IL-12, which also regulate the expression of their cognate receptors.

While IL-23 is an inducer of IL-17, IL-23 activates both STAT-3 and STAT-4 and is a potent inducer of IFN\(\gamma\) and IL-22 in human CD4\(^+\) T cells. In fact, simultaneous IL-17 and IFN\(\gamma\) production was
readily evident in freshly isolated, anti-CD3/anti-CD28–activated human memory CD4+ T cells. Furthermore, in conditions that favored Th1 and Th2 differentiation, IL-17 production was not extinguished as is the case in mouse cells. Thus, while IL-23 is a regulator of Th17 differentiation, there appears to be more flexibility and less evidence of “lineage commitment” for selective cytokine secretion in human CD4+ T cells.

Human memory T cells clearly maintain the capacity to promptly generate IL-17; one would expect this locus to remain accessible in memory CD4+ T cells—even under circumstances that favor IFNγ and IL-4 production. Remodeling of the IL-17 locus in memory cells might also allow for efficient production of this cytokine—this possibility and others will need to be examined in the future. Additionally, we consistently observed the coexistence of IFNγ+,IL-17+ and IFNγ−,IL-17+ cells. In this regard, it is notable that IFNγ+,IL-17+ T cells are also evident in the mouse. Whether these 2 populations represent separate subsets of T helper cells or different stages of lineage polarization is also an important topic for future investigation. Although cytokine loci of opposite lineage are typically silenced in fully polarized murine Th1, Th2, and Th17 cells, this is less evident in human CD4+ cells. Investigating the basis of these differences will be an interesting area of further study as we learn more about the transcriptional and epigenetic regulation of cytokine genes.

Stimulation of human T cells with anti-CD3/anti-CD28 and IL-23 was associated with increased expression of the transcription factor RORγt, a key transcription factor controlling mouse Th17 differentiation (34). However, the expression of RORγt and IL-17 are not as tightly linked as they are in mouse cells, suggesting that IL-17 expression is likely regulated by other transcription factors. Kinetic detection of RORγt protein expression in developing Th17 cells may give some insight into the regulation of IL-17 expression, but the field is hampered by a lack of optimal reagents. Clearly, in human CD4+ T cells, TCR occupancy is very important for IL-17 induction. Accordingly, the proximal promoter of the human IL17A gene contains 2 binding sites for nuclear factor of activated T cells (NF-AT), which appear to be important in the regulation of IL-17 (35). However, our data suggest that factors other than RORγt and NF-AT may be important contributors.

The discordant expression of IL-17 and RORγt is also of interest when viewed in the context of the regulation of FoxP3 in human T cells. In murine T cells, Foxp3 expression is tightly controlled and correlates well with the suppressive activity of Treg cells. However, FoxP3 is more widely expressed in activated human T cells, and its expression is not necessarily indicative of a population of cells with suppressive activity (47). Curiously, FoxP3+,IL-17+ cells were present when T cells were stimulated with TGFβ1 and IL-6; the exact functional significance of such FoxP3+,IL-17+ cells remains to be determined.

Additionally, the mechanisms underlying the distinct regulation of IL-17 and IL-22 are not clear. IL-23–dependent IL-22 production is impaired in STAT-3–deficient T cells, and this is likely due to impaired IL-23R expression (data not shown). However, in preliminary experiments, IL-12–dependent IL-22 production was not impaired in STAT-3–deficient mice, presumably because of the importance of STAT-4.

Recent studies have supported the notion of a role of IL-23 in autoimmune responses, and a preponderance of evidence indicates that this cytokine may be more clinically relevant than its close relative IL-12 in causing autoimmunity (8–11,45). The recent discovery of the relationship between IL-23R single-nucleotide polymorphisms and the prevalence of inflammatory bowel disease highlights the relevance of this cytokine in human autoimmune disease (48). The present data support the importance of an IL-23/IL-17 axis in human CD4+ T cells, consistent with what is seen in murine systems, although IL-23 seems to play a subtly different role. Since IL-23R expression is low in naïve T cells, there are likely to be other unidentified cytokines or signals that act in conjunction with IL-23 to regulate IL-17 expression. However, in human CD4+ T cells, IL-23 is also an extremely potent inducer of IL-22, IFNγ, and TNF, cytokines that all participate in autoimmune diseases (12,49,50). Therefore, the concept that a major driver of autoimmune diseases can be explained solely by a discrete IL-23/IL-17 connection is likely an oversimplification in humans. The actions of IL-23 are clearly of interest, but this cytokine apparently has very diverse effects on human CD4+ T cells.

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AUTHOR CONTRIBUTIONS

Dr. Chen had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Chen, Tato, O'Shea.

Acquisition of data. Chen, Muul, Laurence.

Analysis and interpretation of data. Chen, O'Shea.


Statistical analysis. Chen.

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Enhanced Efficacy of Regulatory T Cell Transfer Against Increasing Resistance, by Elevated Foxp3 Expression Induced in Arthritic Murine Hosts

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Objective. To investigate the efficacy of type II collagen–reactive Foxp3-expressing T cell transfer in suppressing collagen-induced arthritis (CIA) in relation to disease progression.

Methods. CD3-activated CD4 T cells were retrovirally transduced with the Foxp3 gene, and their in vitro suppressive activity on T cell proliferation was assessed for correlation with Foxp3 levels. To suppress CIA, Foxp3-transduced T cells generated with type II collagen– or ovalbumin (OVA)–pulsed dendritic cells (DCs), which were fractionated by Foxp3 levels, were adoptively transferred to mice at various time points.

Results. The in vitro suppressive activity of Foxp3-transduced cells correlated positively with Foxp3 levels. Type II collagen–reactive, but not OVA-reactive, Foxp3-transduced cells significantly suppressed CIA when they were transferred before immunization, and this suppression was accompanied by decreased anti–type II collagen antibody production. Larger cell numbers were required to suppress CIA when transfer occurred 20 days after immunization, indicating that hosts became resistant to suppression. Transfer of $1 \times 10^5$ Foxp3low cells had only a marginal effect on CIA suppression in immunized hosts, while transfer of Foxp3high cells at smaller doses significantly suppressed CIA. Transfer of $1 \times 10^5$ Foxp3high cells after establishment of arthritis attenuated disease progression but did not reverse joint swelling.

Conclusion. Resistance to Foxp3-transduced T cells proceeded as CIA progressed, suggesting that late-stage aggressive arthritis is more resistant to regulatory T cell transfer. An elevated expression level of Foxp3 in type II collagen–specific T cells improved their suppressive function in CIA. Thus, transfer of T cells expressing high levels of Foxp3 could be a strategy to overcome the induced resistance to regulatory T cell therapy.

Recent studies have added to the accumulated evidence that CD4+CD25+ naturally arising regulatory T cells (Treg) play a crucial role in the maintenance of peripheral self tolerance (1,2). Foxp3, a transcription regulator belonging to the forkhead/winged helix transcription factor family (3), is expressed exclusively in T cells with regulatory activities, including naturally arising CD4+CD25+ Treg (4–6) and some adaptive Treg subsets (7–9). Foxp3 is considered as a master gene characterizing phenotypes and functions of Treg. Retrovirus-mediated ectopic Foxp3 expression confers CD4+CD25− non-Treg characteristics similar to those of CD4+,CD25+ Treg, such as expression of CD25, CTLA-4, CD103, and glucocorticoid-induced tumor necrosis factor receptor (GITR) (4). The suppressive activities induced in these cells are independent of soluble factors such as interleukin-10 (IL-10) or transforming growth factor β (4,10).

The involvement of CD4+,CD25+ Treg in auto...
immune diseases in humans and animals has been intensively investigated over the past several years. Analyses of clinical samples demonstrated that patients with multiple sclerosis (11), autoimmune polyglandular syndromes (12), juvenile idiopathic arthritis (13,14), or rheumatoid arthritis (RA) (15–17) had an abnormal number and/or abnormal regulatory function of CD4+,CD25+ T cells. These facts brought forth the idea that supplementation of functionally intact Treg may reverse the activation of autoreactive T cells in patients with these diseases. The development of autoimmunity induced by transfer of CD25-depleted CD4 T cells to athymic nude mice as well as by neonatal thymectomy of normal mice was successfully prevented by adoptive transfer of polyclonal CD4+,CD25+ T cells (18,19).

Similarly, adoptive transfer of naturally arising CD4+,CD25+ Treg as well as Foxp3-transduced T cells was also successful in suppressing autoimmunity in various murine models of lymphopenia, which offer a favorable environment in which the transferred lymphocytes have room to expand. These models included experimental autoimmune encephalomyelitis, autoimmune diabetes, and autoimmune colitis (4,20–23). In some studies, antigen-specific CD4+,CD25+ cells were transferred, while in other studies, nonspecific T cells were transferred. Generally, larger numbers of polyclonal T cells were required to suppress the same disease (i.e., autoimmune diabetes) (21,22). Thus, antigen specificity is a factor promoting the in vivo effects of Treg, probably because they are recruited and/or expand at the sites of local inflammation.

A previous study demonstrated that in vivo depletion of CD25+ cells prior to initiation of collagen-induced arthritis (CIA), a model of RA, worsened arthritis. This finding suggested that CD4+,CD25+ Treg have an inhibitory role in disease development (24). As shown in other animal models of lymphopenia, transfer of non–antigen-specific Treg CD4+,CD25+ T cells inhibited CIA development when host mice received a lethal dose of total body irradiation followed by rescue with syngeneic bone marrow transplantation (25). If larger numbers of CD4+,CD25+ T cells were transferred, a suppressive effect was also observed, without induction of lymphopenia. However, transferring a large number of non–antigen-specific Treg may lead to systemic immune suppression.

The aim of this study was to determine whether antigen-specific Treg transfer efficiently suppresses arthritis in immunocompetent hosts, and whether susceptibility to suppression by Treg changes during the disease course.

MATERIALS AND METHODS

Mice. Male DBA/1J mice were purchased from the Oriental Yeast Company (Tokyo, Japan). The mice were housed in the animal facility under specific pathogen–free conditions at the Research Center for Allergy and Immunology, RIKEN.

Monoclonal antibodies (mAb) and flow cytometry. Fluorescein isothiocyanate–conjugated anti-mouse CD3e (145-2C11 antibody), phycoerythrin (PE)–conjugated anti-mouse CD4 (L3T4 antibody), biotinylated anti-mouse CD25 (mAb 7D4; IgM), and allophycocyanin (APC)–conjugated streptavidin were purchased from BD Pharmingen (San Diego, CA). APC-conjugated anti-mouse CD11c (N418) antibody was purchased from eBioscience (San Diego, CA). Single-cell suspensions were incubated with fluorescent or biotinylated mAb; incubation with biotinylated mAb was followed by incubation with PE-conjugated streptavidin. Data were acquired on a FACSCalibur and were analyzed using CellQuest software (BD Biosciences Immunocytometry Systems, San Jose, CA).

Preparation of retroviral construct and supernatants. Two Foxp3-containing vectors were prepared for these experiments. The first vector consisted of an MIGR1 vector with a mouse Foxp3 complementary DNA insert (MIGR1-Foxp3) (4). The second vector contained the Eco RI–Bgl II fragment from MIGR1-Foxp3, which was ligated into an Eco RI–Bam HI–cleaved pMCs-IG vector (Foxp3-pMCs-IG) (26). MIGR1 and pMCs-IG encode green fluorescent protein (GFP) under the control of an internal ribosomal entry site. Retroviruses were prepared by introducing the empty vectors or the vectors with Foxp3 into the Plat-E packaging cell line (26,27).

Dendritic cell (DC) preparation. Bone marrow cells were collected from 6–7-week-old male DBA/1J mice. The bone marrow cells were cultured in RPMI 1640 medium containing 20 mg/ml granulocyte–macrophage colony-stimulating factor (PeproTech, London, UK), 10% fetal bovine serum, 2 μM L-glutamine, 50 μM 2-mercaptoethanol, and antibiotics for 7 days. The cells were then cultured in the presence of 1 μg/ml lipopolysaccharide (LPS; Sigma-Aldrich, St. Louis, MO) with 50 μg/ml bovine type II collagen (Collagen Research Center, Tokyo, Japan), ovalbumin (OVA; Sigma-Aldrich), or LPS alone for a further 24 hours. These cells were used as mature DCs. A majority of the cells harvested by this method were CD11c positive.

Priming of CD4 T cells with antigen-pulsed DCs, and retroviral infection. DBA/1J mice were immunized twice (1-week interval) with 200 mg of either bovine type II collagen or OVA in Freund’s complete adjuvant (CFA; Difco, Detroit, MI), in the hind footpads. CD4 T cells were isolated from the draining lymph nodes of immunized mice or from the splenocytes of naïve mice, using magnetic-activated cell sorting microbead-coupled mAb and magnetic cell separation columns (Milteny Biotec, Auburn, CA). The cells were cultured with type II collagen–pulsed DCs, OVA-pulsed DCs, or DCs treated with anti-mouse CD3e mAb (145-2C11; BD Pharmingen) in the presence of 100 units/ml recombinant human
IL-2 (Shionogi Pharmaceuticals, Osaka, Japan) for 24 hours. The activated CD4 T cells were mixed with an equal volume of retroviral supernatant and Polybrene (6 μg/ml; Sigma-Aldrich), centrifuged at 1,750g at 32°C for 1 hour, and incubated for a further 7 hours at 37°C in a 5% CO₂ atmosphere. The cells were then cultured in complete medium supplemented with 100 units/ml IL-2. After 2 or 3 days, live GFP-positive fractions of the infected cells were isolated using a BD FACSAria Cell Sorter (BD Biosciences Immunocytometry Systems). To assess antigen-specific priming of CD4 T cells, 1 × 10⁶ CD4 T cells were cultured with antigen-pulsed DCs or unpulsed DCs at various ratios, in 96-well plates. ³H-thymidine uptake was measured after 3 days. For immunoblot analyses and suppression assays, activated CD4⁺,CD25⁺ T cells were prepared by culturing CD4⁺,CD25⁺ T cells with DCs in the presence of anti-CD3 mAb (0.5 μg/ml) and IL-2 (100 units/ml) for 3 days.

**Immunoblot analyses.** Whole cell lysates were prepared using RIPA lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA), and a fraction (10 μg) was loaded to each lane. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis/immunoblot analyses were performed using anti-Foxp3 rabbit antibody (28) as primary antibody and horseradish peroxidase–conjugated anti-rabbit antibody as secondary antibody (Santa Cruz Biotechnology), with detection accomplished using the enhanced chemiluminescence technique (Amersham Biosciences, Uppsala, Sweden). The measurement of band intensity was performed using ImageJ software (29).

**Suppression assay.** To assess the suppressive activities of regulatory T cells, CD4⁺,CD25⁺ responder T cells (5 × 10⁵/well) were cultured in a 96-well plate with Treg at various ratios, in the presence of anti-CD3 mAb (0.5 μg/ml). ³H-thymidine (1 μCi/well) uptake was measured after 3 days.

**Induction of CIA and clinical assessment of arthritis.** Eight-week-old male DBA1/J mice were inoculated intradermally at the tail base with 200 μg of bovine type II collagen in CFA (Difco). The mice received booster immunizations in the same manner, 21 days after the primary immunization. After the booster immunization (day 0), the disease severity in each mouse was recorded using the following scoring system: 0 = normal, 1 = mild swelling in 1 joint, 2 = mild swelling in =2 joints, 3 = severe swelling in the paw or digits, and 4 = severe swelling in entire paw and digits.

**Detection of bovine type II collagen–specific antibodies.** Bovine type II collagen–specific antibodies in mouse serum were measured by enzyme-linked immunosorbent assay. Microtiter plates were coated with 2 μg/ml bovine type II collagen, which was denatured by boiling in phosphate buffered saline (PBS). The plates were washed with 0.05% Tween 20 in PBS (PBST), blocked with 2% bovine serum albumin (BSA) in PBS, and incubated with diluted mouse serum (1:1,000 ratio). Positive reactions were detected by incubation with rabbit anti-mouse IgG1, IgG2a, or IgG2b conjugated to horseradish peroxidase (Zymed, Burlingame, CA) in PBST with 2% BSA. The final reaction was visualized using the TMB Microwell Peroxidase Substrate System (KPL, Gaithersburg, MD). Optical density values were measured at 450 nm. Bovine type II collagen–specific antibody units were determined using a reference serum created from pooled sera from arthritic or nonarthritic mice. A 1:40 dilution of serum from arthritic mice was assigned a value of 1,000 units/ml.

**Histologic assessment of arthritis.** Mice with CIA were killed 14 days after receiving the booster immunization. The knee joints were removed, fixed in formalin, and decalcified in 10% EDTA. The samples were embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

**Statistical analysis.** Experimental groups were analyzed by fitting a repeated-measures model with a random intercept (30), using the linear mixed effects function in the “nlme” (31) package of R, version 2.4.1 (32). Full models were compared with models without the treatment term. P values less than 0.05 were considered significant.

**RESULTS**

**Suppression of T cell proliferation in vitro by Foxp3-transduced cells.** To investigate whether adoptive transfer of Foxp3-expressing T cells can suppress CIA in nonlymphopenic animals, we generated CD4 Treg by retroviral Foxp3 gene transfer. Splenic CD4 T cells from naive mice were mixed with mature DCs at a 10:1 ratio, in the presence of anti-CD3 mAb and IL-2. Stimulated T cells were infected with retroviral vectors including both GFP and Foxp3 genes (Foxp3-pMCs-IG) or GFP gene alone (pMCs-IG). The percentage of GFP-expressing cells was initially 30–50% (Figure 1A) but gradually decreased after 4 days in Foxp3-pMCs-IG–infected cells but not in pMCs-IG–infected cells (data not shown), which indicates that Foxp3 expression repressed cell growth. The Foxp3-expressing cells up-regulated expression of CD25, GITR, and intracellular CTLA-4, which correlated with the GFP expression level (data not shown). Foxp3 was expressed at a higher level in Foxp3-transduced cells than in freshly isolated or anti-CD3–activated CD4⁺,CD25⁺ T cells from naive mice, based on Foxp3:β-actin intensity ratios of 1.2, 0.7, and 1.0, respectively (Figure 1B). In vitro suppressive activity was enhanced in activated cells compared with fresh cells (Figure 1C). CD3-primed Foxp3-transduced T cells significantly suppressed the proliferation of responder T cells, with efficiency that was remarkably higher than that of freshly isolated CD4⁺,CD25⁺ T cells and was comparable with that of activated CD4⁺,CD25⁺ T cells (Figure 1D).

**Suppression of CIA by adoptive transfer of antigen-reactive Foxp3-transduced T cells.** CD4 T cells isolated from the lymph nodes of type II collagen–immunized mice were activated in vitro with type II collagen–pulsed DCs and transfected with retroviruses. Because proliferating cells are susceptible to integration of retroviral genomes, reactivity to type II collagen of the transduced cells should be enriched. The enriched
specificity was confirmed by antigen-specific proliferation of CD4 T cells from lymph nodes of type II collagen–immunized mice reacting with type II collagen–pulsed DCs compared with antigen-unpulsed DCs (Figure 2A).

To evaluate whether Foxp3-transduced T cells effectively suppressed CIA, either 1 × 10^5 or 5 × 10^4 cells were adoptively transferred to the mice 1 day prior to the primary type II collagen immunization for initiation of CIA. Although arthritis should eventually develop after a single immunization, the mice received booster immunizations after 21 days, to synchronize the onset of joint swelling. Both doses of adoptively transferred cells were equally effective for lowering the arthritis scores of treated animals (Figure 2B). This indicated that transfer using this timing was effective in achieving suppression of arthritis, but that the amounts of transferred cells reached optimal levels in this setting.

CD4 T cells from lymph nodes of OVA-immunized mice were activated with OVA-pulsed DCs (Figure 2C) and transfected with Foxp3-expressing retroviruses. The generated OVA-reactive Foxp3-transduced T cells exhibited suppressive activity in vitro against CD3-activated T cell proliferation, which was approximately comparable with that of CD3-primed Foxp3-transduced T cells. Transfer of OVA-reactive Foxp3-transduced T cells prepared in

Figure 1. Foxp3 expression and suppressive activity of regulatory T cells. A, Splenic CD4 T cells from naive mice were stimulated with dendritic cells (DCs) and anti-CD3 monoclonal antibodies (mAb) and infected with Foxp3-pMCs-IG. The green fluorescent protein (GFP)–expressing fraction at 48 hours after infection is shown. B, CD4 T cells were activated and infected with Foxp3-pMCs-IG or pMCs-IG. After 48 hours, GFP-expressing fractions were isolated in order to prepare whole cell lysates for immunodetection of Foxp3 and β-actin. Whole cell lysates were also prepared from freshly isolated and activated CD4+,CD25+ T cells as well as CD4+,CD25− T cells. Activated CD4+,CD25+ T cells were prepared by culturing with DCs treated with anti-mouse CD3e mAb in the presence of 100 units/ml interleukin-1 for 72 hours. C, Freshly isolated and activated CD4+,CD25+ T cells (suppressors; S) were cultured with CD4+,CD25− responder T cells (responders; R) in the presence of irradiated splenocytes and anti-CD3 mAb. Proliferation of responder T cells at the indicated S:R ratios was assessed by measuring [3H]-thymidine uptake. Values are the mean and SD and are representative of 5 individual experiments. D, Foxp3-transduced CD4 T cells were cultured with responder T cells for assessment of suppressive activity, in the same manner. Values are the mean and SD and are representative of 3 individual experiments.

Figure 2. Suppression of collagen-induced arthritis (CIA) by type II collagen (CII)–primed Foxp3-transduced T cells transferred before the primary CII immunization. A and C, CD4 T cells derived from CII-immunized mice (A) or ovalbumin (OVA)–immunized mice (C) were mixed with either CII-pulsed dendritic cells (DCs), OVA-pulsed DCs, or antigen (Ag)–unpulsed DCs at the indicated ratios. Proliferation was measured by [3H]-thymidine uptake after 3 days. Values are the mean ± SD results from triplicate cultures. B and D, Naïve DBA/1 mice received 1 × 10^5 (n = 7) or 5 × 10^4 (n = 7) Foxp3-transduced T cells primed by CII-pulsed DCs (CII-Foxp3+) (B) or 1 × 10^5 Foxp3-transduced T cells primed by OVA-pulsed DCs (OVA-Foxp3+) (D) intravenously, 1 day prior to the primary immunization with CII. A group of mice received no T cell transfer (control; n = 15 in B, n = 7 in D). A booster immunization was administered 21 days after the primary immunization. B, Differences between the control group and the groups receiving CII-primed Foxp3+ (both doses) were significant (P < 0.01 by repeated-measures analysis of variance).
the same manner failed to suppress CIA (Figure 2D), suggesting that antigen specificity of Foxp3-transduced T cells is crucial.

Histopathologic assessment comparing the inflammation-related findings in control CIA mice, such as infiltration of mononuclear cells in the hypertrophic synovial tissue and destruction of joint cartilage (Figure 3A, middle panel), with those in normal mice (Figure 3A, left panel) demonstrated that these findings were suppressed in CIA mice that received transfer of 5 × 10⁴ Foxp3-transduced T cells 1 day prior to the primary immunization (right panel) with the knee joints of normal naive mice (left panel). B, Mice received 1 × 10⁵ Foxp3-transduced T cells primed with CII-pulsed dendritic cells (CII-Foxp3; n = 4) intravenously. 1 day prior to the primary immunization. A group of control mice (n = 4) received no T cell transfer. Serum was collected from each mouse on the day of the primary immunization and 10, 20, 30, and 40 days after the immunization. Unit values for each IgG subclass were measured using enzyme-linked immunosorbent assay. Plots represent the mean values for the 4 samples. Statistically significant differences, by repeated-measures analysis of variance, were observed between control and CII-Foxp3+ groups in the IgG1 and IgG2a subclasses (P < 0.01) and the IgG2b subclass (P < 0.05).

Figure 3. Suppression of joint inflammation and bovine type II collagen (CII)–specific antibody production by Foxp3-transduced T cell transfer. A, Histopathologic examination was performed 14 days after booster immunization to compare the knee joints of control mice with collagen-induced arthritis (CIA) (middle panel) and those of CIA mice that received transfer of 5 × 10⁴ Foxp3-transduced T cells 1 day prior to the primary immunization (right panel) with the knee joints of normal naive mice (left panel). B, Mice received 1 × 10⁵ Foxp3-transduced T cells primed with CII-pulsed dendritic cells (CII-Foxp3; n = 4) intravenously. 1 day prior to the primary immunization. A group of control mice (n = 4) received no T cell transfer. Serum was collected from each mouse on the day of the primary immunization and 10, 20, 30, and 40 days after the immunization. Unit values for each IgG subclass were measured using enzyme-linked immunosorbent assay. Plots represent the mean values for the 4 samples. Statistically significant differences, by repeated-measures analysis of variance, were observed between control and CII-Foxp3+ groups in the IgG1 and IgG2a subclasses (P < 0.01) and the IgG2b subclass (P < 0.05).
Correlation of suppressive activity and expression levels of transduced Foxp3. As described previously, activated naturally arising Treg become more suppressive and express Foxp3 at higher levels than those expressed by freshly isolated cells. This fact led us to examine whether high-level Foxp3 expressers among Foxp3-transduced T cells exert stronger antiarthritic effects. Using a previously performed technique (4), we isolated Foxp3high and Foxp3low fractions based on their GFP expression level (Figure 5A, left). Immunoblot analyses of their cell lysates showed that Foxp3 expression in the fractionated cells correlated well with GFP expression. The low GFP fraction expressed Foxp3 at a level comparable with that expressed by naturally arising CD4\(^+\),CD25\(^+\) T cells (Figure 5A, right). Consistent with previous results (4), the Foxp3\(^{\text{high}}\) fraction suppressed in vitro proliferation of CD4\(^+\),CD25\(^+\) T cells more efficiently than did the Foxp3\(^{\text{low}}\) fraction (Figure 5B).

The differential suppressive activity of the Foxp3\(^{\text{high}}\) and Foxp3\(^{\text{low}}\) cells may derive from differences in their activation levels. Theoretically, highly activated cells may enter the cell cycle more efficiently, thus making themselves more susceptible to retroviral integration. Actually, GFP expression levels of the T cells infected with empty vector (pMCs-IG) correlated positively with their surface CD69 levels (data not shown). To examine directly whether the Foxp3 expression level controls suppressive activity, we additionally used Foxp3-MIGR1, a retrovirus vector that was less efficient at protein expression, joined to the Foxp3 gene. The CD4 T cells activated with anti-CD3 mAb and DCs were divided for transfection with either Foxp3-pMCs-IG or Foxp3-MIGR1 retrovirus vector. Foxp3-transduced T cells generated by Foxp3-pMCs-IG expressed higher levels of Foxp3 (Figure 5C) and had higher suppressive activity (Figure 5B). Thus, the Foxp3 expression level regulated suppressive activity of Foxp3-transduced Treg.

Suppression of CIA by Foxp3\(^{\text{high}}\) T cells versus Foxp3\(^{\text{low}}\) T cells. For treatment of CIA, type II collagen–specific Foxp3\(^{\text{high}}\) and Foxp3\(^{\text{low}}\) T cell populations were generated from type II collagen–primed T cells. First, 1 \times 10^5 cells from each population were transferred to mice 1 day prior to the primary type II collagen immunization. The 2 treatments suppressed arthritis equally, as shown by the arthritis score and measurement of joint swelling (Figure 6A). Transfer of the same number of OVA-reactive Foxp3\(^{\text{high}}\) as well as Foxp3\(^{\text{low}}\) T cells did not affect CIA development (Figure 6B). We next transferred Foxp3\(^{\text{high}}\) or Foxp3\(^{\text{low}}\) T cells to the hosts 20 days after the immunization (Figure 6C). When these mice were subjected to booster immunization for development of overt arthritis, Foxp3\(^{\text{low}}\) T cells exerted only a marginal suppressive effect, demonstrating again that...
immunized hosts became resistant to suppression by Treg. In contrast, Foxp3\textsuperscript{high} T cells overcame the resistance and suppressed CIA efficiently. A titration study demonstrated that as few as 1 $\times$ 10\textsuperscript{4} Foxp3\textsuperscript{high} T cells significantly suppressed CIA (Figure 6D). Because this number of unfracionated Foxp3-transduced cells had no effect, Foxp3\textsuperscript{high} T cells had higher suppressive activity in vivo as well as in vitro.

Transfer of the same number of Foxp3\textsuperscript{high} or Foxp3\textsuperscript{low} fractions into knee joints at the same time did not suppress arthritis as efficiently as did systemic transfer. The effect was observed even in forelimbs (data not shown). This implies that the site of function for the Treg is not local synovial tissue. Finally, type II collagen–specific Foxp3\textsuperscript{high} T cells (1 $\times$ 10\textsuperscript{5} cells) were transferred intravenously 4 days after the booster immunization. The treatment attenuated progression of arthritis slightly but did not reverse the joint swelling (Figure 6E). Thus, arthritic mice gained further resistance to inhibition by Foxp3-expressing T cells.

**DISCUSSION**

The recent introduction of anticytokine therapies, including treatment with anti-TNF\textalpha agents, has improved the clinical outcome of RA that is refractory to conventional treatments (33). However, no current treatment targets pathogenic immune reactions to specific antigens. This often leads to the generalized immune suppression that is responsible for undesirable infections. We addressed this issue by generating antigen-specific Treg via transfer of the Foxp3 gene. Adoptive transfer of these cells to nonlymphopenic animals effectively suppressed CIA. In addition, we found that in vitro and in vivo suppressive activities of the genetically manipulated cells correlate well with the expression level of the Foxp3 gene. This allowed us to demonstrate that CIA becomes increasingly resistant to suppression by Treg during the disease course.

Activation of naturally arising CD4$^{+}$,CD25$^{+}$ T cells augmented their in vitro suppressive function. This is consistent with previous observations that stimulation with anti-CD3 and IL-2 (21,34) or antigen-pulsed DCs (22,35) remarkably enhanced the suppressive activity of CD4$^{+}$,CD25$^{+}$ Treg.

In vivo adoptive transfer experiments revealed that CD4$^{+}$,CD25$^{+}$ T cells specific to disease-relevant antigens were highly effective for disease suppression (21,22), which presumably was closely related to in vivo activation of the Treg. We observed that type II collagen–reactive Foxp3-transduced T cells suppressed CIA at much lower cell numbers compared with the number of non–antigen-specific CD4$^{+}$,CD25$^{+}$ Treg used in a previous study (25). The suppressed production of anti-bovine type II collagen antibodies (Figure 3B), which was not detected in the latter study, supports
the fact that the antigen-specific Treg used in this study suppressed CIA more efficiently, because type II collagen–specific antibodies are known as the direct effectors giving rise to arthritis in the CIA model (36,37). Treg can affect antibody production (38). Generating Foxp3-transduced T cells also circumvented the difficulty in preparing sufficient numbers of CD4<sup>+</sup>,CD25<sup>+</sup> T cells, because this population occurs only as a small portion of peripheral T cells. Non–antigen-specific CD4<sup>+</sup>,CD25<sup>+</sup> T cells (1 × 10<sup>6</sup>) derived from 20 donors were required to treat 1 nonlymphopenic mouse with CIA (25). The Foxp3-transduced T cells needed to treat 1 CIA mouse (1 × 10<sup>5</sup> cells) in this study were generated from 1 donor. At the moment, in vitro generation of Treg appears to be more practical for cell transfer therapy.

BDC2.5 T cell receptor–transgenic CD4<sup>+</sup>,CD25<sup>+</sup> T cells, which presumably recognize an antigen derived from β cells in pancreatic islets, were shown to be capable of suppressing diabetes induced by diabetic NOD mouse splenocytes (21,22). Thus, Treg specific to a single antigen suppress immune responses induced by

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**Figure 6.** Collagen-induced arthritis (CIA) suppression by type II collagen (CII)–primed Foxp3<sup>high</sup>/Foxp3<sup>low</sup> fractions of Foxp3-transduced T cells. A and B, Naive mice received transfer of 1 × 10<sup>5</sup> Foxp3<sup>high</sup>-transduced (n = 5) or Foxp3<sup>low</sup>-transduced (n = 5) T cells primed with CII-pulsed dendritic cells (DCs) (A) or 1 × 10<sup>6</sup> Foxp3<sup>high</sup>-transduced (n = 5) or Foxp3<sup>low</sup>-transduced (n = 5) T cells primed with ovalbumin (OVA)–pulsed DCs (B) intravenously, 1 day prior to the primary immunization. Boosters were administered to the experimental mice as well as control mice, which did not receive Foxp3-transduced T cells (n = 10 in A and B). Joint swelling in the hind limbs was measured at the indicated days after booster immunization, and the mean values for the right and left sides of all mice in the experimental groups were plotted. Differences in CIA scores, ankle width, and paw thickness between the control group and the Foxp3<sup>high</sup> and Foxp3<sup>low</sup> groups were significant (P < 0.01 by repeated-measures analysis of variance [ANOVA]). C–E, Mice received transfer of 1 × 10<sup>5</sup> Foxp3<sup>high</sup> (n = 5) or Foxp3<sup>low</sup> (n = 5) T cells (C), 5 × 10<sup>4</sup> (n = 3) or 1 × 10<sup>4</sup> (n = 5) Foxp3<sup>high</sup> T cells (D), or 1 × 10<sup>5</sup> Foxp3<sup>high</sup> T cells (n = 5) (E) primed with CII-pulsed DCs, intravenously 20 days after the primary immunization (C and D) or 4 days after the booster immunization (E). A group of control mice did not receive T cells (n = 10 in C, n = 8 in D, and n = 10 in E). C, P < 0.01, control versus Foxp3<sup>high</sup>, and P < 0.05, control versus Foxp3<sup>low</sup>, by repeated-measures ANOVA. D, P < 0.01, control versus Foxp3<sup>high</sup> (5 × 10<sup>4</sup>) and Foxp3<sup>high</sup> (1 × 10<sup>5</sup>), by repeated-measures ANOVA. E, P < 0.01, control versus Foxp3<sup>high</sup>, by repeated-measures ANOVA. Data below the figures represent the mean ± SD day of onset (a), the frequency (%) of arthritis induced (b), the mean ± SD maximum score (c), and the mean ± SD day on which the maximum score was reached for each mouse (d) among the mice in each group and are representative of 2 individual experiments. ND = not determined.
polyclonal effectors. We assume that Foxp3-transduced T cells reactive to a single antigen specifically expressed in joint tissue could suppress T cell reactions against multiple autoantigens in the joints; the original target antigens of the arthritis may not need to be determined.

The Foxp3 expression level in Foxp3-transduced T cells directly correlated with their antiarthritic activity. A previous study showed that CD4 T cells from Foxp3-transgenic mice expressed 10–15-fold higher Foxp3 messenger RNA compared with those from wild-type mice. However, their in vitro suppressive activity was slightly reduced relative to that of wild-type CD4+CD25+ T cells (6). Those investigators speculated that excessive Foxp3 expression interfered with the functional activation of Treg. In our experiments, the Foxp3 gene was retrovirally introduced after activating the T cells. This technique, combined with use of 2 different retrovirus vectors, allowed us to observe that the Foxp3 expression level dictated the suppressive activity of Foxp3-transduced Treg, both in vitro and in vivo.

It has been shown that stimulated CD4+,CD25− responder T cells were more resistant to in vitro suppression by Treg than were fresh CD4+,CD25− T cells (39,40). We observed that a larger number of Foxp3-transduced T cells was required to suppress CIA when transfer occurred after the primary immunization. Moreover, Foxp3high cells suppressed arthritis when the same dose of Foxp3low cells failed to have an effect. These findings demonstrate that in vivo resistance to Treg was induced after effector activation. In the CIA model, various other effectors besides T cells are involved in pathogenesis, including synovial fibroblasts. Insensitivity of these effectors to suppression by Treg may also contribute to the observed resistance. It may be possible that transfer of even larger numbers of Foxp3high cells can ameliorate full-blown arthritis. However, based on our results, current techniques would require multiple donors to treat a single host.

Our results suggest a possible problem in terms of the clinical application of Treg transfer therapy for patients with RA. Because advanced-stage disease would be refractory to such therapy, Treg transfer should be most effective for the induction of remission in the early stage of RA and for remission maintenance later. In contrast, Foxp3high T cells overcame, to some extent, the insensitivity of the immunized mice. If Treg are generated using Foxp3 gene transfer, transfer of T cells expressing high levels of Foxp3 would be desirable in Treg therapy for autoimmune diseases.

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AUTHOR CONTRIBUTIONS

Dr. Ohata had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Ohata, Ziegler, Kohsaka, Hori.
Acquisition of data. Ohata, Miura.
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REFERENCES


Inhibition of Toll-like Receptor 4 Breaks the Inflammatory Loop in Autoimmune Destructive Arthritis

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Objective. Degeneration of extracellular matrix of cartilage leads to the production of molecules capable of activating the immune system via Toll-like receptor 4 (TLR-4). The objective of this study was to investigate the involvement of TLR-4 activation in the development and progression of autoimmune destructive arthritis.

Methods. A naturally occurring TLR-4 antagonist, highly purified lipopolysaccharide (LPS) from Bartonella quintana, was first characterized using mouse macrophages and human dendritic cells (DCs). Mice with collagen-induced arthritis (CIA) and mice with spontaneous arthritis caused by interleukin-1 receptor antagonist (IL-1Ra) gene deficiency were treated with TLR-4 antagonist. The clinical score for joint inflammation, histologic characteristics of arthritis, and local expression of IL-1 in joints were evaluated after treatment.

Results. The TLR-4 antagonist inhibited DC maturation induced by Escherichia coli LPS and cytokine production induced by both exogenous and endogenous TLR-4 ligands, while having no effect on these parameters by itself. Treatment of CIA using TLR-4 antagonist substantially suppressed both clinical and histologic characteristics of arthritis without influencing the adaptive anti–type II collagen immunity crucial for this model. Treatment with TLR-4 antagonist strongly reduced IL-1β expression in articular chondrocytes and synovial tissue. Furthermore, such treatment inhibited IL-1–mediated autoimmune arthritis in IL-1Ra−/− mice and protected the mice against cartilage and bone pathology.

Conclusion. In the present study, we demonstrate for the first time that inhibition of TLR-4 suppresses the severity of experimental arthritis and results in lower IL-1 expression in arthritic joints. Our data suggest that TLR-4 might be a novel target in the treatment of rheumatoid arthritis.

Rheumatoid arthritis (RA) is an autoimmune disease of unknown etiology associated with chronic inflammation of peripheral joints. Today it is generally accepted that proinflammatory cytokines play an important role in the pathogenesis of RA (1); however, the mechanisms of initiation and perpetuation of the inflammatory cascade in RA are still unknown.

Toll-like receptors (TLRs) are a family of pattern recognition receptors that are involved in the recognition of conserved pathogen-associated molecular patterns (2). Ligand binding to TLRs initiates a signaling cascade that leads to the activation of the NF-κB and interferon regulatory factor 3 transcription factors and MAPKs, which in turn promote the production of inflammatory cytokines, chemokines, and tissue-destructive enzymes and the expression of costimulatory molecules on antigen-presenting cells (APCs). These costimulatory molecules provide a second signal to T cells to initiate the adaptive immune response (3,4).

Considering the role of TLRs as a critical link between the innate and the adaptive immune responses,
the idea has emerged that continuous activation or dysregulation of TLR signaling might contribute to the pathogenesis of autoimmune diseases such as RA. Indeed, TLR ligands of exogenous origin such as bacterial peptidoglycans and CpG-containing DNA, activating TLR-2 and TLR-9, respectively, have been found in the synovial fluid of patients with RA (5,6). In experimental models of arthritis, TLR ligands have repeatedly been used to induce the disease in susceptible animals; for instance, intraarticular injection of streptococcal cell wall fragments, double-stranded RNA, or CpG-containing DNA, which mainly signal through TLR-2, TLR-3, and TLR-9, respectively, can induce arthritis (7–9). Furthermore, lipopolysaccharide (LPS) from the outer membrane of gram-negative bacteria, signaling through TLR-4, has been used extensively to aggravate or reactivate arthritis in distinct animal models (10–12). In addition, LPS has been demonstrated to circumvent the interleukin-1 (IL-1) dependence of the K/BxN serum transfer model of arthritis (13).

In spite of the arthritogenic properties of a variety of TLR ligands, it is still not clear whether TLR activation is present in RA. Alternatively, TLRs can also be activated by several endogenous ligands that are released from stressed or damaged host tissue. In this respect, TLR-4 can recognize extracellular matrix components such as heparan sulfate (HS) and extra domain A (ED-A) of fibronectin (14,15), and both TLR-2 and TLR-4 can recognize the matrix component biglycan (16). TLR activation by these self antigens can potentially promote the development of autoimmune diseases. A critical role of TLRs in autoimmunity is supported by the finding that autoreactive B cells can be activated by RNA- as well as DNA-associated autoantigens via sequential engagement of the B cell receptor and TLR-7 or TLR-9, respectively (17,18). This finding may have implications for our understanding of the pathogenesis of systemic lupus erythematosus.

Some of the endogenous TLR ligands can be found in arthritic joints. It was recently shown that RNA released from necrotic synovial fluid cells of RA patients can activate TLR-3 on RA synovial fibroblasts (19). The presence of endogenous TLR-4 ligands such as fibronectin fragments and heat-shock proteins has also been demonstrated in rheumatoid synovium (20–22), and it has been reported that rheumatoid synovial fibroblast-like cells synthesize ED-A–containing fibronectin (23). In addition, serum and synovial fluid from RA patients can activate a TLR-4–expressing Chinese hamster ovary cell line, suggesting the presence of TLR-4–activating substances in RA serum and joints (24).

In the present study, we investigated the involvement of TLR-4 activation in the development of chronic destructive arthritis, using the collagen-induced arthritis (CIA) model and the spontaneous arthritis model in IL-1 receptor antagonist–deficient (IL-1Ra−/−) mice. CIA is an autoimmune model of arthritis based on autoantibodies and T cell immunity against type II collagen (CII). Here, we demonstrate for the first time that inhibition of TLR-4 signaling in mice with non-LPS-accelerated CIA substantially suppresses both clinical and histologic characteristics of early-phase as well as established arthritis. The TLR-4 antagonist was the highly purified LPS from the gram-negative bacterium Bartonella quintana, which we previously reported to act as a specific TLR-4 antagonist (25). In the present study, we demonstrate that B quintana LPS can also inhibit cytokine production by endogenous TLR-4 ligands and dendritic cell (DC) maturation by Escherichia coli LPS. Suppression of arthritis by the TLR-4 antagonist was accompanied by a strong reduction of IL-1 expression in the joint. The protective effects of the TLR-4 antagonist in CIA were not mediated by a disruption of the adaptive immune response disturbing the development of autoimmunity in this model. In addition, treatment with the TLR-4 antagonist reduced the clinical and histopathologic features of arthritis in IL-1Ra−/− mice, in which an autoimmune T cell–mediated arthritis develops spontaneously due to unbalanced IL-1 signaling (26,27).

**MATERIALS AND METHODS**

**Animals.** Male DBA/1 mice were purchased from Janvier-Elevage (Le Genest St. Isle, France). IL-1Ra−/− mice on a BALB/c background were kindly provided by Dr. M. Nicklin (Sheffield, UK). The mice were housed in filter-top cages, and water and food were provided ad libitum. Age- and sex-matched animals were used in all experiments. Animal studies were approved by the Institutional Review Board and were performed according to the related codes of practice.

**Preparation and purification of TLR-4 antagonist.** The TLR-4 antagonist was LPS derived from the cell membrane of the gram-negative bacterium *B. quintana*. The *B. quintana* Oklahoma strain was kindly provided by Prof. D. Raoult (Marseilles, France) and cultured on sheep blood agar at 37°C with 5% CO2. The 5-day cultures of *B. quintana* were heat-killed at 56°C for 60 minutes, and LPS was extracted using a 2-step phenol–water extraction method to remove proteins and lipids as previously described (28).

**Isolation, culture, and stimulation of murine resident peritoneal macrophages.** Murine macrophages were isolated from naive DBA/1 mice by lavage of the peritoneal cavity using 10 ml cold medium (Dulbecco’s modified Eagle’s medium plus 10% fetal calf serum). Adherent cells were harvested and
cultured for 4 days before being used. For cytokine production, cells were incubated with purified *E. coli* LPS (10 ng/ml), purified *B. quintana* LPS (1 μg/ml), Pam3CysSerLys4 (Pam3Cys; 10 μg/ml), poly(I-C) (25 μg/ml), IL-1 (10 ng/ml), tumor necrosis factor α (TNFα; 10 ng/ml), ED-A of fibronectin (1 μM), and HS (10 μg/ml) for 24 hours. ED-A and HS were incubated with 10 μg/ml of polymyxin B for 30 minutes prior to use in order to disable possible LPS contamination. When used in combination with TLR-4 antagonist, cells were exposed to 10× higher concentrations of the antagonist for 30 minutes prior to stimulation. Both *E. coli* LPS and *B. quintana* LPS were double-purified by the phenol–water extraction method (28) before use to eliminate potential protein contaminations.

**Measurement of cytokines.** Concentrations of cytokines (except transforming growth factor β [TGFβ]) in culture supernatants and mice sera were determined using the Bioplex (Luminex) cytokine assays from Bio-Rad (Hercules, CA). TGFβ concentrations were measured using enzyme-linked immunosorbent assay (ELISA; R&D Systems, Abingdon, UK), following the manufacturer’s instructions.

**Generation and maturation of monocyte-derived DCs.** Human immature DCs were generated from adherent monocytes as described by Roelofs et al (24). For DC maturation, 1 × 10^6 immature DCs were incubated with 2 μg/ml purified *E. coli* LPS or 1 μg/ml TLR-7 ligand single-stranded polyuridine (ssPolyU) for 48 hours after a preincubation with 10× higher concentrations of TLR-4 antagonist. DC maturation was determined by measuring the up-regulation of class II major histocompatibility complex (MHC) molecules and “de novo” expression of CD83 using fluorescence-activated cell sorting analysis, as described previously (24).

**Induction of CIA.** Arthritis was induced in 10–12-week-old DBA/1 mice. Bovine CII was dissolved in 0.05M acetic acid to a concentration of 2 mg/ml and emulsified in an equal volume of Freund’s complete adjuvant (2 mg/ml of *Mycobacterium tuberculosis* strain H37Ra; Difco, Detroit, MI). Mice were immunized by intradermal injection of 100 μl of the emulsion at the base of the tail and were given an intraperitoneal (IP) booster injection of 100 μl of bovine CII dissolved in phosphate buffered saline without any adjuvant on day 21. Clinical onset and progression of arthritis were macroscopically evaluated by 2 observers in a blinded manner and scored on a scale of 0–2 for each paw, as described by Joosten et al (29).

**Treatment of arthritis with TLR-4 antagonist.** To investigate the effects of TLR-4 inhibition on the development of arthritis, mice with CIA were treated using a total of 3 IP injections of TLR-4 antagonist (400 μg/kg body weight) once every 2 days starting on day 22 after immunization and before clinical onset of disease. For therapeutic treatment, mice received 4 daily injections of 2 mg/kg body weight TLR-4 antagonist after a macroscopic inflammation score of 0.5–1 (maximum possible score = 8) was reached (on day 24–25 after immunization). Development of arthritis was evaluated as described above, BALB/c IL-1Ra<sup>−/−</sup> mice received 400 μg/kg body weight TLR-4 antagonist (or saline as control) IP 3 times a week for 2 weeks, starting after the spontaneous onset of arthritis (mean starting score of 1 on a scale with a maximum possible score of 4).

**Histology.** For histologic assessment of arthritis, total joints were isolated at the end point of the experiments and fixed for 4 days in 4% formaldehyde, then decalcified in 5% formic acid and embedded in paraffin. Tissue sections (7 μm) were stained with hematoxylin and eosin to study inflammatory cell influx and chondrocyte death, or with Safranin O to determine proteoglycan (PG) depletion and cartilage and bone destruction. Each parameter was scored on a 0–3-point scale by 2 observers in a blinded manner (29).

**Immunohistochemistry.** Local expression of IL-1β was evaluated in paraffin sections of the knee joints. Sections were deparaffinized in xylol and rehydrated in serial dilutions of
ethanol. Endogenous peroxidase was blocked using 1% \( \text{H}_2\text{O}_2 \) for 15 minutes. Tissue sections were incubated with 7.5 \( \mu \text{g/ml} \) rabbit anti-mouse IL-1 \( \beta \) antibodies or rabbit normal IgG (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour, followed by incubation with biotinylated swine anti-rabbit antibodies and peroxidase-labeled streptavidin. Color was developed with diaminobenzidine, and tissues were counterstained with hematoxylin. IL-1 \( \beta \) expression was scored on articular chondrocytes (scale 0–2) and synovial tissue around the patella, tibia, and femur (each scored between 0 and 2, then averaged to obtain overall expression in synovium).

**Measurement of anticollagen antibodies.** Concentrations of anti-mouse CII IgG1 and IgG2a antibodies were determined using ELISA. Briefly, 96-well plates were coated with 0.1 \( \mu \text{g} \) of mouse CII (Chondrex, Redmond, WA). Non-specific binding sites were blocked by a 5% solution of milk powder. Serial dilutions of mice sera were added, followed by incubation with isotype-specific goat anti-mouse antibodies (peroxidase labeled) and 5-aminosalicylic acid as substrate. Absorbance was measured at 450 nm.

**Statistical analysis.** Group measures are expressed as the mean \( \pm \) SEM. Statistical significance was assessed with a Student’s unpaired 2-tailed \( t \)-test performed using GraphPad Prism 4.0 software (GraphPad Software, San Diego, CA). \( P \) values less than or equal to 0.05 were considered significant.

### RESULTS

**Characterization of the TLR-4 antagonist.** The TLR-4 antagonist used in this study was the double-extracted LPS derived from the cell membrane of the gram-negative bacterium *B quintana*. This highly purified LPS has been described to act as a potent TLR-4 antagonist, since it strongly blocks the induction of IL-1, TNF, and other proinflammatory cytokines by *E coli* LPS (25). To further characterize the TLR-4 antagonist, we determined the effects of *B quintana* LPS on the production of a wide range of pro- and antiinflammatory cytokines by mouse peritoneal macrophages. The TLR-4 antagonist did not by itself induce the production of proinflammatory cytokines including IL-1, TNF\( \alpha \), IL-6, and IL-12, nor did it induce the production of antiinflammatory cytokines such as IL-4, IL-10, and TGF\( \beta \). The only inflammatory mediator induced by *B quintana* LPS was the chemokine cytokine-induced neutrophil chemoattractant (KC) (343 pg/ml after stimulation with 1 \( \mu \text{g/ml} \) *B quintana* LPS). In comparison, stimulation of

![Figure 2](image_url)

**Figure 2.** Suppression of clinical and histologic signs of collagen-induced arthritis by prophylactic treatment with Toll-like receptor 4 (TLR-4) antagonist. Mice received a total of 3 intraperitoneal injections of TLR-4 antagonist (400 \( \mu \text{g/kg} \) body weight) or 0.2 ml saline once every 2 days, starting before the onset of arthritis. **A,** Mean and SEM macroscopic arthritis score (scale of 0–2 for each paw). \( * \) = \( P < 0.05 \), saline versus TLR-4 antagonist. **B,** Histologic analysis of the knee joints on day 6 of treatment, expressed as the mean \( \pm \) SEM score (scale of 0–3) from 2 independent experiments. All parameters were scored by 2 observers in a blinded manner (\( \approx 10 \) mice per group per experiment). \( * \) = \( P < 0.05 \) versus saline-treated mice. **C,** Representative histologic images. Tissue sections were stained with hematoxylin and eosin (top panels) to study inflammatory cell influx and chondrocyte death (arrow) or with Safranin O (bottom panels) to determine proteoglycan (PG) depletion and cartilage and bone destruction (arrows). PG depletion is apparent from loss of red staining. \( P \) = patella; \( C \) = cartilage; \( JS \) = joint space; \( F \) = femur; \( B \) = bone. (Original magnification \( \times 100 \) in top panels; \( \times 200 \) in bottom panels.)
mouse peritoneal macrophages with only 10 ng/ml of purified *E. coli* LPS resulted in the production of as much as 2,000 pg/ml KC (Figure 1A). Furthermore, the TLR-4 antagonist did not suppress cytokine production upon stimulation with IL-1, TNFα, and the synthetic TLR-2 and TLR-3 ligands Pam3Cys and poly(I-C), respectively (data not shown).

In addition, in contrast to *E. coli* LPS, *B. quintana* LPS did not induce the maturation of human monocyte-derived DCs in terms of induction of CD83 expression and class II MHC up-regulation. More important, *B. quintana* LPS had no inhibitory effect on DC maturation that was induced by mechanisms other than TLR-4 activation (e.g., via stimulation of TLR-7) (Figures 1B and C).

**Inhibition by TLR-4 antagonist of proinflammatory cytokine induction by endogenous TLR-4 ligands.** There is growing evidence that extracellular matrix components, which are generated by tissue damage during chronic inflammation, can activate TLR-4. Therefore, we examined the ability of the TLR-4 antagonist to block the inflammatory signal induced by some of these endogenous TLR-4 ligands (i.e., ED-A of fibronectin and HS). Mouse peritoneal macrophages were preincubated with the TLR-4 antagonist and then stimulated with LPS, ED-A, or HS. As we expected, stimulation of macrophages with ED-A or HS, which were premixed with excessive amounts of polymyxin B to inhibit the putative LPS contaminant, resulted in the production of IL-1β and TNFα. Preincubation of cells with the TLR-4 antagonist clearly inhibited TNF production upon stimulation with LPS and the endogenous TLR-4 ligands (Figure 1D), and similar results were obtained for IL-1β (data not shown).

**Inhibition of disease progression by prophylaxis against CIA using TLR-4 antagonist.** To investigate the role of TLR-4 in the development of arthritis, we inhibited TLR-4 in an experimental model of arthritis. DBA/1 mice with CIA were treated with the TLR-4 antagonist before clinical manifestation of the disease. The disease incidence was not significantly reduced; however, macroscopic evaluation of the paws showed a
significant reduction of disease severity in mice treated with the TLR-4 antagonist (Figure 2A).

Subsequently, we investigated the effect of prophylactic TLR-4 blocking on the inflammatory cell influx and various hallmarks of cartilage and bone damage. Analysis of the paraffin sections of the knee joints revealed that specific TLR-4 inhibition significantly suppressed PG depletion from the cartilage matrix, the earliest sign of cartilage damage in experimental arthritis. Furthermore, destruction of the cartilage matrix was markedly reduced in mice treated with the antagonist ($P = 0.014$ for both parameters).

The microscopic score of chondrocyte death was also decreased by the treatment, although nonsignificantly ($P = 0.19$). TLR-4 blocking had no effect on the inflammatory cell influx and the severity of bone erosion in this setting (Figure 2B). Representative images of histologic analysis demonstrating the effects of anti-TLR-4 treatment before the onset of CIA are shown in Figure 2C.

**Strong suppression of joint pathology in ongoing disease by therapeutic treatment of CIA using TLR-4 antagonist.** To investigate whether TLR-4 blocking could ameliorate the ongoing disease in mice, mice with CIA with a macroscopic inflammation score of 0.5–1 were treated with the TLR-4 antagonist. As shown in Figure 3A, therapeutic treatment of CIA resulted in an ~50% suppression of the clinical score for arthritis.

Histologic examination of the knee joints revealed that treatment with the TLR-4 antagonist strongly prevented PG depletion and destruction of the cartilage matrix. Chondrocyte death and infiltration of inflammatory cells into the joint space were also dramatically inhibited ($P < 0.05$ for all parameters). Furthermore, another hallmark of CIA, bone erosion, was suppressed by inhibition of TLR-4 signaling, although nonsignificantly ($P = 0.067$) (Figure 3B). Figure 3C shows representative images of the knee joints of mice with CIA treated with the TLR-4 antagonist compared with those treated with saline.
Serum cytokine and anti-CII antibody levels. Treatment of mice with the TLR-4 antagonist before the onset of CIA did not result in any difference in serum concentrations of cytokines and chemokines. In contrast, inhibition of TLR-4 after the onset of arthritis led to a reduction in serum levels of IL-6 and KC, which corresponded well to a reduction of inflammatory cell influx into the joint (data not shown). IL-1β and TNFα concentrations were not different in the 2 groups. Importantly, serum levels of anti-mouse CII antibodies at the end point of the treatment with TLR-4 antagonist did not differ from those in the control groups, in both the prophylactic and therapeutic settings (Figures 4A and B). This finding confirms that treatment with the TLR-4 antagonist did not interact with the development of autoimmune responses driving the initiation and expression of CIA.

Reduction of local production of IL-1β in the joint by treatment with TLR-4 antagonist. IL-1 is considered the main mediator of cartilage PG depletion and destruction and bone erosion during CIA (30). Immunohistochemical staining of IL-1β showed that IL-1 was highly expressed in the synovium of control mice, especially at the sites of bone erosion. Inhibition of TLR-4 before as well as after the onset of CIA resulted in lower expression of IL-1β protein in the joints. IL-1β expression was reduced in chondrocytes of articular cartilage and also in synovial tissue surrounding patellar, tibial, and femoral surfaces of the knee joint (Figures 4C–E).

Blockage of progression of ongoing arthritis in IL-1Ra−/− mice by TLR-4 inhibition. Spontaneous development of arthritis in IL-1Ra−/− mice reflects an IL-1–mediated autoimmune process that progresses with age (26). Arthritis in these mice is represented by an aggressive pannus-forming synovitis accompanied by cartilage and bone destruction. To confirm the relevance of TLR-4 activation in driving IL-1–mediated joint pathology during the chronic phase of arthritis, IL-1Ra−/− mice with ongoing disease were treated with a TLR-4 antagonist. Consistent with the findings in CIA, the severity of joint inflammation was clearly reduced in anti–TLR-4–treated mice compared with saline-treated control mice, in which arthritis became more aggravated in time (Figure 5A).

Histologic analysis of the ankle joints revealed that inhibition of TLR-4 significantly protected the cartilage against PG depletion and chondrocyte death (P = 0.033 and P = 0.041, respectively). Furthermore, treatment with the TLR-4 antagonist resulted in substantial suppression of cartilage destruction and bone erosion perceptible in several joints of the ankle (P = 0.028 and P = 0.032, respectively). Synovial inflammation (lining cell proliferation and invasion of inflammatory cells into the joint) was also reduced, although nonsignificantly (P = 0.09) (Figure 5B).

No induction of corticosteroids, antinflammatory cytokines, or local chemotactic events in vivo by the TLR-4 antagonist. To exclude the possibility that the inhibitory effect of the TLR-4 antagonist on progression of arthritis is mediated through the induction of antinflammatory cytokines or corticosteroids, naive male DBA/1 mice were injected IP with 2 mg/kg body weight of the antagonist, 400 μg/kg body weight E. coli LPS, or an equal volume of saline. Corticosterone and cytokine concentrations were measured in serum 90 minutes, 4 hours, and 24 hours after injection.

Table 1 shows that systemic injection of B. quintana LPS, in contrast to E. coli LPS, did not induce the
production of TNFα, IL-1β, IL-6, and IL-10, indicating that the binding of B quintana–derived LPS to the TLR-4 receptor complex does not lead to the common NF-κB activation. Furthermore, no substantial levels of KC were found in serum after injection of B quintana LPS. These cytokines were not detectable in serum 24 hours after injection, and IL-4 was not detected at all. Injection of E coli LPS also led to the production of high levels of corticosterone within 90 minutes, whereas animals treated with B quintana LPS had corticosterone levels comparable with those of saline-treated animals. Furthermore, examination of the peritoneal cell population 24 hours after IP injection of B quintana LPS excluded a potential chemotactic activity of TLR-4 antagonist at the site of injection as a mechanism for the suppression of inflammation and tissue damage in arthritic joints, since both the total count and type of cells were not affected by the TLR-4 antagonist (data not shown).

**DISCUSSION**

We report for the first time that inhibition of TLR-4 activation suppresses the clinical manifestations (i.e., swelling and redness) and histologic manifestations of arthritis in early-phase as well as established disease in mice. Inhibition of joint inflammation and cartilage damage was accompanied by reduced IL-1β expression. Our observations strongly suggest a proinflammatory role for TLR-4 in 2 chronic non–LPS-driven models of autoimmune arthritis.

TLRs were originally thought to have a function only in sensing pathogen-associated molecules. Activation of TLRs by these molecules has been proven to play a key role in the development and progression of various chronic infectious diseases depending on the expression of TLRs at sites of contact with bacteria. For instance, the Asp299Gly polymorphism of TLR-4, which is expressed on intestinal epithelial cells, was recently associated with Crohn’s disease (31), and a synthetic TLR-4 antagonist was shown to inhibit the development of 2 experimental models of inflammatory bowel disease (32). Despite the concerns regarding possible LPS contamination, it is currently believed that some damage-associated components of extracellular matrix can activate TLR-4, and it was therefore hypothesized that TLR-4 activation may also be involved in several non–infectious disease conditions based on the “danger model” of autoimmunity (33). Consistent with this hypothesis, TLR-4–deficient mice have been shown to exhibit less myocardial and hepatic ischemia-reperfusion injury compared with wild-type animals (34,35). Very recently, it was demonstrated that interaction of hyaluronan degradation products with TLR-2 and TLR-4 provides signals to initiate inflammation after noninfectious lung injury, whereas TLR-2 and TLR-4 serve to maintain epithelial cell integrity and tissue repair by sensing native high-molecular-mass hyaluronan (36).

In the present study, we explored the contribution of TLR-4 to the pathogenesis of RA, using the CIA and the spontaneous IL-1Ra−/− models of arthritis. TLR-4 is expressed by macrophages and fibroblasts in synovial lining and is up-regulated in moderately inflamed synovium of RA patients (37). CIA was chosen as a model of chronic joint inflammation that is accompanied by gradual cartilage and bone erosion and possibly leads to the production of endogenous TLR-4 agonists.

We blocked TLR-4 in this model using LPS from an intracellular gram-negative bacillus, B quintana. The antagonistic activity of B quintana LPS has been examined extensively in parallel studies, in which microarray analysis revealed that highly purified B quintana LPS does not affect gene expression in human peripheral blood mononuclear cells, and it completely blocks gene

### Table 1. Cytokine and corticosterone levels after systemic injection of Bartonella quintana LPS or Escherichia coli LPS*

<table>
<thead>
<tr>
<th></th>
<th>90 minutes after injection</th>
<th>4 hours after injection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline</td>
<td>E coli LPS</td>
</tr>
<tr>
<td>TNFα, pg/ml</td>
<td>10.4 ± 3.7</td>
<td>1,965.2 ± 26.1</td>
</tr>
<tr>
<td>IL-1β, pg/ml</td>
<td>ND</td>
<td>15.2 ± 5.3</td>
</tr>
<tr>
<td>IL-6, pg/ml</td>
<td>40.1 ± 22.3</td>
<td>8,941.7 ± 108.8</td>
</tr>
<tr>
<td>KC, pg/ml</td>
<td>18.9 ± 5.2</td>
<td>5,302.0 ± 235.6</td>
</tr>
<tr>
<td>IL-10, pg/ml</td>
<td>ND</td>
<td>232.2 ± 13.9</td>
</tr>
<tr>
<td>Corticosterone, nmoles/liter</td>
<td>295.3 ± 112.9</td>
<td>1,846.7 ± 523.5</td>
</tr>
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</table>

* Values are the mean ± SD. Naive DBA/1 mice were injected intraperitoneally with 400 μg/kg E coli lipopolysaccharide (LPS) or 2 mg/kg B quintana LPS, and corticosterone and cytokine concentrations were measured in serum 90 minutes and 4 hours after injection. TNFα = tumor necrosis factor α; IL-1β = interleukin-1β; ND = not detectable; KC = cytokine-induced neutrophil chemoattractant.
regulation by *E. coli* LPS (25). Although *B. quintana* LPS seems to induce KC production weakly in murine macrophages, it is still an effective TLR-4 antagonist in mice. The most likely explanation of the mechanism of TLR-4 inhibition by *B. quintana* LPS is that it competes with TLR-4 agonists for a common binding site on TLR-4 or the TLR-4–myeloid differentiation 2 receptor complex; however, only ligand-binding assays can prove this hypothesis. The antagonistic activity of this LPS is probably caused by the cylindrical conformation of its lipid A due to the presence of a long-chain fatty acid, a characteristic that has been described for *Bartonella henselae* LPS and is known to reduce endotoxicity of LPS (38). Long-chain fatty acids are also present in other types of LPS with TLR-4–antagonistic properties, such as LPS of *Helicobacter pylori* and *Rhodobacter capsulatus* (39,40). Further support for the TLR-4–blocking activities of *B. quintana* LPS was provided by inhibition of cytokine release upon stimulation with endogenous TLR-4 ligands in the presence of excessive amounts of polymyxin B and by inhibition of DC maturation upon stimulation with *E. coli* LPS.

Inhibition of the TLR-4 pathway using this TLR-4 antagonist substantially suppressed clinical and histologic manifestations of CIA. The dose of the antagonist in the prophylactic CIA study (400 μg/kg body weight) was based upon the reported dose of other TLR-4 antagonists with similar structure (lipid A analog E5564) (41). For therapeutic treatment, we enhanced the dosage and shortened the intervals, because we expected generation and release of more inflammation-and damage-associated TLR-4 agonists during existing disease. Inhibition of TLR-4 after onset of CIA proved to be more effective than such inhibition before onset. This might be explained by the presence of more endogenous TLR-4 agonists in ongoing disease.

We performed a broad range of control studies to exclude nonspecific mechanisms of inhibition of joint inflammation and damage such as induction of antiinflammatory cytokines and corticosteroids, as well as recruitment of polymorphonuclear cells to the peritoneal cavity after IP injection of the antagonist. None of these mechanisms was found to be responsible for the suppression of CIA in our experiments. Furthermore, the development of normal titers of autoantibodies against murine CII after treatment with TLR-4 antagonist showed that TLR-4 inhibition did not interfere with the humoral immune response against foreign CII and the cross-reactivity to self collagen. In addition, a recent study demonstrated that TLR-4–knockout mice exhibit normal delayed-type hypersensitivity and lymph node cell proliferation in response to a retinal antigen (42), indicating that the lack of TLR-4 activation does not disrupt the Th1-mediated immune response that is an essential participant in the induction of CIA.

To confirm the relevance of TLR-4 activation in arthritis, TLR-4 antagonist was administered to IL-1Ra<sup>−/−</sup> BALB/c mice, in which a chronic polyarthritis closely resembling RA develops spontaneously. Arthritis in these mice arises from disturbed immune homeostasis due to excessive IL-1 signaling, which results in the induction of costimulatory molecules such as OX40 and CD40 ligand on T cells, thereby enhancing APC–T cell interaction and mediating T cell autoimmunity (26,27). For therapeutic treatment of IL-1Ra<sup>−/−</sup> mice, we chose a lower dose but a longer treatment course than that for existing CIA because of the milder joint destruction and the more chronic progression of arthritis in this model. Here we show that anti–TLR-4 treatment substantially improves both the clinical inflammation score and the histologic characteristics of arthritis in this model. While the inhibition of inflammatory cell influx into the joint was less pronounced, disruption of TLR-4 signaling had strong protective effects on cartilage and bone. Our recent studies, in which we crossed IL-1Ra<sup>−/−</sup> mice into TLR-4<sup>−/−</sup> animals on a BALB/c background, show that IL-1Ra<sup>−/−</sup> TLR-4<sup>−/−</sup> mice develop a clearly less progressive arthritis compared with IL-1Ra<sup>−/−</sup> TLR-4<sup>+/+</sup> littermates at later stages of the disease (43). This underscores the involvement of TLR-4 activation in the chronic phase of the disease, when the existing inflammation allows the formation of endogenous TLR-4 agonists.

Synovial macrophages and fibroblasts may be the first responders to non–pathogen-associated endogenous TLR-4 agonists, thereby contributing to the development and progression of arthritis. TLR-4 activation of APCs stimulates these cells to produce neutrophil- and lymphocyte-attracting chemokines (44,45) and to activate T cells by providing costimulatory signals (46,47). The infiltrated cells also express TLR-4 and can be activated by products of extracellular matrix degradation, leading to persistent activation of the innate and adaptive immune systems. In an attempt to establish the presence of endogenous TLR-4 activation in RA, we cultured synovial biopsy specimens from patients with active RA with the TLR-4 antagonist *B. quintana* LPS, and we found that inhibition of TLR-4 clearly reduced levels of IL-1 and TNF produced by cultured synovium (43).

An important mediator of cartilage and bone degradation is IL-1, which is produced upon TLR-4
activation (30). In cooperation with other cytokines, IL-1 promotes the production of nitric oxide and tissue-destructive enzymes, the activation of osteoclasts, and other catabolic events in the arthritic joint (1). Therefore, the protective effects of anti–TLR-4 treatment are, at least in part, attributed to reduced IL-1 production, as demonstrated by the lower expression of IL-1 in articular chondrocytes and synovial tissue of the knee joints after treatment with TLR-4 antagonist. This suggests that TLR-4 might function upstream of proinflammatory mediators such as IL-1 in certain stages of arthritis. Whether continuation of anti–TLR-4 treatment would have the same effects in later phases is an issue for further investigation. Another important question is whether short-term inhibition of TLR-4 would affect the further long-term progress of arthritis. Since endogenous TLR-4 agonists are expected to be more abundant in longstanding disease, and inhibition of IL-1 has been proved to be effective in late stages of CIA (29), we expect that anti–TLR-4 treatment at later phases will still suppress the disease in part.

To our knowledge, these data are the first to demonstrate the protective effects of anti–TLR-4 treatment in 2 autoimmune models of arthritis that are not driven by exogenous TLR-4–activating microbial adjuvants. The immunologic mechanisms of these protective effects involve reduced cytokine production and the inhibition of their deleterious effects in the joint. Further studies are warranted to investigate the exact source and nature of TLR-4–activating molecules and their arthropathogenic capacity in RA joints, and especially the potential of TLR-4 blockade as a therapeutic strategy in chronic inflammatory diseases such as RA.

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AUTHOR CONTRIBUTIONS

Dr. Abdollahi-Roodsaz had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Abdollahi-Roodsaz, Joosten, van der Meer, Netea, van den Berg.

Acquisition of data. Abdollahi-Roodsaz, Joosten, Roelofs, Popa, Netea, van den Berg.

Analysis and interpretation of data. Abdollahi-Roodsaz, Joosten, Roelofs, Radstake, Popa, van der Meer, Netea, van den Berg.

Manuscript preparation. Abdollahi-Roodsaz, Joosten, Matera, van der Meer, Netea, van den Berg.

Statistical analysis. Abdollahi-Roodsaz.

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Circulating C3 Is Necessary and Sufficient for Induction of Autoantibody-Mediated Arthritis in a Mouse Model

Paul A. Monach,1 Admar Verschoor,2 Jonathan P. Jacobs,3 Michael C. Carroll,3 Amy J. Wagers,3 Christophe Benoist,1 and Diane Mathis1

Objective. For the inflammation characteristic of rheumatoid arthritis, the relative contribution of mediators produced locally in the synovium versus those circulating systemically is unknown. Complement factor C3 is made in rheumatoid synovium and has been proposed to be a crucial driver of inflammation. The aim of this study was to test, in a mouse model of rheumatoid arthritis, whether C3 synthesized within the synovium is important in promoting inflammation.

Methods. Radiation bone marrow chimeras between normal and C3−/− mice were constructed in order to generate animals that expressed or lacked expression of C3 only in hematopoietic cells. Parabiotic mice were made by surgically linking C3−/− mice to irradiated wild-type mice to obtain animals having C3 only in the circulation. Arthritis was induced by injection of serum from arthritic K/BxN mice.

Results. In bone marrow chimeras, synthesis of C3 by radioresistant cells was necessary and sufficient to confer susceptibility to serum-transferred arthritis. Parabionts having C3 only in the circulation remained sensitive to arthritis induction, and the cartilage of these arthritic mice contained deposits of C3.

Conclusion. In a mouse model in which the alternative pathway of complement activation is critical to the induction of arthritis by autoantibodies, circulating C3 was necessary and sufficient for arthritis induction.

The complement cascade is essential for the induction of inflammatory arthritis by autoantibodies in at least 2 mouse models (1–3). The role of complement in human rheumatoid arthritis (RA) has been more difficult to assess, but a contribution of this pathway is suggested by several findings. First, complement components are depleted (4,5) and complement degradation products are generated (6,7) in the synovial fluid in RA but not other types of inflammatory arthritis. Second, C3 is deposited on the surface of cartilage and synovium in RA (8,9), as it is in various rodent models (10–12).

The details of complement involvement are particularly clear in the K/BxN mouse serum-transfer model. K/BxN mice uniformly develop severe, symmetric, inflammatory arthritis due to activation of the KRN transgene-encoded T cell receptor by a peptide from the glycolytic enzyme glucose-6-phosphate isomerase (GPI) presented by the class II major histocompatibility complex molecule Ag7 (13), leading to massive production of anti-GPI antibodies. These antibodies can effectively induce arthritis upon transfer into other mice (14). Because a wide range of natural mutant and gene-disrupted mouse strains can be used as recipients, this serum-transfer model has allowed the delineation of many genes and cell types required downstream of autoantibody production (1,15–18). With regard to the complement cascade, factors B, D (Monach PA: unpublished observations), C3, C5, and the receptor for C5a (C5aR) are required, whereas Clq, C4, C6, and the complement receptors CR1, CR2, and CR3 are not (1,19). Thus, induction of arthritis requires the alterna-
tive pathway of complement activation, leading to production of the chemoattractant and activating mediator C5a. Recently, a similar requirement for alternative but not classical pathway elements was found for induction of arthritis by antibodies directed against type II collagen (20). Most studies of complement in RA have not differentiated between activation of the classical and alternative pathways, but one that did so indicated that local activation of the alternative pathway in synovial fluid is particularly characteristic of RA (21).

The details of C3 involvement in inflammatory arthritis are of particular interest, not only because this protein is involved in all of the major pathways of complement activation and subsequent activation of effector mechanisms, but also because both systemic and local synthesis have been well documented. A few years ago, one might have assumed that the obligatory source of C3 and other essential complement components would be the liver. The liver is thought to be the source of the vast majority of circulating C3, and although this protein has a relatively short half-life, its concentration in plasma is the highest of any complement protein, at 1.0–1.4 mg/ml. However, not only has the synthesis of complement proteins by leukocytes now been clearly demonstrated (22–24), but leukocyte-derived C3 was found to be sufficient for the generation of antibody responses to a model antigen (25) and to be both necessary and sufficient for optimal antibody responses to intradermal herpes simplex virus infection in mice (26,27). Production of C3 by the inflamed synovium from patients with RA has also been demonstrated (28), and both hematopoietic and nonhematopoietic cells were implicated as potential sources (29,30), leading to the proposal that local synthesis of C3 might be important in propagating inflammation (30).

Because it is not currently possible to test this hypothesis in human RA, we did so in the K/BxN mouse serum-transfer system by using bone marrow chimeras and parabiotic mice.

**MATERIALS AND METHODS**

**Mice.** C3<sup>−/−</sup> mice (31) were maintained locally; C57BL/6 (B6) mice and B6 mice congenic for the CD45.1 isoform were purchased from The Jackson Laboratory (Bar Harbor, ME). Animals were maintained under specific pathogen–free conditions, and all procedures were performed in accordance with Institutional Animal Care and Use Committee–approved protocols ARCM-03204 and ARCM-03912.

**Bone marrow chimeras.** Recipient mice were lethally irradiated (6.5 Gy administered twice, 6 hours apart) and reconstituted intravenously with unfractionated bone marrow cells (BMCs) freshly obtained from the femurs of donor mice. Staining for the CD45.1 and CD45.2 isoforms on peripheral blood leukocytes (PBLs) showed that >95% of PBLs were of donor origin. Circulating C3 was measured by enzyme-linked immunosorbent assay (ELISA) and correlated perfectly with the capacity of the recipient to synthesize C3 (26). Chimeric animals were tested for arthritis susceptibility 6–12 weeks after reconstitution.

**Parabiotic mice.** Wild-type (WT) mice were lethally irradiated (10 Gy) immediately prior to parabiosis surgery. As described previously (32,33), animals were anesthetized and the skin incised from elbow to knee on one side. The elbows and knees of a pair of mice were sutured together through the musculature, then the skin incision was stapled and sutured such that the animals were joined by the dorsal and ventral skin. Parabions were given parenteral analgesia for 2 days and trimethoprim/sulfamethoxazole in the drinking water for 2 weeks. In a pilot experiment using pairs of WT mice mismatched for CD45 isoforms, leukocytes from the nonirradiated partner were readily detectable in the irradiated partner by 7 days after surgery and comprised >90% of the circulating.
leukocytes. Parabionts were tested for susceptibility to arthritis 5 weeks after surgery.

**Arthritis induction.** K/BxN mice were bred as described previously (34). Serum was collected at 7 weeks of age and stored at −20°C. Serum was injected intraperitoneally by our standard protocol (14), as follows: 0.15 ml on day 0 and again on day 2. The 4 paws were assessed by the clinical score (0–3-point scale for each paw, where 0 = no swelling, 1 = either swelling confined to 1 or 2 digits or mild swelling of the larger structures, 2 = severe arthritis involving the wrist or ankle but extending along the dorsum of the paw to the bases of the digits, and 3 = intermediate severity [score between 1 and 3]). In addition, thickening of the right ankle (or of the unconstrained ankle in parabiotic mice) was measured using a precision caliper (Kafer dial thickness gauge with flat anvils; Long Island Indicator Service, Hauppauge, NY). Disease was routinely evaluated twice per week.

**Histologic assessment and immunofluorescence staining.** For frozen sections, the skin over an ankle was removed immediately after the animal was killed, and a piece of tissue ~10 mm long encompassing the ankle and midfoot joints was immersed in Tissue-Tek OCT medium (Sakura Finetek, Torrance, CA), flash-frozen in dry ice/ethanol, and stored at −80°C until sectioned. Ankles were sectioned, without prior decalcification, using a tape transfer method (Instrumedics, Hackensack, NJ), as described previously (1,16). Sections were stored at −20°C until used, at which time they were fixed for 5 minutes in acetone at 4°C, briefly allowed to dry, rehydrated with phosphate buffered saline (PBS) for 15 minutes, blocked with PBS containing 2% bovine serum albumin (BSA) and 0.1% Tween 20 for 45 minutes, stained with fluorescein-labeled antibody to C3 (F[ab’]2 goat anti-mouse C3; ICN Biochemicals, Irvine, CA), diluted 1:200 in PBS/0.1% Tween 20 for 60 minutes, then washed 3 times with PBS/Tween 20 before placing coverslips using Gel/Mount medium (Biomeda, Foster City, CA).

For paraffin sections, fresh tissue containing the ankle and midfoot joints was fixed in 4% paraformaldehyde at 4°C overnight, decalcified with 3 changes of 0.375 M EDTA (pH 7.5) at 4°C for 2 weeks, and then processed for paraffin sectioning and hematoxylin and eosin staining by standard techniques.

Microscopy was performed using a Zeiss Axioplan 2 instrument equipped with a Spot RT Slider camera (Diagnostic Instruments, Sterling Heights, MI) and IPLab imaging software (Scanalytics, Billerica, MA).

**Measurement of C3.** Circulating C3 was measured by ELISA, as previously described (26).

**RESULTS**

A requirement for C3 synthesis by radioresistant cells. The role of leukocyte-derived C3 was assessed in radiation bone marrow chimeras. This experimental
approach leads to virtually complete replacement of a mouse's hematopoietic cells with cells from a donor mouse, whereas nonhematopoietic cells remain of host origin. Thus, WT or C3-deficient recipients were lethally irradiated, then reconstituted with unfractionated bone marrow cells from either WT or C3⁻/⁻ donors (Figure 1A).

Arthritis development after K/BxN serum transfer, whether evaluated by a clinical index or by ankle measurement, correlated perfectly with the ability of the recipients' radioresistant cells to produce C3 (Figure 1B). Furthermore, when 2 mice from each of the critical groups (C3⁻/⁻ BMCs into WT mice, and WT BMCs into C3⁻/⁻ mice) continued to receive injections of K/BxN serum for a total of 4 weeks and arthritis was evaluated until day 42, the WT recipients continued to show severe inflammation, while disease never developed in the C3-deficient recipients (results not shown).

Histopathologic assessment of ankle joints confirmed the clinical impression of the presence or absence of inflammatory arthritis with the typical features of a dense mononuclear cell infiltrate of the subsynovial connective tissue, neutrophilic joint effusion, loss of cartilage, and marginal erosion of bone (Figure 2). According to immunofluorescence staining, arthritic ankles from WT recipients had prominent deposits of C3...
on the cartilage surface and in the synovium, whereas the nonarthritic ankles from C3−/− recipients did not (Figure 2). In short, production of C3 by leukocytes was not required for K/BxN serum–induced arthritis, even with prolonged administration of arthritogenic serum.

**No need for C3 synthesis by joint-resident cells.** The next question was whether C3 could be produced by radiosensitive cells locally within the joint, or whether it could be made by parenchymal cells at distant sites and transported to the joints via the circulation. Therefore, we generated mice whose joint-resident cells could not synthesize C3 but whose circulation carried C3 that had been synthesized by cells distant from the joint. C3-deficient mice were surgically joined with WT animals immediately after the latter had been subjected to lethal irradiation in order to ablate circulating blood cells. As illustrated in Figure 3A, the irradiated WT partner in these pairs harbored parenchymal cells that produce C3 and introduce it into the circulation, but lacked hematopoietic cells that might produce C3. The C3−/− partner lacked C3 synthesis by both parenchymal and hematopoietic cells but would be exposed to C3 through its circulation due to anastomosis with the circulation of the WT partner (Figure 3A). Measurement of circulating C3 confirmed equal concentrations of C3 in both partners, at levels ~25% of those seen in B6 mice (results not shown). Upon transfer of K/BxN serum, clear manifestations of inflammatory arthritis were observed in 4 of the 5 C3−/− partners and all 5 of the irradiated WT partners (Figure 3B). Immunofluorescence staining of ankle joints confirmed that C3 was prominently deposited on the cartilage surface and in the synovium, even in animals whose only source of C3 was through the circulation (Figure 4).

**DISCUSSION**

Circulating C3, produced by parenchymal cells distant from the joint, is both necessary and sufficient for induction of disease in the K/BxN mouse model of antibody-mediated arthritis. The serum-transfer system allowed us to focus specifically on the role of C3 in the inflammatory effector phase, after high levels of autoantibodies had been produced. It remains possible that C3 made either by leukocytes or by joint-resident parenchymal cells plays an important role in the initiation phase of autoimmunity, or that these 2 sources make a nonessential contribution to the effector process.

It is reasonable to propose that C3 plays a role in human RA similar to that in the K/BxN serum-transfer system, particularly in the early stages of inflammation or in the reactivation of acute inflammation in previously quiescent joints. Although the specific autoantibody responsible for arthritis in the K/BxN mouse model is observed in only a minority of patients with RA (35), particularly in those with Felty’s syndrome or other extraarticular manifestations (36,37), there is now considerable evidence supporting the pathogenicity of autoantibodies in RA. First, the B cell–depleting monoclonal antibody rituximab is highly effective in treating many patients with RA (38). Second, a newly described mouse model links an RA-specific autoantibody (anti-citrulline-containing protein) to murine arthritis (39). The role of complement has not yet been assessed in this context, but findings made previously in other models are likely to remain relevant to RA; that is, the ability of diverse autoantibody responses to generate pathology resembling RA in mice (40) indicates a general sensitivity of joints to antibody-mediated inflammation and supports the generalizability of the principles determined using the K/BxN mouse model and other models.

The hypothesis that local C3 production is important in RA arose from the finding that more C3 is produced in rheumatoid synovium than in osteoarthritic synovium, as determined qualitatively or quantitatively using in situ hybridization to detect messenger RNA (mRNA) for C3 (28,30). Subsequently, several microarray analyses of synovial gene expression in either human patients or rodent models have yielded conflicting data on this point, sometimes showing an increase in synthesis of C3 (41,42) and sometimes not showing such an increase (43–45). Importantly, we also detected mRNA for C3 in mouse synovium by both complementary DNA microarray and quantitative polymerase chain reaction (data not shown), although we did not observe an increase as arthritis unfolded.

The mechanism and kinetics by which circulating C3 gains access to joints are uncertain but not difficult to envision. Because the synovial “membrane” (functionally, a combination of the subsynovial vasculature and the synovial lining) is rather permeable at baseline, roughly in inverse proportion to the mass of the macro-molecule (46,47), and because C3, at 185 kd, is not extremely large, it would be expected that an abundant circulating protein of this size would have some passive access to normal synovial fluid. Indeed, although we are not aware of any reports in which C3 has been measured in normal synovial fluid, it has been readily detected in human osteoarthritic joint effusions at concentrations similar to those seen in RA, i.e., ~0.2–0.5 mg/ml (48,49).

The reason that concentrations in RA are not higher is probably related to local consumption (4–7,48),
because inflamed synovium otherwise loses its size-selective filtration and becomes about equally permeable to all macromolecules (46). Even if some increase in synovial fluid C3 is required for it to be locally activated and promote inflammation, increased local vascular permeability may be a very early feature of inflammatory arthritis. For example, studies in the K/BxN mouse serum-transfer model have shown that IgG-containing immune complexes selectively increase the permeability of periarticular vessels to IgG itself (50) and to a higher molecular weight (400 kd) tracer (51). Interestingly, complement is not required for this early vascular permeability (51); therefore, further research on the role of complement in this model will likely focus on downstream events, such as the role of complement receptors, particularly C5aR, on different populations of inflammatory cells.

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AUTHOR CONTRIBUTIONS

Dr. Mathis had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Targeting of Gr-1+,CCR2+ Monocytes in Collagen-Induced Arthritis

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Objective. The chemokine receptor CCR2 is highly expressed on monocytes and considered a promising target for treatment of rheumatoid arthritis. However, blockade of CCR2 with a monoclonal antibody (mAb) during progression of collagen-induced arthritis results in a massive aggravation of the disease. In this study we investigated why CCR2 antibodies have proinflammatory effects, how these effects can be avoided, and whether CCR2+ monocytes are useful targets in the treatment of arthritis.

Methods. Arthritis was induced in DBA/1 mice by immunization with type II collagen. Mice were treated with mAb against CCR2 (MC-21), IgE, or isotype control antibodies at various time points. Activation of basophils and depletion of monocyte subsets were determined by fluorescence-activated cell sorter analysis and enzyme-linked immunosorbent assay.

Results. Crosslinkage of CCR2 activated basophils to release interleukin-6 (IL-6) and IL-4. In vivo, IL-6 release occurred only after exposure to high doses of MC-21, whereas application of low doses of the mAb circumvented the release of IL-6. Regardless of the dose level used, the antibody MC-21 efficiently depleted Gr-1+,CCR2+ monocytes from the synovial tissue, peripheral blood, and spleen of DBA/1 mice. Activation of basophils with high doses of MC-21 or with antibodies against IgE resulted in a marked aggravation of collagen-induced arthritis and an increased release of IL-6. In contrast, low-dose treatment with MC-21 in this therapeutic setting had no effect on IL-6 and led to marked improvement of arthritis.

Conclusion. These results show that depletion of CCR2+ monocytes may prove to be a therapeutic option in inflammatory arthritis, as long as the dose-dependent proinflammatory effects of CCR2 mAb are taken into account.

CCR2, a chemokine (CC motif) receptor, is considered a promising target for disorders such as multiple sclerosis, type II diabetes, and rheumatoid arthritis, and Phase I and II clinical trials are currently in progress (1). Data on CCR2 expression in humans and results from studies of CCR2-deficient mice (2–4) support the blockade of CCR2 as an effective strategy in the treatment of multiple sclerosis and diabetes. In patients with rheumatoid arthritis, we and other investigators have found an increased frequency of CCR2+ cells in the synovial fluid and synovial tissue (5–7). In addition, increased levels of the CCR2 ligand monocyte chemoattractant protein 1 (MCP-1; CCL2) were found in patients with rheumatoid arthritis (8). Monocytes are thought to play a major role in joint destruction, and their recruitment to sites of inflammation is crucially dependent on CCR2, as shown in several murine disease models (9–12).

However, thus far, data from murine models of collagen-induced arthritis do not support CCR2 as a target for treatment of rheumatoid arthritis. We have previously shown that treatment with a blocking mono-
clonal antibody (mAb) against CCR2, MC-21, administered at 500 μg every third day, improves arthritis, when the treatment is started before the first immunization with collagen (13). In contrast, when the treatment is started later, a marked aggravation of arthritis occurs. This negative effect is most pronounced when the MC-21 treatment is started at day 21 after the first immunization with collagen (13); however, even when the treatment is initiated at days 4 or 9 after the first immunization, worsening of arthritis is still observed (results not shown). A similar result was obtained in CCR2-deficient DBA/1 mice that displayed a more severe form of arthritis than that in wild-type mice (14).

To understand why the blockade of CCR2 during the progression phase of collagen-induced arthritis aggravates the disease, we analyzed the expression of CCR2 on murine leukocytes. We have previously reported that CCR2 is expressed on murine regulatory T cells, which may be compromised by treatment with MC-21 (13). In addition, we noted that DBA/1 mice treated with MC-21 during disease progression had massively elevated levels of interleukin-6 (IL-6) in their plasma (Brühl H, et al: unpublished observations).

IL-6 plays an important role in the pathogenesis of collagen-induced arthritis, as has been demonstrated in IL-6–deficient mice (15,16) and in studies using blocking antibodies against the IL-6 receptor (17). We therefore analyzed whether MC-21 is directly responsible for the increase in IL-6 release. We identified basophils as the cells that release IL-6 after exposure to MC-21, and found that direct activation of basophils with IgE antibodies led to high plasma levels of IL-6 and worsening of arthritis in DBA/1 mice. In addition, we demonstrated that the MC-21–induced IL-6 release could be avoided by injecting low doses of MC-21 (10 μg per day), which efficiently depleted Gr-1+, CCR2+ monocytes and significantly improved the severity of arthritis. These findings may help contribute to the development of a CCR2- and monocyte–based therapy for inflammatory joint diseases.

MATERIALS AND METHODS

Flow cytometry. The following reagents were used for flow cytometry: leukocyte common antigen CD45–fluorescein isothiocyanate (FITC) (30-F11), CD11b-FITC (M1/70), CD11b-phycocerythrin (PE) (M1/70), CD19-FITC (1D3), CD19-PE (1D3), Gr-1–antigen-presenting cell (APC) (RB6-8C5), c-kit–PE (2B8), DX5-PE, DX5-biotin, anti-IgE-FITC (R35-72), mouse anti-rat IgG2b–biotin (R12-3), streptavidin–PE-Cy5 and a combination of directly labeled antibodies were added. Before basophils were stained with anti–IgE-FITC, blood, spleen, or bone marrow samples were washed 3 times in phosphate buffered saline (PBS) to remove unbound IgE. Finally, red blood cells were lysed with fluorescence-activated cell sorter (FACS) lysis solution (BD Biosciences). All FACS analyses were performed on freshly isolated cells, and samples were analyzed on a FACS Calibur (BD Biosciences) within 24 hours.

Cell preparations and cell culture. Blood was collected from the retroorbital plexus of anesthetized mice and anticoagulated with 1 mM EDTA. Plasma was obtained by centrifugation of blood for 10 minutes at 500g. Single cell suspensions of spleens were prepared in cold RPMI 1640 medium with 10% fetal calf serum (FCS) and filtered to remove cell debris. Bone marrow was obtained from the femur and tibia by flushing out the cells with cold RPMI 1640 medium with 10% FCS.

Basophils were depleted from total bone marrow cells with microbeads (Miltenyi Biotec). Cells were stained with anti–IgE-FITC, and then incubated with anti-FITC microbeads and applied to LS columns according to the manufacturer’s recommendations (Miltenyi Biotec). More than 99% of IgE-expressing cells were depleted from the bone marrow. One million total bone marrow cells or basophil-depleted bone marrow cells were incubated in flat-bottom 96-well plates for 24 hours in RPMI 1640, 10% FCS, and 1% penicillin/streptomycin. Soluble antibodies were then added. For coating of culture plates with the antibodies, the plates were incubated overnight at 4°C with 100 μl antibody solution (10 μg/ml). Plates were washed 2 times with PBS before adding the cells.

Isolation and stimulation of basophils. Basophils were isolated from single cell suspensions of bone marrow and spleen from C57BL/6 wild-type mice and CCR2-deficient mice (18) that had been backcrossed for 10 generations to C57BL/6. Using microbeads against DX5 on LD columns (Miltenyi Biotec), basophils were first enriched and then stained with CD45–FITC and DX5–APC, and further separated from natural killer (NK) cells and other contaminating cells by FACS analysis (FACS Aria; BD Biosciences). Basophils were identified by their light-scatter properties, by moderate expression of CD45, and by high expression of DX5. The resulting cell population consisted of >95% basophils as determined by staining with anti–IgE-FITC. Basophils (10,000 per well) were cultured in round-bottom 96-well plates for 24 hours with the various antibodies. Coating of the plates with the antibodies was performed as described above.

Collagen-induced arthritis. Bovine type II collagen (Sigma C1188; St. Louis, MO) was dissolved overnight in 0.1M acetic acid at a concentration of 2 mg/ml and, for the first injection, was emulsified in an equal volume of Freund’s complete adjuvant. Male DBA/1 mice were immunized with an intra-/subcutaneous injection of 100 μl emulsion at the base of the tail on day 0, and an intra-peritoneal (IP) injection of 100 μg type II collagen without adjuvant at day 21. The
clinical score of arthritis was evaluated as follows: 0 = normal; 1 = swelling in 1 joint; 2 = swelling in >1 joint; 3 = swelling of the entire paw; 4 = deformity and/or ankylosis. Animal experiments were performed in accordance with the legal requirements of the government of Bavaria (Az. 55.2-1-54-2531-109-05).

**Histology.** Hind paws were fixed in 3.7% formalin for 24 hours, washed in PBS, and decalcified with RDO rapid decalcifier (Medite, Burgdorf, Germany). The tissue was then embedded in paraffin and cut in 5-μm-thick sections. After staining with hematoxylin and eosin, the following parameters were evaluated, in a blinded manner, on at least 10 sections of the tarsometatarsal joints, using a scale from 0 (normal) to 2 for all categories: synovial inflammation (1 = focal inflammatory infiltrates; 2 = inflammatory infiltrates dominating the histologic characteristics of the cell), synovial hyperplasia (1 = a single joint with continuous synovial lining of at least 3 layers in thickness; 2 = several joints with continuous, at least 3-layer–thick synovial lining), pannus formation and cartilage loss (1 = cartilage partially covered by pannus, no cartilage loss; 2 = cartilage loss), and bone destruction (1 = small areas of bone destruction; 2 = widespread bone destruction).

**Isolation and staining of leukocytes from synovial tissue.** Mice with an arthritis score of at least 3 in at least 1 paw were treated for 2 days with low-dose MC-21 (10 μg/day), high-dose MC-21 (100 μg/day), or MC-67 (10 μg/day) as control antibody, and tissue sections were analyzed 24 hours after the last antibody injection. The skin was removed from the inflamed paws and the remaining tissue was carefully recovered with a scalpel. All bones were left intact and were removed before digestion of the tissue with type I collagenase (Sigma) for 20 minutes at 37°C. Cells were stained with antibodies against CD11b, CD45, and Gr-1.

**Enzyme-linked immunosorbent assay (ELISA).** Levels of IL-4 and IL-6 in the cell culture supernatants and plasma of mice were measured with commercially available kits (OptEIA; BD PharMingen, San Diego, CA). Antibodies against type II collagen were quantified by ELISA. Type II collagen (20 μg/ml) was coated overnight on ELISA plates. Plasma samples were applied in a dilution of 1:1,000–1:20,000 in PBS/3% bovine serum albumin followed by a horseradish peroxidase (HRP)–labeled rabbit anti-mouse polyclonal antibody (P260; Dako, Carpinteria, CA) or HRP-labeled mAb specific for murine IgG1 (clone LO-MG1-2; Serotec, Oxford, UK) or for murine IgG2a (clone R19-15; BD PharMingen).

**Statistical analysis.** All statistical analyses were performed using Student’s unpaired t-test. Results are expressed as the mean ± SEM.

**RESULTS**

**Expression of CCR2 on murine leukocytes.** It has recently been shown that murine monocytes can be separated into 2 different populations by the presence or absence of surface marker Gr-1 (Ly6C/G) (19,20). The results of the present study showed that staining with the CCR2 mAb MC-21 selectively identified the subpopulation of Gr-1+ monocytes in the peripheral blood and spleen tissue from DBA/1 mice (Figure 1A). Monocytes were identified by their specific light-scatter properties and expression of CD11b. Moreover, monocytes could be clearly distinguished from NK cells by the higher expression of CD11b and absence of DX5 in monocytes. Neutrophils and eosinophils were excluded on the basis of their light-scatter properties and the higher expression of Gr-1 on neutrophils compared with that on monocytes. Identical results (not shown) were obtained when monocytes were identified by their expression of CD115 instead of CD11b. Apart from monocytes, small populations of T cells, preferentially CD4+ T cells, express CCR2. We have previously shown that the...
majority of CD4+CCR2+ T cells also stain positive for the regulatory T cell markers CD25 and CD103 and fulfill functional criteria of regulatory T cells in vitro (13).

We also observed a homogeneous expression of CCR2 by basophils, which were identified by their high surface expression of IgE (Figure 1B) as well as their expression of CD123 and absence of c-kit (results not shown) (21). No CCR2 expression was found on B cells, NK cells, neutrophils, or eosinophils in the peripheral blood or spleen tissue from the mice.

Depletion of Gr-1+ monocytes by the anti-CCR2 antibody MC-21. In several cases, rat mAb with the isotype IgG2b have been described as capable of depleting their target cells in murine models. We analyzed whether the rat anti-mouse CCR2 mAb MC-21, which has the IgG2b isotype, is able to deplete CCR2+ cells from the peripheral blood and spleen of mice. By 8–24 hours after the first application of MC-21, a complete depletion of Gr-1+ monocytes was evident in the peripheral blood (Figure 2A), as well as the spleen, of DBA/1 mice and was also observed in other mouse strains (not shown). This complete depletion of Gr-1+ monocytes could be achieved with doses of MC-21 as low as 10 μg, administered IP. Following a single injection of 10 μg MC-21, monocytes reappeared after 48 hours and reached almost normal levels after 72 hours. After repeated daily injections of 10 μg MC-21, the depletion of monocytes could be sustained up to 5 days, whereas after 8 days of MC-21 treatment, no monocyte depletion was detectable (Figure 2B). Most likely, a humoral immune response against MC-21 is responsible for the failure of MC-21 to deplete monocytes after prolonged application, since mouse anti-rat antibodies became detectable after 1 week of treatment with MC-21.

Although the Gr-1− monocytes did not express CCR2, their numbers were also reduced (~50%) by the MC-21 treatment after a few days (Figure 2B). It has been described that Gr-1+ monocytes can develop into Gr-1− monocytes (19,22–24). For this reason, depletion of Gr-1+ monocytes also might eventually result in a reduced frequency of Gr-1− monocytes.

Apart from the effects on monocytes, a small reduction (~30%) in the number of basophils occurred after treatment with MC-21, whereas the number of CD4+,CD25+ T cells remained constant. In lymph nodes, a partial depletion of Gr-1+ monocytes was observed. In contrast, in the bone marrow, no depletion of Gr-1+ monocytes occurred (results not shown).

Both the low dose (10 μg/day) and the high dose (100 μg per day) of MC-21 resulted in an identical depletion of cells. Depletion of Gr-1+ monocytes is mediated by antibody-dependent, cell-mediated cytotoxicity, as confirmed by studies showing that blockade of the low- and medium-affinity Fc receptor CD16/32 almost completely prevents the MC-21–induced depletion of monocytes (results not shown).

Induction of release of IL-6 by MC-21 via CCR2-dependent activation of basophils. In previous experiments we noted that the treatment of mice with high doses of MC-21 (500 μg every third day) during the progression phase of arthritis resulted in a high plasma level of IL-6. We therefore analyzed whether MC-21 per se induces a release of IL-6. Injection of high amounts of MC-21 (e.g., 100 μg IP) resulted in a rapid release of
IL-6, with plasma levels in the range of 50 pg/ml 2 hours after injection, while the injection of lower amounts (e.g., 10 µg MC-21 IP) did not lead to a measurable plasma level of IL-6 (Figure 3A). Injection of an isotype control antibody (100 µg MC-67 IP), which was produced under the same conditions as MC-21, did not result in a measurable release of IL-6. Both the MC-21 antibody and the isotype control antibody were virtually free of lipopolysaccharide (LPS) (LPS content <0.5 ng/mg antibody). The appearance of IL-6 within 2 hours after IP injection of MC-21 suggests that preformed IL-6 is released. IL-4 was undetectable in the plasma after stimulation with MC-21.

To identify the cells responsible for the IL-6

**Figure 3.** Activation of basophils by the CCR2 antibody MC-21. A, Plasma levels of interleukin-6 (IL-6) were assessed 2 hours after injection of high doses (100 µg) or low doses (10 µg) of MC-21, isotype control antibody MC-67 (100 µg), or phosphate buffered saline (PBS), administered intraperitoneally. Representative results from an individual mouse are shown. B, MC-21–induced release of IL-6 from basophils was assessed in the bone marrow of mice. Total bone marrow cells or basophil-depleted bone marrow cells were incubated for 24 hours with 10 µg/ml soluble MC-21 (MC-21 sol.), 10 µg/ml soluble MC-67 (MC-67 sol.), 0.5 µg/ml anti-IgE antibody (clone R35-72), plate-bound MC-21, or plate-bound MC-67. C, Basophils were isolated from the bone marrow of CCR2-deficient or wild-type (WT) C57BL/6 mice, using magnetic beads and fluorescence-activated cell sorting. Cells were incubated with plate-bound or soluble MC-21 or MC-67 (each at 10 µg/ml) for 24 hours, followed by measurement of the release of IL-6 or IL-4. Results in B and C are the mean and SEM, representative of at least 3 experiments.
release, we incubated murine leukocytes in vitro with MC-21. Incubation of splenocytes with MC-21 resulted in only a marginal release of IL-6 (results not shown). However, incubation of bone marrow cells with MC-21 led to a pronounced increase in IL-6 (Figure 3B). In view of the CCR2 expression on basophils and the preferential localization of basophils in the bone marrow, we analyzed basophils as a potential source of the MC-21–induced IL-6 release. Depletion of basophils from the bone marrow almost completely prevented the MC-21–induced, as well as the IgE antibody–induced, IL-6 release (Figure 3B). However, when the cells were stimulated with LPS (100 ng/ml), depletion of basophils from the bone marrow did not result in reduced IL-6 release (1,080 pg/ml and 1,189 pg/ml IL-6 in total and basophil-depleted bone marrow cells, respectively), indicating that LPS-induced IL-6 release is not dependent on basophils.

To further demonstrate that basophils are responsible for the IL-6 release, we isolated basophils from the bone marrow with magnetic beads and FACS analysis, as described in Materials and Methods. Incubation of basophils with plate-bound MC-21, but not with plate-bound MC-67 control antibody, induced a release of both IL-6 and IL-4 (Figure 3C). Soluble MC-21 was unable to induce cytokine release from isolated basophils, suggesting that the activation of basophils occurs due to crosslinkage of CCR2.

Using basophils from CCR2-deficient mice, we investigated whether activation of basophils by the antibody MC-21 is mediated via CCR2 or is the result of a cross-reactivity of MC-21 with other surface molecules on basophils. No release of IL-6 or IL-4 was detectable when CCR2-deficient basophils were stimulated with MC-21, thus indicating the crucial involvement of CCR2 (Figure 3C). Whereas both IL-6 and IL-4 were detectable after the stimulation of isolated basophils with MC-21, no release of IL-4 was detectable when total bone marrow cells were activated with MC-21. This correlates with the finding that after injection of MC-21 in vivo, only IL-6, but not IL-4, was detectable in the plasma.

Influence of basophil activation and monocyte depletion on the course of collagen-induced arthritis. For in vivo blockade of CCR2, high amounts (500 μg every third day) of the CCR2 antibody MC-21 had to be injected to achieve plasma levels of the antibody that are above the 50% inhibitory concentration of MC-21 (9,13). However, depletion of Gr-1+ monocytes is possible with much lower doses (e.g., 10 μg), administered once per day. Injection of low doses of MC-21 circumvents the potentially unfavorable release of IL-6, and thus provides an opportunity to analyze the depletion of CCR2+ monocytes as a treatment strategy in arthritis.

Depletion of monocytes with low doses of MC-21 requires daily injection of the antibody, as indicated by our results showing that Gr-1+ monocytes reappeared after 48 hours. Therefore, we analyzed the effects of a daily injection of high amounts (50 μg/day) or low amounts (10 μg/day) of MC-21 during the progression phase of collagen-induced arthritis. In our previous experiments, injection of 500 μg MC-21 every third day beginning at day 21 after the first immunization significantly increased the severity of arthritis as compared with the effects of injection of an isotype control antibody (MC-67) (13) or PBS (results not shown). Similarly, daily injection of 50 μg MC-21 beginning at day 21...
after the first immunization resulted in a marked increase in arthritis as compared with the effects of MC-67 (Figure 4A). In contrast, when a lower dose of MC-21 (10 µg/day) was injected beginning from day 21, a clear reduction of arthritis was achieved (Figure 4A).

To determine whether the activation of basophils with reagents other than MC-21 also results in increased arthritis, we treated mice from day 21 to day 32 with daily injections of an anti-IgE antibody (20 µg/day), a high dose of MC-21 (50 µg/day), or the MC-67 isotype control antibody (50 µg/day) (Figure 4B). Activation of basophils with the anti-IgE antibody resulted in an even more pronounced aggravation of arthritis than that resulting from injection of 50 µg MC-21 (Figure 4B).

We also measured the plasma levels of IL-6 in mice treated with MC-21 (50 µg/day), IgE antibody (20 µg/day), or MC-67 isotype control antibody. On day 30 after the first immunization with type II collagen, mice treated with high doses of MC-21 had several-fold higher plasma IL-6 levels than did mice treated with the isotype control antibody (Figure 4C). As expected, mice treated with IgE antibodies also had very high plasma IL-6 levels.

In a separate set of experiments, we analyzed whether the depletion of monocytes with low doses of MC-21 is still beneficial when treatment is started after the clinical onset of arthritis (beginning, on average, 30.5 days after the first immunization with type II collagen). Mice with established arthritis (clinical arthritis score of ~3.0) were randomly assigned to receive treatment with the antibody MC-21 or the isotype control antibody MC-67 (Figure 5). The time point of the first application of MC-21 or isotype control antibody was designated day 0, and daily treatment was thereafter continued for 6 days. Treatment with the control antibody resulted in a highly significant progression of arthritis, with a rise in the clinical arthritis score of 2.9 on day 0 to 5.1 on day 6. In contrast, no significant progression of arthritis occurred in the MC-21–treated group (clinical arthritis score 3.1 at day 0 and 3.4 at day 6) (Figure 5A). As soon as day 2 of treatment, the clinical arthritis score in the MC-21–treated mice was significantly lower than that in the control group, and remained lower until the end of the experiment at day 6.

After treating mice for 3–5 days with low-dose MC-21 or control antibodies, we determined the percentage of Gr-1+ monocytes in the peripheral blood and the plasma levels of IL-4, IL-6, anti-type II collagen IgG1, and anti-type II collagen IgG2a. The percentage of blood CD11b+ monocytes was significantly lower in mice treated with low-dose MC-21 (5.7% of total leukocytes) compared with mice treated with the control antibody (9.5% of total leukocytes) (Figure 5B). The majority of monocytes expressed Gr-1 in both groups. The plasma levels of IL-4 and IL-6 were equally low in both groups (Figure 5B). Moreover, the anti-type II collagen IgG1 and IgG2a levels were not different between groups (results not shown).

We also examined the histologic changes that occurred in the hind paws in mice treated for 6 days after

![Figure 5. Improvement of established arthritis in mice following treatment with low-dose MC-21. A, DBA/1 mice were immunized with type II collagen on day 0 and day 21, and then observed for the development of arthritis. Starting on days 28–33 (1–2 days after arthritis onset), mice were randomly assigned to receive daily intra-peritoneal treatment with low-dose MC-21 (10 µg) or isotype control antibody MC-67 (10 µg) (n = 17 per group). At each time point, equal numbers of mice were evaluated in the 2 groups. The day of the first application of MC-21 or isotype control antibody was designated day 0, and the treatment was continued for 6 days. Mice were killed on day 6 for histologic assessment. From day 0 to day 6, the arthritis score significantly increased in the control group only (\( P < 0.01 \)). Results are representative of at least 2 experiments. Differences between the groups were significant (\( P < 0.05 \)) on days 2–6. B, Blood was withdrawn from the mice on days 3–5 after treatment with the antibodies. The percentage of CD11b+ monocytes in relation to total leukocytes, and plasma levels of interleukin-4 (IL-4) and IL-6 were compared between the groups. \( * = P < 0.05 \) versus MC-67–treated mice. Results are the mean and SEM.

\( P < 0.05 \) in mice treated with low-dose MC-21 (5.7% of total leukocytes) compared with mice treated with the control antibody (9.5% of total leukocytes) (Figure 5B). The majority of monocytes expressed Gr-1 in both groups. The plasma levels of IL-4 and IL-6 were equally low in both groups (Figure 5B). Moreover, the anti-type II collagen IgG1 and IgG2a levels were not different between groups (results not shown).
the onset of arthritis with low doses of MC-21 or MC-67 (each at 10 μg/day) (Figures 6A and B). Mice were killed on day 6 of antibody treatment, and the tarsometatarsal joints were evaluated after decalcification and staining with hematoxylin and eosin. In correlation with the changes in the clinical arthritis score, the joints of MC-21–treated mice displayed significantly less leukocyte infiltration and synovial hyperplasia as well as
reduced bone and cartilage erosion as compared with the control group.

We also analyzed the cellular composition of the infiltrating cells. Mice with an arthritis score of at least 3 in at least 1 paw were treated for 2 days by daily injection of 10 μg MC-67 or 10 μg MC-21, and 24 hours later the synovial tissue leukocytes were analyzed by FACS (Figures 6C and D). In mice treated with MC-21, the subpopulation of Gr-1⁺ monocytes was markedly reduced in the synovial tissue, whereas there was little influence on Gr-1⁻ monocytes or neutrophils. Gr-1⁺ monocytes were also depleted in the peripheral blood and, to a lesser degree, in the spleens after treatment with MC-21 (results not shown).

Taken together, these data indicate that treatment with low doses of MC-21 starting at day 21 after the first immunization with type II collagen or even after the clinical onset of arthritis significantly improves arthritis. Treatment of mice with high doses of MC-21 or with antibodies against IgE results in release of IL-6, which is attributable to the activation of basophils, and leads to a marked increase in the severity of arthritis.

**DISCUSSION**

In the present study we analyzed why the blockade of CCR2 with the antibody MC-21 during the progression phase of collagen-induced arthritis has proinflammatory effects, how these proinflammatory effects can be avoided, and what are the mechanisms of action of CCR2⁺ monocytes in the progression of arthritis. Our findings showed that the CCR2 antibody MC-21 activates basophils to release IL-6 and IL-4. In vivo, IL-6 release was observed only at high doses of the CCR2 antibody (e.g., 50–100 μg) and could be avoided by injecting lower doses of MC-21 (e.g., 10 μg). Activation of basophils by daily application of high doses of MC-21 or by injecting antibodies against IgE resulted in a markedly aggravated arthritis and an increased IL-6 release. In contrast, the application of low doses of the MC-21 CCR2 antibody (10 μg/day) circumvented the release of IL-6, allowed the depletion of Gr-1⁺ monocytes from the peripheral blood, spleen, and synovial tissue, and markedly improved established arthritis.

An aggravation of arthritis due to activation of basophils has not been previously described in the literature. However, it is known that mast cells and IL-6 play an important role in the development of arthritis. Mast cell–deficient or IL-6–deficient mice are, to a large extent, protected from antibody- or collagen-induced arthritis, respectively (15,16,25). Some reports describe the expression of CCR2, as determined by reverse transcriptase–polymerase chain reaction, on cultured bone marrow–derived murine mast cells (26), and an activation of pulmonary mast cells by the CCR2 ligand MCP-1 (27). We found only a marginal expression of CCR2 on c-kit⁺ cells in the bone marrow, using the antibody MC-21 (mean channel fluorescence 18.5 on c-kit⁺ cells versus 490 on monocytes).

We also showed that depletion of basophils from total bone marrow, with magnetic beads directed against IgE, almost completely prevented the MC-21–induced IL-6 release. The only cells in freshly isolated bone marrow that expressed surface IgE were basophils, but not mast cells. This observation helps rule out the possibility of a substantial contribution of mast cells to the MC-21–induced release of IL-6.

Thus far, it is not known how many basophils are present in the synovial tissue, either in mice or in patients with arthritis. However, in the bone marrow, large numbers of basophils are present. In histologic sections of joint tissue from mice with severe collagen-induced arthritis, we clearly observed expansion of synovial tissue and inflammatory infiltrates extending into the bone marrow. In addition, a recent study in humans with rheumatoid arthritis has shown that inflamed tissue is present in the bone marrow, and that the inflammatory process extends to the bone marrow cavity (28). It is therefore conceivable that cytokines released from bone marrow cells (e.g., IL-6 produced by basophils) might contribute to the severity of arthritis.

It is not widely recognized that basophils also release IL-6 (21). In our experiments with isolated basophils, we observed a release of IL-6 and IL-4 after stimulation with anti-IgE or MC-21 antibodies. When total bone marrow cells were stimulated with anti-IgE or MC-21, and when plasma cytokines were measured after injection of MC-21, only IL-6, but not IL-4, was detectable. This might be due to the detection limit of our ELISA or due to a consumption or degradation of IL-4.

The stimulation of basophils with the CCR2 antibody MC-21 is dependent on the crosslinkage of CCR2. When isolated basophils were assessed, only plate-bound MC-21, but not soluble MC-21, induced a release of IL-6 and IL-4. When total bone marrow cells were assessed, both plate-bound and soluble MC-21 induced basophil activation. We assume that the large number of Fc receptor–positive cells present in the total bone marrow immobilizes the MC-21 antibody, and thereby allows crosslinkage of CCR2. It is well accepted that the oligomerization of chemokine receptors affects their activation state. With antibodies against CCR5, we
have previously shown that crosslinkage results in partial activation of CCR5 (29). In several assays (calcium flux, cell migration, receptor down-modulation), we were unable to detect agonistic activity of the CCR2 antibody MC-21. However, it is important to note that partial activation might become detectable only if multiple signal transduction pathways are analyzed in several cell types. Eotaxin/CCL11, a natural antagonist of CCR2, was recently shown to induce signal transduction via the ERK-1/2 pathway (30). Human basophils also express CCR2 (31) and can be activated via CCR2, resulting in shape changes (31) and the release of cytokines and leukotriene C4 (32).

In vivo, the release of IL-6 by MC-21 was dose-dependent and was only detectable after administration of high doses of MC-21, whereas the depletion of Gr-1+ monocytes from the peripheral blood, spleen, and synovial tissue was almost identical after high doses (100 µg) or low doses (10 µg) of MC-21. This enabled us to study the effects of monocyte depletion without activating basophils. The application of low doses of MC-21 resulted in a significant improvement of arthritis, using 2 different treatment regimens. Starting the treatment at day 21 after the first immunization, and even starting the treatment after the onset of arthritis, resulted in an improved clinical arthritis score in the MC-21-treated group. Following treatment with MC-21, the number of Gr-1+ monocytes was reduced in the peripheral blood, in the spleen, and in the synovial tissue of mice with arthritis. We were somewhat surprised that the depletion of peripheral blood monocytes in DBA/1 mice with arthritis was not as complete as in naive DBA/1 mice (>95% depletion of Gr-1+ monocytes). This difference might result from a higher turnover of monocytes in DBA/1 mice with arthritis. This finding and the fact that the depletion of monocytes can only be sustained for a few days may explain why we missed the MC-21–induced depletion of monocytes in our previous experiments, in which the depletion of monocytes was analyzed only after 10 days of MC-21 application in DBA/1 mice with arthritis (13).

Several lines of evidence suggest that the depletion of CCR2+ monocytes, but not the blockade of CCR2, is responsible for the improvement of arthritis after low-dose MC-21 treatment. First, the application of 10 µg MC-21 is unlikely to fully block CCR2. Second, CCR2-deficient DBA/1 mice show increased progression of arthritis as compared with wild-type mice (14). The underlying mechanisms of the disease in CCR2-deficient mice are unclear. Apart from findings of an impaired activation-induced cell death (14), the increased number of Gr-1+ monocytes in the bone marrow of CCR2-deficient mice (33) might contribute to the aggravation of arthritis, provided that the leukocytes in the bone marrow have access to the inflamed joints, which appears possible in view of the extensive destruction of the bone architecture in this model. In addition, a recent clinical trial showed that blockade of the MCP-1–CCR2 interaction by a humanized antibody against MCP-1 was associated with a worsening of rheumatoid arthritis (34). Indeed, CCR2 might not be the ideal target for an antibody-mediated depletion of monocytes as a therapeutic approach in arthritis, since crosslinkage of CCR2 on basophils activates basophils to release IL-6, and activation of basophils markedly aggravates arthritis. Other surface structures on monocytes may be more suitable for the recognition and depletion of monocytes.

By low-dose treatment with an anti-CCR2 antibody, we were able to significantly improve collagen-induced arthritis in mice by depleting CCR2+ monocytes. Potential proinflammatory effects resulting from the blockade of CCR2 and the activation of basophils were prevented. The beneficial effect of depleting CCR2+ monocytes may thus help in the design of new strategies for the treatment of arthritis.

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AUTHOR CONTRIBUTIONS

Dr. Mack had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Brühl, Mack.

Acquisition of data. Brühl, Cihat, Plachy, Kunz-Schughart, Niedermeier, Denzel, Gomez, Talke, Luckow, Stangassinger, Mack.

Analysis and interpretation of data. Brühl, Kunz-Schughart, Niedermeier, Denzel, Gomez, Talke, Luckow, Stangassinger, Mack.


Statistical analysis. Brühl, Mack.

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Correlation of the Development of Knee Pain With Enlarging Bone Marrow Lesions on Magnetic Resonance Imaging

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Objective. Results of cross-sectional studies have suggested that bone marrow lesions (BMLs) visualized on magnetic resonance imaging (MRI) are related to knee pain, but no longitudinal studies have been done. This study was undertaken to determine whether enlarging BMLs are associated with new knee pain.

Methods. Subjects ages 50–79 years with knee osteoarthritis (OA) or at high risk of knee OA were asked twice at baseline about the presence of knee pain, aching, or stiffness (classified as frequent knee pain) on most days; absence of knee pain was the baseline eligibility criterion. At 15 months’ followup, subjects were again queried twice about frequent knee pain. A case knee was defined as absence of knee pain at baseline but presence of knee pain both times at followup. Controls were selected randomly from among knees with absence of pain at baseline. All MR images were scored for volume of BMLs in the medial, lateral, and patellofemoral compartments. We focused on the maximal change in BML score among the knee compartments from baseline to 15 months. Multiple logistic regression, with adjustments for demographic and clinical variables, was used to assess whether an increased BML score is predictive of the development of knee pain.

Results. Among case knees, 54 of 110 (49.1%) showed an increase in BML score within a compartment, whereas only 59 of 220 control knees (26.8%) showed an increase \( (P < 0.001 \text{ by chi-square test}) \). A BML score increase of at least 2 units was much more common in case knees than in control knees (27.5% versus 8.6%; adjusted odds ratio 3.2, 95% confidence interval 1.5–6.8). Among case knees with increased BMLs, most already had BMLs at baseline, with enlarging BMLs at followup, but among the subset of knees with no BMLs at baseline, new BMLs were more common in case knees (11 [32.4%] of 34) than in control knees (9 [10.8%] of 83).

Conclusion. Development of knee pain is associated with an increase in BMLs as revealed on MRI.

Approximately one-fourth of adults ages ≥55 years have frequent knee pain (1). Although many of these individuals have a diagnosis of knee osteoarthritis (OA), at least half do not. Furthermore, even in those subjects with knee OA, the source of the joint pain is unknown.

 Whereas cartilage is aneural, bone is richly innervated and the involvement of bone in the pathologic features of OA is well recognized (2–4). Because of this innervation, bone could be a source of pain in at least some subjects with knee OA. In a previous cross-sectional study, we compared the findings on magnetic resonance imaging (MRI) of the knee in subjects with pain-free knee OA with the findings in subjects with knee pain and radiographic knee OA (5). We found that the subjects with knee pain were much more likely to have bone marrow lesions (BMLs) (5).

Subsequent studies of the association of BMLs with knee pain, all of which have been cross-sectional, have either corroborated our initial findings (6) or contradicted them (7,8). This has left uncertainty as to the relationship between BMLs and knee pain. The
KNEE PAIN AND BONE MARROW LESIONS

All MOST subjects were recruited from 2 communities in the US, Birmingham, Alabama and Iowa City, Iowa, through mass mailing of letters and study brochures, supplemented by media and community outreach campaigns. Each center also recruited ethnic minorities according to their representation in the recruitment population. Subjects were excluded from the MOST if they screened positive for rheumatoid arthritis (10), had ankylosing spondylitis, psoriatic arthritis, or reactive arthritis, experienced problems with the kidneys that resulted in the need for hemo- or peritoneal dialysis, had a history of cancer (except for nonmelanoma skin cancer), had undergone bilateral knee replacement surgery, were unable to walk without the help of another person or walker, or were planning to move out of the area in the subsequent 3 years. The study protocol was approved by the institutional review boards at the University of Iowa, University of Alabama, Birmingham, University of California, San Francisco, and Boston University Medical Center.

In the present study we used a subsample of subjects from the parent study. Using a nested case–control study design (11), we investigated knees that were considered eligible for MRI and that had been examined both at baseline and at 15 months’ followup. At baseline, all study subjects were asked a question regarding knee pain, as follows: “During the past 30 days, have you had pain, aching, or stiffness in your knee on most days?” This question was posed to subjects both by phone interview and during a clinic visit 1 month thereafter. If the subject answered no regarding the presence of pain, aching, or stiffness in the knee at each of these time points at baseline, that knee was considered eligible for analysis in the current nested case–control study.

At 15 months’ followup, this same question regarding knee pain was posed to subjects, both in a phone interview and at a clinic visit. If the subject answered yes to the question at both of these followup time points, the knee with new pain was considered to be a case knee (n = 110). Because this question was intended to identify pain, aching, or stiffness “on most days,” and because the 2 time points were, on average, 1 month apart, we characterized a positive response at both time points as “consistent frequent knee pain.” Furthermore, although our question included the symptoms of stiffness and aching, we labeled a positive response as related to pain. Control knees (n = 220) were selected randomly from among the knees examined by MRI at baseline and for which the subject indicated absence of pain at both baseline time points.

In addition, at the baseline clinic examination, subjects completed surveys on medication use and filled out a questionnaire on symptoms of depression, the Center for Epidemiologic Studies Depression Scale (CES-D) (12). In addition, subjects were weighed (without shoes) on a balance-beam scale to determine the body mass index (BMI), computed as weight (kg)/height (m$^2$). The same survey on medication use was administered at the 15-month visit. Subjects also completed the Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC), a survey of joint pain, stiffness, and limitation of physical function (13), for each knee at both the baseline and 15-month visits.

MRI. MR images were obtained with a 1.0T dedicated MR system (OrthOne; Oni, Wilmington, MA) with a circumferential extremitiy coil. All MRIs were performed using fat-suppressed, fast spin-echo, proton density–weighted sequences in 2 planes, the sagittal plane (repetition time [TR] 4,800 msec, time to echo [TE] 35 msec, slice thickness 3 mm, interslice gap 0 mm, field of view [FOV] 14 cm, matrix 288 × 192 pixels, number of excitations [NEX] 2) and the axial plane (TR 4,700 msec, TE 13.2 msec, slice thickness 3 mm, interslice gap 0 mm,
Two musculoskeletal radiologists (AG and FR), who were blinded to the case/control status and clinical data, read the MR images for evidence of BMLs and effusions, according to the Whole-Organ MRI Score (WORMS) (14). Using the WORMS, BMLs were scored 0–3 based on the size, or volume, of the lesion in each of 5 subregions of the medial and lateral compartments and in 4 subregions of the patellofemoral compartment. Similarly, effusion volume was scored 0–3. The MR images were read as paired images from both the baseline and followup visits. In addition, bone attrition was scored 0–3 for each subregion, with results expressed as the maximal score for a compartment. Periarticular lesions (e.g., anserine bursitis) have also been cross-sectionally associated with knee pain (15); however, no change in these lesions was observed in our subjects’ knees.

Given that the results of previous studies (5,6) have suggested that the size of BMLs is associated with pain, we focused on the change in size, or volume, of the lesion as our measure of BML change. We summed the BML score for all 5 subregions of the medial compartment (score range 0–3 in each subregion; across all 5 subregions, score range 0–15), all 5 subregions of the lateral compartment (score range 0–15), and all 4 subregions of the patellofemoral compartment (score range 0–12). This yielded 3 BML scores for each knee.

In each knee compartment, change was defined as change in the BML score from baseline to followup. Interreader agreement for this change score was an intraclass correlation coefficient (ICC) of 0.93 ($P < 0.001$). Given the compartment-specific nature of OA and the likelihood that a compartment would develop new or worse disease, we focused on change in BML score in each compartment. For each subject, we thus obtained 3 compartment-specific BML change scores, and of these 3 scores, we focused on the change score that showed the maximal unit increase; we have previously used this compartment-specific approach for scoring of BMLs to identify a strong relationship between BMLs and knee malalignment (16). In a sensitivity analysis, we summed all of the BML scores for the whole knee and created a global knee change score by subtracting the baseline score from the followup score. Results were the same as those obtained using the compartment-specific approach.

Radiographic assessment. At baseline, all subjects underwent weight-bearing, posteroanterior (PA), fixed-flexion radiographic evaluation of the knee using the protocol described by Peterfy et al (17). Body weight was equally distributed between the 2 legs, and the big toes of the feet and the front of the thighs were placed in contact with the front plate of the plexiglass frame. The external rotation of the feet was fixed at 10 degrees, using a V-shaped foot angulation support on the frame. The central radiographic beam was directed to the midpoint between the back of the knees, at a caudal angle of −10 degrees, to allow the anterior and posterior lips of the medial tibial plateau to be optimally superimposed (film-focus distance 183 cm).

A musculoskeletal radiologist (PA) and a rheumatologist (JTF), who were experienced in reading study films and were blinded to both the case/control status and clinical data, graded all of the PA radiographs according to the Kellgren/Lawrence (K/L) scale of radiographic knee OA (18). Radiographic OA was considered present if knees were assessed a K/L grade ≥2.

Full-limb radiographs of both legs were obtained at baseline, using the method of Sharma et al (19). The mechanical axis was defined as the angle formed by the intersection of a line from the center of the head of the femur to the center of the tibial spines, and a second line from the center of the talus to the center of the tibial spines. The interobserver ICC for determination of the mechanical axis was 0.99 ($P < 0.0001$).

Statistical analysis. To test the frequency of change in BMLs in the case knees as compared with the control knees, we used a chi-square test, both comparing knees and comparing subjects (given the occurrence of 8 cases in which both the left and the right knee of a subject were affected). We used multiple logistic regression with generalized estimating equations (to adjust for the correlation of knees) to evaluate the relationship between an increase in BML score and incident knee pain, in models adjusted for age, sex, race (white versus African American), BMI, CES-D score, baseline quadriceps strength, K/L grade, effusion score, baseline BML score, and change in effusion score, and with or without adjustment for mechanical knee alignment. Additional analyses in which we examined change in pain medication use (including use of nonsteroidal antiinflammatory drugs) as a covariate yielded similar findings, as did additional analyses in which we adjusted for the presence or absence of bone attrition (a rare finding in the knees studied). All $P$ values (2-tailed) were calculated using SAS for Windows, version 9.1 (SAS Institute, Cary, NC). $P$ values less than or equal to 0.05 were considered significant.

RESULTS

Case knees (n = 110), i.e., knees with incident pain, aching, or stiffness at the 15-month followup visit, were identified in study subjects who were slightly older and more often female than were the subjects in the control group (n = 220). In addition, case knees were identified as having radiographic OA at more frequently than control knees (Table 1).

In analyses focusing on the knee compartment with greatest change in BML score, we found that 54 of 110 case knees (49.1%, 95% confidence interval [95% CI] 40.2–58.9%) showed an increase in BML score in a compartment, whereas only 59 of 220 control knees (26.8%, 95% CI 21.0–32.7%) showed an increase in BML score in a compartment ($P < 0.001$ by chi-square test, between knees and between subjects). Since each compartment had 4 or 5 subregions, we compared case knees and control knees according to the number of subregions of a compartment that showed an increase in BML score. We found that an increase in BML score occurred in 1 subregion in 27.3% of case knees (95% CI 19.0–35.6%) compared with 23.6% of control knees (95% CI 18.0–29.3%). Of note, however, the BML score
increased in more than 1 subregion in 25.5% of case knees (95% CI 17.3–33.6%) but in only 8.2% of control knees (95% CI 4.6–11.8%).

Compared with control knees, case knees showed larger increases in BMLs ($P = 0.0003$), even after adjustment for age, sex, race, BMI, baseline quadriceps strength, CES-D score, baseline K/L grade, baseline BML score, effusion score, and change in effusion score (Table 2). There were no knees or compartments within a knee in which the BML score, either at baseline or at followup, was at the highest possible (ceiling) level. Additional adjustments for alignment of the mechanical axis across the knee yielded similar results.

We then assessed whether change in BML score (as well as magnitude of change in score) might be associated with new knee pain (Table 3). The results showed that new knee pain was modestly, but nonsignificantly, associated with a 1-unit increase in the BML score in any compartment of the knee (adjusted odds ratio [OR] 1.5, 95% CI 0.8–3.1). However, new knee pain was strongly and significantly associated with a ≥2-unit increase in BML score (adjusted OR 3.2, 95% CI 1.5–6.8). In fact, among the control knees, only 8.6% had a BML score increase of ≥2, as compared with 27.5% of case knees showing this magnitude of increase in BML score.

Most of these findings were accounted for by an increase in BML score in knees that were already reported to have BMLs at baseline. Nevertheless, among the subset of knees with no BMLs in any compartment of the knee at baseline, new BMLs were more common in case knees (11 [32.4%] of 34 knees, 95% CI 17.3–49.4%) than in control knees (9 [10.8%] of 83 knees, 95% CI 4.2–17.5%).

In analyses focusing on the site of increase in BMLs, we found that increases in BML scores of ≥2 units occurred in all compartments of the knee more often among case knees than among control knees. There was no predilection for these increases to occur in any compartment.

We then examined whether the increase in BML score involved single large lesions or multiple small lesions. The results showed that more than one-half of the BML increases were a reflection of the development of single large lesions, in which the BML score would change from a score of 0 (absence of lesions) to scores of 2 or 3 or from a score of 1 to a score of 3.

### Table 1. Characteristics of subjects with knee osteoarthritis (OA) among the case and control groups*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Cases (n = 110 knees; n = 102 subjects)</th>
<th>Controls (n = 220 knees; n = 220 subjects)</th>
<th>$P^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean ± SD years</td>
<td>62.9 ± 8.3</td>
<td>61.2 ± 8.4</td>
<td>0.08</td>
</tr>
<tr>
<td>Female, no. (%)</td>
<td>79 (65.5)</td>
<td>132 (60.0)</td>
<td>0.04</td>
</tr>
<tr>
<td>African American, no. (%)</td>
<td>12 (10.9)</td>
<td>30 (13.6)</td>
<td>0.29</td>
</tr>
<tr>
<td>BMI, mean ± SD kg/m²</td>
<td>29.5 ± 4.6</td>
<td>29.7 ± 4.3</td>
<td>0.60</td>
</tr>
<tr>
<td>CES-D score, mean ± SD</td>
<td>7.3 ± 7.5</td>
<td>6.7 ± 6.6</td>
<td>0.43</td>
</tr>
<tr>
<td>Quadriceps strength, mean ± SD newtons</td>
<td>84.5 ± 39.0</td>
<td>96.0 ± 42.9</td>
<td>0.02</td>
</tr>
<tr>
<td>Knee OA (K/L grade ≥2), no. (%)</td>
<td>33 (30.0)</td>
<td>47 (21.4)</td>
<td>0.08</td>
</tr>
<tr>
<td>Bone marrow lesion score at baseline, mean ± SD‡</td>
<td>2.1 ± 2.3</td>
<td>1.7 ± 2.1</td>
<td>0.07</td>
</tr>
<tr>
<td>No. (%) of knees with effusion score ≥1 at baseline§</td>
<td>40 (36.4)</td>
<td>65 (29.6)</td>
<td>0.21</td>
</tr>
<tr>
<td>No. (%) of knees with change in effusion score from baseline to followup§</td>
<td>27 (24.6)</td>
<td>23 (10.5)</td>
<td>0.0008</td>
</tr>
</tbody>
</table>

* All values are from the baseline examination, except change in effusion score. Control knees were randomly selected from the same source population as case knees but were without pain, aching, or stiffness at baseline. Case knees were those reported to have frequent pain, aching, or stiffness at 15 months' followup. BMI = body mass index; CES-D = Center for Epidemiologic Studies Depression Scale; K/L = Kellgren/Lawrence.

† Calculated by chi-square test or 2-sample t-test, as appropriate.

‡ Calculated using the Whole-Organ Magnetic Resonance Imaging Score (scale 0–3).

§ Calculated from findings on magnetic resonance imaging (scale 0–3). Change was defined as an increase in score.

### Table 2. Association of 1-unit increase in BML score with incident knee pain*

<table>
<thead>
<tr>
<th>OR (95% CI)†</th>
<th>Adjusted OR (95% CI)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>1 (referent)</td>
</tr>
<tr>
<td>Cases</td>
<td>1.74 (1.38–2.18)</td>
</tr>
</tbody>
</table>

* Increase in bone marrow lesion (BML) score was defined as the maximal increase in BML score among the compartments of a knee. OR = odds ratio; 95% CI = 95% confidence interval.

† Adjusted only for Kellgren/Lawrence (K/L) grade of radiographic knee osteoarthritis.

‡ Adjusted for age, sex, race (white versus African American), body mass index, quadriceps strength, Center for Epidemiologic Studies Depression Scale score, K/L grade, baseline BML score, and presence of knee malalignment, all at baseline. Also adjusted for change in effusion score determined on magnetic resonance imaging.

§ $P = 0.0003$ versus controls.
DISCUSSION

In the present study, which we believe is the first to examine the relationship between BMLs on MRI and presence of new knee pain in subjects over time, we found that an increase in the size of BMLs in a knee compartment was associated with the development of knee pain. The relationship persisted after statistical adjustment for a large number of factors that have been linked to knee pain, including effusion score (20). Our observations provide strong evidence that BMLs are one source of pain in knees with or without radiographic OA.

Although our data suggest a consistent and moderately strong association of both the development of BMLs (new lesions) and enlargement of existing BMLs with the development of frequent knee pain, the findings do not identify BMLs as being the sole source of knee pain. For example, of those subjects in the incident knee pain group, only 49.1% had an increase in BML score, compared with 26.8% of control knees, suggesting that such an increase in BMLs is frequent and, in some knees, may not be associated with pain. Thus, although we provided additional evidence of the importance of BMLs as a likely source of knee pain, our results suggest that they are not the sole source, and many subjects may develop knee pain without any increase in BMLs in their knee and must therefore develop pain for other reasons. One of these reasons might be inflammation within the joint or enlargement of effusions.

To meet criteria for incident knee pain, subjects must have reported consistent knee pain at followup and no knee pain at baseline. However, those subjects who noted no frequent knee pain at baseline often reported some knee pain on the baseline WOMAC questionnaire. Thus, incident knee pain in such subjects would represent an increase in the frequency and severity of the knee pain and not necessarily completely new knee pain.

There were important limitations to this study. First, we were unable to tell whether BMLs ought to be defined by the largest lesions or the sum of all lesions within a knee. The strong relationship between knee pain and an increase of least 2 units in the BML score suggests that the size of the lesions is strongly related to knee pain. Moreover, since most subjects who developed incident pain did not have WOMAC knee pain scores of 0 at baseline, their “incident” knee pain actually represents the development of more frequent pain. We cannot say that incident knee pain was truly incident in our subjects. Many of our subjects could have had knee pain before the baseline examination and could have experienced fluctuating pain thereafter. We can conclude, however, that BMLs are likely to appear or get larger when pain occurs.

A similar relationship between changing BMLs and pain has been reported in an observational manner in subjects with transient bone marrow edema syndrome, a phenomenon sometimes seen in diabetics, in which the resolution of BMLs is associated with the resolution of pain (21). Transient BMLs also occur in subjects with acute knee trauma (22,23). Another limitation of our study is that we did not study the resolution of pain, but rather its increase. We have previously reported that BMLs are unlikely to disappear in knees with OA, but rather, BMLs often tend to either increase or appear over time (24,25), and this has been confirmed by other investigators (26). We therefore did not believe that it would be productive to focus on the resolution of pain and its association with the resolution of BMLs, since little such resolution occurs.

Observational studies investigating pain in OA will continue to be constrained by the cooccurrence of multiple pathologic features, each of which may be associated with pain. The OA knee is a joint that is

<table>
<thead>
<tr>
<th>Table 3. Incident knee pain in relation to incremental change in bone marrow lesion (BML) score over 15 months’ followup*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control knees, no. (%)</td>
</tr>
<tr>
<td>No increase in BML score</td>
</tr>
<tr>
<td>Increase of 1 in BML score</td>
</tr>
<tr>
<td>Increase of ≥2 in BML score</td>
</tr>
</tbody>
</table>

* OR = odds ratio; 95% CI = 95% confidence interval.
† One case had missing information on bone marrow change because we had difficulty assessing change given the lack of comparable images at baseline and followup.
‡ Adjusted only for Kellgren/Lawrence (K/L) grade of radiographic knee osteoarthritis.
§ Adjusted for age, sex, race (white versus African American), body mass index, quadriceps strength, Center for Epidemiologic Studies Depression Scale score, K/L grade, presence of knee malalignment, and BML score, all at baseline. Also adjusted for change in effusion score on magnetic resonance imaging.
¶ P for trend = 0.002.
failing, with pathologic features in many structures, including the bone, ligaments, and joint capsule, as well as inflammation in the synovium. The pathologic features in these structures are not independent, but rather reflect the whole disease process, and therefore it will continue to be difficult to distinguish the contribution of different structures to pain. One approach is to assess the disease earlier in its process in subjects who have little structural disease. Many of our subjects had minimal, if any, radiographic disease and had no BMLs at baseline. In this subgroup, we similarly found a strong relationship of the development of new bone lesions with new knee pain. We anticipate that future studies will focus on earlier stages of the disease, at which point it might be easier to identify specific structural pathologic features and their relationship to symptoms.

In conclusion, in this study evaluating the development or enlargement of BMLs in individuals and the association of change in BMLs with knee pain, we found that subjects without frequent knee pain who developed knee pain in followup were more likely to experience an increase in BMLs on MRI than were a control group of subjects with similar knee pain decline and with similar baseline MRI characteristics in the knee. This provides further evidence that bone marrow lesions are likely to develop or enlarge in individuals and the knee pain in followup are more likely to experience an increase in BMLs on MRI than were a control group of subjects drawn from the same cohort. This provides further evidence that bone marrow lesions are likely to be a source of the knee pain in some subjects.

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AUTHOR CONTRIBUTIONS

Dr. Felson had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Felson, Torner, Lewis, Nevitt.

Acquisition of data. Felson, Guermazi, Roemer, Aliabadi, Clancy, Torner, Lewis.

Analysis and interpretation of data. Felson, Niu, Guermazi, Roemer, Torner.


Statistical analysis. Niu.

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Varus Foot Alignment and Hip Conditions in Older Adults

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Objective. Mechanical strain on the hip can result from varus malalignment of the foot. This study was undertaken to explore the cross-sectional relationship between varus foot alignment and hip conditions in a population of older adults.

Methods. The Framingham Osteoarthritis Study cohort consists of a population-based sample of older adults. Within this sample, we measured forefoot and rearfoot frontal plane alignment using photographs of a non–weight-bearing view of both feet of 385 men and women (mean age 63.1 years). Each foot segment was categorized according to the distribution of forefoot and rearfoot alignment among cases of ipsilateral hip pain, trochanter tenderness, hip pain or tenderness, and total hip replacement (THR). The relationship of foot alignment to these conditions was examined using logistic regression and generalized estimating equations, adjusting for age, body mass index, sex, and physical activity.

Results. The mean ± SD rearfoot varus alignment was 0.7 ± 5.5 degrees, and the mean ± SD forefoot varus alignment was 9.9 ± 9.9 degrees. Subjects in the highest category of forefoot varus alignment had 1.8 times the odds of having ipsilateral hip pain (P for trend = 0.06), 1.9 times the odds of having hip pain or tenderness (P for trend < 0.01), and 5.1 times the odds of having undergone THR (P for trend = 0.04) compared with those in the lowest category. No significant associations were found between rearfoot varus alignment and any hip conditions.

Conclusion. Forefoot varus malalignment may be associated with ipsilateral hip pain or tenderness and THR in older adults. These findings have implications for treatment, since this risk factor is potentially modifiable with foot orthoses.

Approximately 14% of adults >60 years of age report having hip pain on most days (1). Despite this prevalence, we know little about the etiology of most hip conditions. Medical management remains largely dependent on palliative drugs and, in the case of radiographic findings, total hip replacement (THR). In spite of costly treatment, some pain or tenderness often persists. In the effort to develop safer, cheaper, and more effective treatment options, it is essential that we deepen our appreciation of etiologic pathways and draw attention to modifiable risk factors.

One potentially important source of repetitive stress on the hip is varus foot malalignment. Forefoot and rearfoot varus malalignments are frontal plane malalignments that alter the foot's orientation to the ground. Excessive forefoot or rearfoot varus alignment can disrupt the closed chain functioning of the lower limb and strain proximal tissues. Excessive varus alignment of the forefoot has been associated with overuse injuries of the foot (2), shank (3), and knee (4). Biomechanical models (5,6) anticipate that varus malalignment may present even greater risk for overload of the hip. However, no previous studies have evaluated the relationship of forefoot or rearfoot varus alignment to hip conditions. Moreover, all previous studies of foot alignment and proximal pain (2–4) have sampled younger adults, in whom the risk of hip pain is minimal. The purpose of this study was to assess the relationship of...
forefoot and rearfoot varus alignment to hip pain, trochanter tenderness, and THR in a population of older adults.

SUBJECTS AND METHODS

Study sample. The Framingham Osteoarthritis (OA) Study cohort consists of a population-based sample of older adult residents of Framingham, Massachusetts. Members of the cohort originate from 2 groups. The first is the Heart Study Offspring group, which consists of surviving descendants of the original Framingham Heart Study cohort. The second group was selected from the Framingham community using random-digit dialing and census tract data. To be included, subjects had to be at least 50 years old and ambulatory. Subjects with rheumatoid arthritis were excluded. From this combined OA Study cohort, we consecutively sampled all clinic attendees between May 2004 and June 2005.

Assessment of foot alignment. Forefoot and rearfoot alignment was measured using a digital photograph taken while the subject lay in the prone position. Positioning of the legs and ankles followed a strict protocol and was performed by a single trained examiner who was blinded with regard to the study question. The medial malleoli were aligned with the inferior edge of an examination table and the legs were fixed in neutral rotation so that the posterior aspect of the calcaneus was uppermost. A maximum allowable distance of 16.5 cm between the sagittal midline of the body and each lateral malleolus was marked on the table. The examiner brought both ankles to neutral dorsiflexion (0 degrees) using gentle thumb pressure over the third metatarsal head. The subject maintained both feet in this position while a single digital photograph, using a PowerShot camera (Canon, Lake Success, NY), was obtained from above. The camera remained fixed to the wall throughout the study in order to ensure consistent positioning.

Rearfoot alignment was defined by the angle between a vertical bisection of the calcaneus and a horizontal reference line drawn across the inferior edge of the examination table (Figure 1). Forefoot alignment was defined by the angle between the same horizontal reference line and a line connecting the first and fifth metatarsal heads (7) (Figure 1). A single investigator (KDG), who was blinded with regard to outcome status, used Canvas software, version 9.0.3 (ACD Systems, Vancouver, British Columbia, Canada) to measure the alignment of each foot segment to the nearest 0.1 degree. Measurements of varus alignment were recorded as positive values, while measurements of valgus alignment were assigned negative values.

A comparison of measurements taken before and after subject repositioning revealed a test–retest reliability (intraclass correlation coefficient [ICC]) of 0.88 for the forefoot and 0.81 for the rearfoot. Repeated assessment of the photographs 6–12 months later showed that the ICC for intrarater reliability was 0.91 for the forefoot and 0.87 for the rearfoot. Comparison with measurements obtained by an outside rater showed that the ICC for interrater reliability was 0.93 for the forefoot and 0.85 for the rearfoot.

Identification of hip conditions. To identify hip pain, subjects indicated whether they felt “pain, aching, or stiffness on most days” by placing a written mark over the anterior hip on a labeled body chart. To identify trochanter tenderness, the examiner calibrated 3 pounds of palpatory pressure with a dolorimeter prior to examining each hip while the subject lay on his or her side. Training ensured that the examiner’s performance matched that of an experienced rheumatologist (DTF). Digital pressure was applied to the bony prominence of the greater trochanter and to the soft tissues immediately posterior and superior to the greater trochanter. Tenderness in any of these neighboring areas indicated a positive response (8). A case of hip pain or tenderness was identified by a positive response to either of the above. Finally, subjects responded “yes” or “no” to the examiner’s inquiry about whether or not they had undergone a THR. If they answered “yes,” then they were asked to indicate which hip had been replaced.

Covariates. Age, sex, and body mass index (BMI) were assessed in all subjects. BMI was calculated as weight (kg) divided by height (m²). Height was measured to the nearest 0.25 inch using a stadiometer. Weight was measured to the nearest 0.25 pound using a balance scale.

Each subject completed a Physical Activity Scale for the Elderly (PASE) questionnaire (9). The derived score was a weighted sum of 12 categories of physical activity related to daily living, recreation, and work. Activities performed while seated were not counted. In adults 67–80 years of age, PASE scores correlated with average daily measurements obtained using an accelerometer (10). The ICC for test–retest reliability was 0.75 (9).

Statistical analysis. Five categories of forefoot or rearfoot varus alignment were created using the quintile distribution among hip pain cases to define category cut points. We examined the relationship of forefoot or rearfoot varus alignment to the prevalence of ipsilateral hip pain in each category using a logistic regression model that adjusted for age, sex, BMI, and PASE score. The same approach was used to examine the relationship of forefoot or rearfoot varus alignment to trochanter tenderness and to hip pain or tenderness. When assessing the relationship of forefoot or rearfoot varus alignment to THR, only 3 categories were created, using the tertile distribution among THR cases. We did this because the number of THR cases was small (n = 11). In all analyses, generalized estimating equations were used to adjust for the correlation between 2 hips in the same subject. The lowest category served as the reference group for calculation of an odds ratio (OR).

RESULTS

Four hundred ten subjects in the Framingham OA Study were consecutively sampled. Twenty-five were
excluded because of an inability to lie in a prone position. Clinical and demographic characteristics of the subjects are presented in Table 1. Among 385 eligible adults, 54.6% were women and 94.8% were white. Compared with all other members of the Framingham OA Study cohort, subjects in this study were slightly younger (mean ± SD age 63.1 ± 8.0 years versus 65.8 ± 9.1 years) and had a greater prevalence of hip pain (16.0% versus 11.6% of hips). The mean PASE score of the cohort (132 ± 7.25) did not differ significantly from that of the parent cohort (P = 0.75) or from the reported mean PASE scores of a sample of adults of similar age (11). In this study, trochanter tenderness was noted in 17.3% of hips, and either pain or tenderness was noted in 24.8%. THR had been performed in 1.4%. The mean forefoot varus alignment was 9.9 ± 9.9 degrees, while the mean rearfoot varus alignment was 0.7 ± 5.5 degrees.

Table 1. Characteristics of the study subjects*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean ± SD years</td>
<td>63.1 ± 8.0</td>
</tr>
<tr>
<td>BMI, mean ± SD</td>
<td>28.2 ± 5.1</td>
</tr>
<tr>
<td>Sex, % female</td>
<td>54.6</td>
</tr>
<tr>
<td>Race, % white</td>
<td>94.8</td>
</tr>
<tr>
<td>PASE, mean ± SD score</td>
<td>132 ± 72.5</td>
</tr>
<tr>
<td>Forefoot varus angle, mean ± SD degrees</td>
<td>9.9 ± 9.9</td>
</tr>
<tr>
<td>Rearfoot varus angle, mean ± SD degrees</td>
<td>0.7 ± 5.5</td>
</tr>
<tr>
<td>Hip findings, no. of hips/no. studied (%)</td>
<td></td>
</tr>
<tr>
<td>Pain</td>
<td>120/750 (16.0)</td>
</tr>
<tr>
<td>Trochanter tenderness</td>
<td>112/649 (17.3)</td>
</tr>
<tr>
<td>Pain or tenderness</td>
<td>191/770 (24.8)</td>
</tr>
<tr>
<td>THR</td>
<td>11/770 (1.4)</td>
</tr>
</tbody>
</table>

* BMI = body mass index; PASE = Physical Activity Scale for the Elderly; THR = total hip replacement.

Table 2. Hip conditions, by quintile of forefoot and rearfoot varus alignment*

<table>
<thead>
<tr>
<th>Quintile</th>
<th>Hip pain</th>
<th>Trochanter tenderness</th>
<th>Hip pain or tenderness</th>
<th>THR†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<tr>
<td>1</td>
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<td>196</td>
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<td>151</td>
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<td>180</td>
<td>134</td>
</tr>
<tr>
<td>5</td>
<td>90</td>
<td>96</td>
<td>150</td>
<td>133</td>
</tr>
</tbody>
</table>

Adjusted OR (95% CI)

<table>
<thead>
<tr>
<th>Quintile</th>
<th>Hip pain</th>
<th>Trochanter tenderness</th>
<th>Hip pain or tenderness</th>
<th>THR†</th>
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<td>1.00</td>
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<tr>
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<td>0.91</td>
<td>0.91</td>
<td>1.00</td>
</tr>
<tr>
<td>3</td>
<td>0.96</td>
<td>0.96</td>
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<tr>
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<td>1.83</td>
<td>1.83</td>
<td>1.83</td>
</tr>
</tbody>
</table>

* OR = odds ratio; 95% CI = 95% confidence interval; THR = total hip replacement.
† Tertile distribution of THR cases was used because of the small number of cases.
The relationship of forefoot and rearfoot varus alignment to hip pain is shown in Table 2. Generally, with increasing forefoot varus alignment, there was increasing prevalence of hip pain (P for trend = 0.06). In the highest quintile of forefoot varus alignment, more than one-fourth of the subjects (25.6%) had ipsilateral hip pain on most days. The odds of hip pain in this quintile (≥22 degrees of varus alignment) were 1.8 (95% confidence interval [95% CI] 0.9–3.6) times those in the lowest quintile (<3 degrees varus alignment). No association was discernible in the relationship of rearfoot varus alignment to hip pain (P for trend = 0.27).

A linear trend was suggested in the relationship of forefoot varus alignment to ipsilateral trochanter tenderness, but this association failed to reach statistical significance (P for trend = 0.09). No relationship was found between trochanter tenderness and rearfoot varus alignment (P for trend = 0.20) (Table 2).

Table 2 also shows the relationship of forefoot and rearfoot varus alignment to hip pain or tenderness. Among 762 hips, we found a highly significant association (P for trend < 0.01) between increasing forefoot varus alignment and the prevalence of ipsilateral hip symptoms. Among subjects in the highest quintile of forefoot varus alignment, 36.9% of hips were either painful on most days or tender to palpation yielding an OR of 1.9 (95% CI 1.1–3.3) compared with the lowest quintile. There was no association between rearfoot varus alignment and hip pain or tenderness (P for trend = 0.12).

Despite the small number of cases (11 hips), we found a significant association between increasing forefoot varus alignment and the prevalence of THR (P for trend = 0.04) (Table 2). In the highest tertile of forefoot varus alignment, the OR for THR was 5.1 (95% CI 1.0–26.5). The relationship of rearfoot varus alignment to THR was less evident (P for trend = 0.05).

**DISCUSSION**

These findings suggest that forefoot varus alignment is associated with hip pain or tenderness and THR in the population of older adults. No associations were found between rearfoot varus alignment and any hip conditions. These data therefore support the assertion that varus malalignment of the forefoot presents a more powerful risk for hip conditions than comparable varus malalignment of the rearfoot.

Because of its frequent presence in overuse injuries, clinical commentators have referred to forefoot varus malalignment as “the destructive foot” (6). Its role as a risk factor for patellofemoral conditions (4) is consistent with the predictions of biomechanical models (5,6). However, prior to this study, the association of forefoot varus malalignment with hip conditions had remained speculative.

When the foot initially contacts the ground during routine walking, it does so with the ankle in neutral dorsiflexion. Muscles control the forefoot’s descent. With impact, a ground reaction force is imparted that results in an external moment to drive the foot into pronation (Figure 2) and the lower limb into internal rotation (5). This obligatory coupling of internal rotation with foot pronation (Figure 3) loads the hip by pulling taut the powerful lateral rotator muscles of the

![Figure 2](image1.png)

**Figure 2.** Effect of ground reaction force on foot pronation and rotation. Ground reaction force is applied at the center of ground contact. In a normally aligned foot, the ground reaction force generates a small moment for pronation (left). As ground contact is shifted laterally in varus foot malalignment, there is an increase in the moment for pronation (right).

![Figure 3](image2.png)

**Figure 3.** Coupling of pronation to internal rotation. This action results in the transfer of stress from the foot to the hip.
greater trochanter and drawing the femoral head more deeply into the acetabulum.

Evidence (12) suggests that a foot aligned in varus will, as expected, strike the ground further laterally on its plantar surface (Figure 2). A lateral shift in the ground reaction force implies an exaggerated moment to drive both foot pronation and limb rotation (5). The findings of Lafortune and colleagues (13) confirm that internal rotation increases when walking with the feet in excessive varus alignment with the ground. Where malalignment extends into the forefoot, the mechanical incentives for pronation and internal rotation are not only amplified, but also persist. As the knee locks in extension during midstance, the hip is obliged to absorb all continued demands for rotation (13). It is an obligation that renders the hip vulnerable to eventual overload.

As a cross-sectional study, this investigation cannot confirm that forefoot varus malalignment truly precedes the onset of hip conditions. Confidence comes from knowing that forefoot varus malalignment is frequently observed in young adults as well as in older adults (2). Varus foot malalignment is believed to originate in the failure of the talar neck to fully derotate from its fetal position, a process that is completed during childhood.

In skeletally mature adults, screening for persistent forefoot varus malalignment is carried out with subjects in a prone position (14). Additional examination when the subjects are standing and walking is required to determine how forefoot varus malalignment interacts with other anatomic, environmental, and task constraints to alter the posture and motion of the limb during weightbearing. Foot and ankle flexibility, joint axis orientation, shoes, walking surface, tibiofemoral and coxofemoral joint alignment, and history of injury or dysplasia are among many relevant factors that were not assessed during this study’s brief screening examination. To the extent that these factors modify the relationship of foot alignment to hip conditions, they should be kept in mind when evaluating this study’s conclusions and their relevance to individual patients. Nevertheless, screening for foot malalignment with subjects in a prone position is a salient component of the clinical examination for several lower extremity conditions (6,14). Forefoot varus malalignment is not observable when the patient is standing.

While screening for foot malalignment continues among clinicians, disagreement has arisen among researchers as to exactly how the foot should be prepositioned for measurement. Various methods attempt to place the rearfoot in a theoretical “subtalar joint neutral” position. Unfortunately, these methods are known to have poor reliability (15). In addition, since the subtalar joint neutral position is not achieved while walking, the functional relevance of the posture remains unclear. By simplifying our protocol to avoid subtalar joint neutral prepositioning, we were able to obtain clinically reproducible and functionally relevant measurements with high intrarater, interrater, and test–retest reliability. As suggested by Chen et al (7), the presentation of measured malalignment relative to a horizontal facilitates recognition of the likely functional consequences as well as the needed features of an accommodating orthosis. Caution is indicated when comparing the results of studies in which measurement procedures differ.

A final comment pertains to the condition defined by the presence of either hip pain or trochanter tenderness. While the inclusion of this composite end point increased the study’s power to detect a true association, clinical interpretation of this finding may be problematic. Self-reported anterior hip pain and confirmed trochanter tenderness often arise from distinct diagnoses. Although linear trends were suggested in the relationship of forefoot varus alignment to each separate finding, the association was stronger for hip pain ($P$ for trend $= 0.06)$ than for trochanter tenderness ($P$ for trend $= 0.09$). One explanation is that forefoot varus malalignment is more closely associated with diagnoses such as osteoarthritis, which commonly produce anterior hip pain, and is less closely associated with diagnoses such as trochanteric bursitis, which commonly produce tenderness. We made no attempt in this study to distinguish these diagnoses or to rule out the possibility of pain referral from extrinsic sources.

We encourage further studies to clarify the association of forefoot varus malalignment with distinct diagnoses at the hip and to evaluate the response of these diagnoses to orthotic intervention.

**AUTHOR CONTRIBUTIONS**

Dr. Gross had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

**Study design.** Gross, Zhang, Holt, Hunter.

**Acquisition of data.** Gross, Felson, McLennan, Hunter.

**Analysis and interpretation of data.** Gross, Niu, Zhang, Felson, Hannan, Holt, Hunter.

**Manuscript preparation.** Gross, Hannan, Holt, Hunter.

**Statistical analysis.** Gross, Niu, Zhang.
REFERENCES

Inverse Relationship Between Matrix Remodeling and Lipid Metabolism During Osteoarthritis Progression in the STR/Ort Mouse

James W. Watters, Chun Cheng, Maureen Pickarski, Gregg A. Wesolowski, Ya Zhuo, Tadashi Hayami, Wei Wang, John Szumiloski, Robert L. Phillips, and Le T. Duong

Objective. The biologic changes associated with osteoarthritis (OA) are incompletely understood. The aim of this study was to elucidate the molecular mechanisms underlying OA progression in an STR/Ort murine model of spontaneous disease.

Methods. Global patterns of gene expression were assessed using microarray analysis of articular cartilage/subchondral bone from the tibial plateaus of STR/Ort mice at 3, 9, and 12 months of age. The age-dependent severity of osteophyte formation and extent of cartilage damage were determined in the corresponding femurs using microfocal computed tomography and the Mankin histologic scoring system. Pathway analysis was used to identify the functions of genes associated with OA progression, and changes in gene expression were confirmed using immunohistochemistry.

Results. Six hundred twenty-one genes were associated with both osteophyte formation and cartilage damage in the STR/Ort joints. Genes involved in the development/function of connective tissue and in lipid metabolism were most significantly enriched and regulated during disease progression. Genes directly interacting with peroxisome proliferator-activated receptor α (PPARα)/PPARγ were down-regulated, whereas those genes involved with connective tissue remodeling were up-regulated during disease progression. Associations of down-regulation of myotubularin-related phosphatase 1 (a phosphoinositide 3-phosphatase involved in lipid signaling) and up-regulation of biglycan (a member of the small leucine-rich protein family known to modulate osteoblast differentiation and matrix mineralization) with OA progression were confirmed by immunohistochemistry.

Conclusion. Since adipogenesis and osteogenesis are inversely related in the developing skeletal tissue, these results suggest that a shift in the differentiation of mesenchymal cells from adipogenesis toward osteogenesis is a component of the OA pathophysiologic processes occurring in the tibial plateau joints of STR/Ort mice.

Osteoarthritis (OA), a disease associated with reduced synovial joint function and increased pain, is a major cause of disability in humans (1). There are no consistently effective methods for preventing OA or slowing its progression, and symptomatic treatments provide limited benefit for many patients. Gross changes in the structure and content of articular cartilage, subchondral bone, synovial membrane, joint ligaments, and tendons have been described for many years in patients with OA (2). However, the molecular changes associated with OA in these tissues have only recently begun to be elucidated (for review, see refs. 3–8).

Important features of OA include the degradation of articular cartilage and remodeling of subchondral bone (9). Cartilage damage is thought to be mediated through excess synthesis and release of catabolic factors including proinflammatory cytokines, matrix metalloproteinases (MMPs), and nitric oxide, as well as a reduced synthesis of anabolic factors such as insulin-like growth factor 1 (IGF-1) (10,11). However, other studies
metabolism were shown to be the biologic functions that development and function of the connective tissue and lipid wide expression profiling and functional analysis, devel-
oped at age 1 year (25). Using genome-

PPAR also regulate inflammatory responses. For example, PPAR has been shown to be a negative regulator of macrophage activation (21). Moreover, PPAR activation inhibits the production of interleukin-1β (IL-1β), tumor necrosis factor α (TNFα), and IL-6 in monocytes (22). In addition, PPAR agonists can inhibit the production of MMP-13 in human chondrocytes and MMP-1 in human synovial fibroblasts (23,24).

It has therefore been suggested that PPAR agonists exert their therapeutic effects on surgically induced or collagen-induced arthritis through the suppression of these inflammatory mediators (17,18). However, these animal models involve an external mechanism of inflammation in the development of OA, and therefore the disease in these models may not be representative of generalized OA in humans. As such, the current level of understanding has not yielded a satisfactory hypothesis as to how spontaneous OA is initiated or what major signaling mechanisms are involved in the progression of spontaneous, idiopathic OA.

In order to identify the molecular mechanisms underlying the progression of spontaneous OA, we analyzed the changes in gene expression that occur in affected joints during OA progression in the STR/Ort mouse, a strain derived from the common inbred strain STR/1N. STR/Ort animals spontaneously develop histologic lesions resembling those of human OA, with ~85% of male STR/Ort mice developing the disease in the medial tibial plateau at age 1 year (25). Using genome-wide expression profiling and functional analysis, development and function of the connective tissue and lipid metabolism were shown to be the biologic functions that are most significantly up-regulated and down-regulated, respectively, during OA progression. Furthermore, genes regulated by PPARα and PPARγ were down-regulated in a coordinated manner during disease progression. These results suggest that PPAR signaling is down-regulated during the progression of OA in STR/Ort animals, and that a shift away from adipocyte formation and toward osteoblast differentiation in mesenchymal precursor cells is an important component in this spontaneous, idiopathic model of OA in mice.

MATERIALS AND METHODS

Collection of joint samples and tissue preparation. A colony of STR/Ort mice was established from 3 original breeding pairs obtained from Dr. R. M. Mason (Imperial College of Medicine, London, UK) (25). The joints from both hind limbs were collected from 3 groups of male STR/Ort mice at 3, 9, and 12 months of age. After careful disarticulation, the femur and corresponding tibia from each joint were collected for histology and RNA preparation, respectively. The femur was fixed in 4% paraformaldehyde (Fisher Scientific, Fair Lawn, NJ) in phosphate buffered saline (PBS). The corresponding tibia was carefully cleaned of attached ligaments and muscle.

Tibial articular cartilage and subchondral bone were microdissected together by collecting a 200-μm-thick section measured from the cartilage surface of the tibial plateau. From each group of mice, the cartilage/bone sections from the left and the right tibia were quickly frozen and stored in liquid nitrogen until processing for RNA. In addition, the dorsal segments of the ribs, from approximately equal lengths (2 mm) of cartilage and bone, were collected from each mouse. The rib tissues were pooled for RNA preparation.

Mankin histologic scores of cartilage changes. Semi-quantitative histopathologic grading was performed as described previously (9), according to a modified Mankin scoring system established for grading OA changes (26–28). The 5 subcategories of the Mankin score evaluated were structure, chondrocyte number, chondrocyte clustering, proteoglycan content, and subchondral plate and/or tidemark changes. After being scanned by microfocal computed tomography (micro-CT), the femurs were decalcified in 0.5M EDTA, pH 7.6, and embedded in paraffin in the same orientation.

OA develops focally in STR/Ort mice, and it is therefore very difficult to compare the histopathologic characteristics of OA cartilage between mouse joints. This limitation is frequently attributable to the technical inability to collect histologic sections at the same depth of tissue in the joints. We therefore collected 20 sections, each of 5 μm in thickness and 50 μm apart, from each femur. Three toluidine blue–stained sections were carefully selected from each sample on the basis of whether they were of comparable orientation and tissue depth. After microscopic evaluation of each section of cartilage, the modified Mankin score was assessed, as previously described (29). Briefly, scores were assessed for the severity of cartilage surface structural damage (scores 0–10), changes in cellularity (scores 0–4), cell clustering (scores 0–4), pericellu-
lar staining (scores 0–4), and matrix proteoglycan staining (scores 0–4), and the maximum score was 26 (28).

**Immunohistochemistry.** Tissue sections were deparaffinized in xylene, hydrated in graded ethanol, and then treated with 500 units/ml testicular hyaluronidase (Sigma, St. Louis, MO) at 37°C for 15 minutes. Endogenous biotin and biotin binding activity were blocked with an avidin–biotin blocking kit (Zymed, Burlingame, CA) followed by serum blocking. Tissue sections were then incubated with either anti-human biglycan antibodies (R&D Systems, Minneapolis, MN) or anti-human myotubularin-related phosphatase 1 (MTMR-1) antibodies (Abgent, Bioggio-Lugano, Switzerland) for 30 minutes, followed by streptavidin–horseradish peroxidase conjugate (Zymed) for 10 minutes. These sections were again rinsed with PBS, developed using the aminoethylcarbazole chromogen of the Histostain SP kit (Zymed), and counterstained with hematoxylin. For immunostaining to detect biglycan, sections were rinsed in PBS with 0.3% Tween 20 and then incubated with biotin-conjugated anti-rabbit antibodies (Vector, Burlingame, CA) for 30 minutes, followed by streptavidin–horseradish peroxidase conjugate (Zymed) for 10 minutes. Sections were rinsed and developed to a brown color with 500 units/ml testicular hyaluronidase (Sigma, St. Louis, MO) at 37°C for 15 minutes. Endogenous biotin and biotin binding activity were blocked with an avidin–biotin blocking kit for quality control purposes. All probes on the microarrays were synthesized in situ with inkjet technology (Agilent Technologies, Palo Alto, CA) (30). After hybridization, arrays were scanned, and fluorescence intensities for each probe were recorded. Ratios of transcript abundance (experimental to control) were obtained following normalization and correction of the array intensity data. Gene expression data were analyzed using Rosetta Resolver gene expression analysis software (version 5.1; Rosetta Biosoftware, Seattle, WA).

**Identification of genes associated with disease phenotypes.** In order to identify genes associated with the osteophyte score, joints were placed into 2 groups (group 1 comprising samples with osteophyte scores ≤1; group 2 comprising samples with osteophyte scores ≥4), and an analysis of variance (ANOVA) calculation was performed to identify probes differentially expressed between groups. Correlation analysis was used to identify genes associated with the Mankin score. An estimated false discovery rate (FDR) of 5%, based on 500 permutations of the data, was used to determine the thresholds for significant values in the ANOVA and correlation analyses. The significance of the size of overlap between genes associated with each disease phenotype was calculated using the hypergeometric distribution.

**Gene function and network analysis.** Genes identified as being positively or negatively associated with the Mankin score and the osteophyte score were used for network and gene function analyses. These genes comprised the seed set. Locus identification numbers were imported into the Ingenuity Pathway Analysis (IPA) system (Ingenuity Systems, Mountain View, CA), and genes were then categorized based on the published findings regarding biochemical, biologic, or molecular functions. Calculations of the P value for enrichment of gene functions were based on the hypergeometric distribution.

The identified genes were also mapped to interaction networks as described previously (32). Briefly, the construction of interaction networks involves 1) overlay of genes identified as significant from the experimental data onto the IPA interactome, 2) determination of the specificity of connections between genes by calculating the percentage of each gene’s connections to other significant genes (networks are grown from genes with the highest specificity connections), and 3) assessment of the significance of the identified networks by determining the probability that a collection of genes with a sample size equal to or greater than the number in the network could be achieved by chance alone. The resulting networks are ranked by score, with a score of 3 indicating that there is a lower than 1 in 1,000 chance that the focus genes are in a network due to random chance. Networks with a ranking score >3 were considered significant.

**RESULTS**

**Gross morphologic and histologic features of individual joints.** Male STR/Ort mice were killed for morphology and histology. OA-like pathologic features were surveyed by histologic evaluation of the individual joints from mice at 3, 9, and 12 months of age (n = 15–18 samples per group). Typically, the normal femoral con-
dyles (Figure 1A, panel a) and tibial plateaus (results not shown) of the joints of 3-month-old STR/Ort mice had intact cartilage integrity (Figure 1A, panel a, inset), occasionally accompanied by limited penetration of calcified tissue from the subchondral surface (Figure 1A, panel a, arrows). In contrast, OA-associated lesions could be readily observed in joints from mice as early as 3 months of age (Figure 1A, panel b, inset), including a loss of cartilage cellularity and proteoglycan staining, increase in focal surface damage, bone growth into the cartilage, and growth of small osteophytes (Figure 1A, panel b, arrows). As the animals aged, surface erosion became more severe, accompanied by increases in proteoglycan loss, osteophyte formation, and subchondral bone sclerosis (Figure 1A, panel c). By 12 months of age, complete loss of cartilage and bone eburnation could be observed in the animals (Figure 1A, panel c).

At each time point, joint damage was assessed by measuring the presence and severity of osteophytes and extent of cartilage damage. Micro-CT scans were performed on individual joints, and presence/severity of osteophytes was scored by 2 independent observers. A grading system was developed whereby joints were assigned a score of 0–5 depending on the number and size of the observed osteophytes. Figure 1B shows micro-CT images of the coronal and axial planes of a typical normal femur (Figure 1B, panels a and b) compared with those from an age-matched STR/Ort mouse (Figure 1B, panels c and d). The osteophyte score (range 0–5) was assigned based on the overall size and extent of the osteophytes as assessed on all axial plane images for each femur. Asterisks in d indicate subchondral sclerosis.

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Interestingly, disease progression was not observed in all of the joints assessed. No osteophytes were observed in 4 of 18 joints from 3-month-old animals, 2 of 18 joints from 9-month-old animals, and 1 of 15 joints from 12-month-old animals (Figure 2A). This observation is consistent with previous reports of incomplete disease penetration at 1 year of age (25). Whereas 4 of the 18 joints from 3-month-old animals showed absence of osteophyte development, all 18 joints from the 3-month-old animals showed Mankin scores ≥1 (Figure 2B), indicating that mild cartilage damage precedes osteophyte development in the STR/Ort murine disease model. This is consistent with the observations in the rat ACL transection model of OA, in which cartilage damage has also been shown to precede the development of osteophytes (29).

**Joint anabolic and catabolic factors.** Data on gene expression were generated from the same joints used to assess disease phenotypes. Joint tissue, comprising articular cartilage and subchondral bone, was obtained from the mouse tibial plateaus for profiling on Agilent Technologies oligonucleotide-based DNA microarrays. Due to the small size of rodent joints and the inherent difficulties in separating articular cartilage from bone, the tibial plateaus that contained both articular cartilage and subchondral bone tissue were microdissected together in a 200-μm section. The tibial tissue section from each animal was processed for profiling studies.

To assess the catabolic and anabolic processes that occur in STR/Ort joints, we first analyzed the expression of candidate genes involved in biologic functions thought to be important for the initiation or progression of OA. We chose candidate genes that are commonly described in the literature, from 4 general categories: 1) cartilage components (Col2a1, Agc1, Hapln1, and Comp), 2) cartilage catabolism (Adamts5, Il1b, and Il6), 3) osteoclast function (Ctsk and Mmp9), and 4) bone anabolism (Igf1 and Tgfb1). Figure 3 shows the regulation of these genes in the STR/Ort joints by age groups.

The expression levels of cartilage components, cartilage catabolic factors, and genes indicative of osteoclast function were significantly higher in the joints of mice at 3 months of age compared with 12 months of age (P < 0.05). This is again consistent with the observations in the rat ACL transection model of OA, in which subchondral bone resorption and cartilage loss are
Regulation of candidate genes involved in murine osteoarthritis. Genes were assigned to 1 of 4 categories: 1) cartilage components (Col2a1, Age1, Hapln1, and Comp), 2) cartilage catabolism (Adams5, Il1b, and Il6), 3) osteoclast function (Ctsk and Mmp9), and 4) bone anabolism (Igf1 and Tgfb1). Data represent the log_{10} of the ratio of expression in each joint sample relative to the pooled reference sample from mouse rib tissue. Genes that were up-regulated relative to the reference sample are shown in magenta, while genes that were down-regulated relative to the reference sample are shown in cyan. Asterisks denote genes whose average expression in the joints from mice at age 3 months was significantly higher than that in mice at age 12 months (P < 0.05 by analysis of variance).

Functions of genes in relation to disease progression. In order to gain insights into the biologic processes and signaling networks involved in OA progression, we performed analyses of the biologic functions and contributory pathways in the STR/Ort joints, in order to uncover the relationships among genes associated with disease progression in this model. Instead of focusing on individual genes or a group of candidate genes that were previously demonstrated to change with disease progression, we utilized a different approach to allow a more unbiased assessment of the biologic processes underlying disease progression in the STR/Ort model. This approach involved use of the IPA software tool as described in Materials and Methods (see the Ingenuity Systems Web site at http://www.ingenuity.com), which enabled us to identify the gene functions that were statistically significantly enriched among the 621 genes described as being associated with the osteophyte and Mankin histologic scores. Note that others have previously used the IPA tool to identify biologic networks involved in complex processes, including inflammation, glucocorticoid receptor signaling, and cancer (32,34,35).

The general function most significantly enriched among the 331 genes up-regulated during disease progression was the development and function of the connective tissue (P = 9.32 × 10^{-5}–4.55 × 10^{-2}). The specific function most significantly associated with the group of up-regulated genes was patterning of bone (P = 9.32 × 10^{-5}) for the genes Bgn, Cdx1, Enpp1, Ggt1, Nog, Ptn, Ptp4r, Src, and Wnt3a), which is consistent with the development of osteophytes with increasing age in STR/Ort animals (Figure 2A). The general function
most significantly enriched among the group of 290 down-regulated genes was lipid metabolism ($P = 8.08 \times 10^{-7}$–$4.93 \times 10^{-2}$), and modification of fatty acid was the most significantly enriched specific function ($P = 8.08 \times 10^{-5}$ for the genes $Acas2$, $Acox1$, $Amarc$, $Ech1$, $Gpam$, $Hadha$, $Hadhb$, $Phyh$, and $Sle27a2$). These findings suggest that a general down-regulation of genes involved in lipid metabolism may play a key role in OA pathogenesis. The inverse time-dependent regulation of genes identified as being involved in the development and function of the connective tissue and in lipid metabolism is summarized in Figure 4.

**Interaction networks.** In order to further assess the biologic processes associated with lipid metabolism during OA progression, we investigated, with the use of the IPA platform, interaction networks formed by the above-described 290 genes identified as being down-regulated in association with disease progression. This system identifies biologic interaction networks among genes of interest by mining published findings regarding biochemical, biologic, or molecular interactions (see Materials and Methods).

Interestingly, 2 networks that contain PPAR transcription factors as central nodes were observed to be highly significant. As shown in Figure 5A, PPAR$_\alpha$ was identified as a central node of the most significant interaction network identified among the down-regulated genes (significance score of 23). This network includes 3 transcriptional targets of PPAR$_\alpha$ ($Acox1$, $Fgg$, and $Acas2$, as highlighted in Figure 5A). In addition, a second significant interaction network (significance score of 14) contained PPAR$_\gamma$ as a central node (Figure 5B). This network includes 4 transcriptional targets of PPAR$_\gamma$ ($Fasn$, $Vnn1$, $Cyp4b1$, and $Tpm2$, as highlighted in Figure 5B) and 1 gene known to physically interact with PPAR$_\gamma$ ($Smarcd3$). These results suggest that reduction of PPAR signaling is an important mechanism underlying the progression of OA in the STR/Ort mouse. This is supported by the results of previous studies that have suggested a therapeutic role for PPAR$_\gamma$ activation in animal models of surgically induced OA (17).

**Relative protein expression of MTMR-1 and biglycan.** To provide an additional level of validation of these changes in mRNA expression, we performed a qualitative assessment of the protein levels in the STR/Ort joints by immunohistochemistry. We chose to evaluate protein expression of the signature genes for which immunohistochemistry reagents are available for use in a murine system. Among the signature genes that correlated with OA progression in the STR/Ort mice, the protein expression of MTMR-1 and biglycan, as assessed by immunohistochemical methods, was further verified (Figure 6).

MTMR-1, a phosphoinositide 3-phosphatase involved in lipid signaling, was found to be highly expressed in the joints of 3-month-old mice compared with 12-month-old mice (Figures 6A and B, brown stain). In the joints of 3-month-old mice, MTMR-1 appeared to be highly expressed in the hypertrophic chondrocytes and, to a lesser extent, in chondrocytes near the articular surface (results not shown). MTMR-1 expression was not detected in the ligaments and muscles surrounding the joints. The regulation of MTMR-1 protein expression was consistent with our findings of the general down-regulation of genes involved in lipid metabolism occurring in conjunction with OA progression (as shown in Figure 4).

Conversely, biglycan, a small leucine-rich proteoglycan that plays a critical role in the formation of collagen fibrils, was highly expressed in the joints of
12-month-old mice compared with 3-month-old mice (Figures 6D and E). In the joints of 12-month-old mice, we detected high levels of biglycan protein in the hypertrophic cartilage/bone interphase and in cells lining the surfaces of articular cartilage and subchondral trabecular bone. This increase in protein expression of biglycan supports our observations of the up-regulation of genes involved in connective tissue development and function occurring in parallel with OA progression (also shown in Figure 4).

**DISCUSSION**

OA is currently considered to be a complex joint disease in which all tissues in the joints play an important role in disease initiation and/or progression. It has long been suggested that the progression of articular cartilage degeneration is concomitant with intense remodeling of the subchondral bone and increased bone stiffness, leading to abnormal mechanical stress across the overlying cartilage (36,37). Indeed, increased subchondral bone activity in OA patients, as determined by enhanced uptake of scintigraphic technetium-labeled disphosphonate, has been shown to precede detectable cartilage loss (38), and the bone formation marker osteocalcin was...
reported to be higher in synovial fluid from patients with severe scintigraphic signals compared with that from patients with mild alterations on knee scans (39). Unlike patients with osteoporosis, OA patients tend to have a high body mass index together with an elevated rate of bone turnover, resulting in increased bone density (40). This suggests that the process of new bone synthesis exceeds degradation in susceptible individuals.

Studying the pathogenesis of OA in humans is hampered by inherent difficulties such as a lack of availability of normal and diseased tissue at early stages of the disease. Therefore, animal models of OA are essential for understanding disease etiology and for the development of effective therapies. Although numerous animal models have been developed and characterized (for review, see ref. 41), many of these models involve surgical intervention or inflammatory stimuli to induce disease. In models of OA involving surgically induced joint instability in several species, both bone and cartilage changes occur concomitantly (29). In contrast, in animal models in which a spontaneous OA-like disease develops (resembling human disease), increased bone density and osteoid volume are often more severe than cartilage changes. For example, Dunkin-Hartley guinea pigs (42) and cynomolgous macaques (43) have age-related changes in bone that precede those in cartilage. Evidence demonstrating that subchondral bone remodeling is linked to cartilage destruction in both humans and animals is well accepted; however, the mechanisms by which the changes in subchondral bone influence articular cartilage are incompletely understood.

In this study, we sought to elucidate the molecular mechanisms underlying disease progression in the spontaneous STR/Ort mouse model. STR/Ort mice share physical characteristics similar to those in human subjects with OA, such as high body weight and high bone mineral density. In addition, a number of biochemical features of OA in STR/Ort mice are similar to the changes observed in human disease, such as matrix proteoglycan depletion by MMPs and aggrecanase (25). Due to recent progress in mouse genomics, the STR/Ort model provides a unique opportunity to investigate the events associated with the initiation and progression of spontaneous OA.

We used gene expression profiling to understand how biologic pathways change with disease progression in the STR/Ort model. By associating gene expression changes with cartilage damage and micro-CT scores in SRT/Ort joints, we were able to identify the genes that were up- or down-regulated with disease progression. Rather than focusing on individual candidate genes, we analyzed biologic functions in an unbiased manner by leveraging informatics tools to identify ascribed biologic functions that were statistically significantly associated with the disease. In this way, we can build a more pathway-centric view of disease biology that leverages information across many genes and is not reliant on individual genes that are chosen for analysis based on preexisting knowledge of other disease models.

This study is the first to provide evidence that altered lipid metabolism through a reduction in PPAR signaling is an important component of spontaneous, idiopathic OA. While PPARγ and PPARα themselves were not found to be significantly altered at the mRNA level, multiple direct targets of the PPARs were altered. We speculate that there is a non–mRNA-based means of down-regulating the activity of the PPARs, for example, a posttranslational modification or a binding to other coactivators or corepressors that alters the activity of PPAR signaling without actually altering the mRNA for PPARs. The PPARγ agonist rosiglitazone has recently been shown to be therapeutic for surgically induced OA in guinea pigs (17), an effect hypothesized to be mediated through the inhibition of proinflammatory signals in the affected joint.

While no direct link between PPARα and OA has been reported, PPARα has also been shown to have antiinflammatory properties (44,45). Therefore, one possible explanation for the relationship between PPAR signaling and OA progression is that decreased PPAR signaling leads to an elevation of inflammation in the joint microenvironment that favors catabolic signals over anabolic signals, resulting in the observed cartilage damage and osteophyte formation. An alternative explanation involves the developmental link between adipogenesis, chondrogenesis, and osteogenesis. These cells share a common mesenchymal cell precursor that can be induced to differentiate into one of these cell types in vitro by adjustment of the culture microenvironment (46,47). It is thus possible that an early event in the initiation and progression of OA is a preferential shift toward osteoblastogenesis resulting from the down-regulation of PPAR signaling. This is supported by the observed increase in subchondral bone formation and sclerosis associated with disease progression in humans and animal models of OA.

Cumulative evidence suggests that the dysregulation of subchondral bone metabolism is different in OA, possibly due to an altered osteoblast phenotype. Indeed, osteoblasts isolated from the subchondral bone of patients with OA demonstrated altered phenotypes (48,49). In comparison with normal osteoblasts, OA osteoblasts produce more alkaline phosphatase, osteocalcin, IGF-1, urokinase plasminogen activator, cyto-
kines, and eicosanoids, including IL-1β, IL-6, prostaglandin E₂, and leukotrienes. All of these factors could promote subchondral bone remodeling and are also involved in deposition and turnover of matrix. Because of the development of microcracks, vascular channels or neovascularization may provide a link between subchondral bone tissue and cartilage, potentially enabling these factors to influence the abnormal metabolism of articular chondrocytes and remodeling of OA cartilage.

Currently, no single factor responsible for osteoblast-induced cartilage degradation has been identified. Unlike in other animal models of experimentally induced OA, IGF-1 and transforming growth factor β1 (TGFβ1) were not associated with disease phenotypes in our STR/Ort model, as determined by microarray profiling and reverse transcription–polymerase chain reaction analysis on the same RNA samples used in profiling studies (results not shown). This suggests that alternative mechanisms are important for the development of spontaneous OA in the STR/Ort model. Nevertheless, even with the lack of observed changes in IGF-1 and TGFβ1 mRNA levels during disease progression, we cannot rule out the possibility that posttranscriptional modifications or storage of these growth factors in the extracellular matrix and release via osteoclastic bone resorption during subchondral bone remodeling are alternative mechanisms through which IGF-1 and TGFβ1 might affect OA. However, their lack of association with disease progression is consistent with recent reports that IGF-1 and TGFβ1 mRNA levels are not different between OA and non-OA human bone (50).

In summary, the involvement of bone formation in OA initiation and progression has been recognized for many years (36), and our previous work in the rat ACL transection model of OA supports an early role of bone remodeling in disease development (9,29). Based on the data presented herein, we hypothesize that a shift of mesenchymal cell differentiation from adipogenesis toward osteogenesis in the subchondral region is an important component of the pathogenesis of spontaneous OA. Due to the observed inverse relationship between lipid metabolism and matrix remodeling in this spontaneous disease model, it is likely that up-regulation of PPAR signaling abrogates OA progression by inhibiting early bone formation, which constitutes a therapeutic strategy that could be applicable to human OA.

**AUTHOR CONTRIBUTIONS**

Dr. Duong had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.


47. Gori F, Thomas T, Hicok KC, Spelsberg TC, Riggs BL. Differen-
genic protein-2 increases OSF2/CBFA1, enhances osteoblast expres-


Low Molecular Weight Isoforms of the Aggrecanases Are Responsible for the Cytokine-Induced Proteolysis of Aggrecan in a Porcine Chondrocyte Culture System

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**Objective.** The major proteases responsible for aggrecan turnover in articular cartilage are the aggrecanases (ADAMTS-4 and ADAMTS-5). Although several studies have demonstrated C-terminal truncation of these aggrecanases, the mechanism and importance of this processing are poorly understood. The objective of this study was to further investigate ADAMTS-4 and ADAMTS-5 C-terminal truncation in a porcine model in vitro culture system.

**Methods.** Chondrocyte–agarose cultures with well-established extracellular matrices were treated with or without interleukin-1 (IL-1), for a variety of different culture time periods. Cultures were analyzed for release of sulfated glycosaminoglycan, aggrecanase-generated interglobular domain (IGD)–aggrecan cleavage, and the presence of ADAMTS-4 and ADAMTS-5 isoforms. Inhibition of aggrecanase activity with monoclonal antibodies, tissue inhibitor of metalloproteinases 3 (TIMP-3), and cycloheximide pretreatment were used to identify ADAMTS isoforms involved in IGD–aggrecan catabolism.

**Results.** Multiple isoforms, including possible zymogens, of ADAMTS-4 and ADAMTS-5 were sequenced within the extracellular matrix formed by 3-week chondrocyte–agarose cultures. IL-1 exposure induced production of a low molecular weight (37 kd) isoform of ADAMTS-4. This isoform was capable of degrading exogenous aggrecan at the IGD–aggrecanase site, was inhibited by TIMP-3, was blocked after preincubation with an antibody to a sequence in the catalytic domain of ADAMTS-4, and required de novo synthesis in the presence of IL-1 for its generation.

**Conclusion.** In porcine chondrocyte–agarose cultures, a 37-kd ADAMTS-4 isoform appears to be the major matrix protease responsible for the IGD–aggrecanase activity detected in response to exposure to IL-1. This conclusion contradicts that of recent studies of transgenic knockout mice and highlights the need to determine the roles of the different aggrecanase(s) in human disease.

Degradation of cartilage is one of the major pathologic features of arthropathies such as osteoarthritis and rheumatoid arthritis and involves proteolysis of the major structural elements of cartilage, aggrecan, and type II collagen. Aggrecanolysis has been attributed to ADAMTS-4 and ADAMTS-5 (1). Aggrecan comprises 3 globular domains (G1, G2, and G3) intersected by 2 rod-like segments, the interglobular domain (IGD), and the 2 glycosaminoglycan (GAG) attachment regions, CS1 and CS2, respectively, whose charge density provides the tissue with its water-imbibing properties (2). Aggrecan degradation occurs as an early event in the pathogenesis of osteoarthritis. Cleavage occurs within the IGD at Glu373–Ala374, resulting in release of the GAG-rich regions to the synovial fluid (3). Both ADAMTS-4 and ADAMTS-5 (and, to a lesser extent, ADAMTS-1, ADAMTS-8, and ADAMTS-9) have demonstrated proteolytic cleavage at this IGD–aggrecan site (4–7).

ADAMTS-4 was first isolated from interleukin-1 (IL-1)–stimulated bovine explant culture medium as a
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62-kd protein (8). Thezymogen form of the protein was predicted to have a molecular weight of 90.2 kd, and the furin-cleaved isoform was predicted to have a molecular weight of 67.9 kd (9). Furin-cleaved recombinant human ADAMTS-4 (rHuADAMTS-4) has been reported as having a molecular weight of 68 kd (10) and a molecular weight of 70 kd (11). The thrombospondin motif of ADAMTS-4 is required for aggrecan binding and cleavage (mediated via the GAG chains of aggregan) (12). However, C-terminal autocatalysis of a 68-kd recombinant ADAMTS-4 isoform resulted in smaller 53-kd and 40-kd isoforms that exhibited a reduced affinity for sulfated GAG (sGAG) despite retention of their thrombospondin motifs (10). This finding suggests that the presence of the cysteine-rich and/or spacer domains of ADAMTS-4 also affect the substrate-binding specificity of furin-cleaved ADAMTS-4. This C-terminal truncation of furin-cleaved ADAMTS-4 (molecular weight 68 kd) has been proposed to be mediated by membrane type 4 matrix metalloproteinase (MT4-MMP) (13) rather than via autocatalysis (10). In addition, previous studies have indicated that cleavage at the aggregan–IGD site occurs more readily following removal of the cysteine-rich and spacer domains of ADAMTS-4 (14,15). Removal of these C-terminal domains and the thrombospondin 1 motif appears to lead to a broader substrate specificity of the enzyme (15).

At present, the mechanisms and significance of ADAMTS processing/truncation are poorly understood. Furthermore, much of the published information has been derived using recombinant enzymes generated using nonchondrocytic or transformed (chondrosarcoma) cells (10,13–15). Whether or not similar ADAMTS processing occurs in cartilage or primary chondrocytes in response to physiologically relevant stimuli (e.g., IL-1) remains to be determined. Therefore, in the present study, we used primary articular chondrocytes cultured in agarose to generate a cartilaginous extracellular matrix, in order to investigate the effects of IL-1 exposure on the presence and function/activity of different ADAMTS-4 and ADAMTS-5 isoforms and their role in aggregcan catabolism.

MATERIALS AND METHODS

Materials. Pronase from Streptomyces griseus was obtained from Boehringer Mannheim (Lewes, UK). Type II collagenase, prepared from Clostridium histolyticum, was obtained from Worthington (Freehold, NJ). Dulbecco’s modified Eagle’s medium (DMEM), fetal calf serum (FCS), gentamicin, and 4–12% Tris–glycine gels were obtained from Invitrogen (Karlsruhe, Germany). Phosphitan C was obtained from Showa Denko (Tokyo, Japan). SeaPlaque agarose was obtained from Fisher Scientific (Leicestershire, UK). Human recombinant IL-1α was obtained from Totam Biologicals (Cambridgeshire, UK). Blue Sepharose 6 Fast Flow and enhanced chemiluminescence (ECL) reagents (catalog no. RP21108) were obtained from Amersham Biosciences (Little Chalfont, UK). Recombinant human ADAMTS-4 was kindly donated by Dr. Carl Flannery (Wyeth Pharmaceuticals, Boston, MA). Keratanase and keratanase II were obtained from AMS Biotechnology (Whitney, UK). Nitrocellulose membrane was obtained from Schleicher & Schuell (Dassel, Germany). Alkaline phosphatase–conjugated goat anti-mouse secondary antibody and substrate used in Western blot analysis (catalog no. W3920) were obtained from Promega (Mannheim, Germany). Monoclonal antibody (mAb) BC-3 was purchased from Abcam (Cambridge, UK). Anti–TS-4N was prepared as culture medium (see below). Polyclonal antibodies RP1-ADAMTS-4 and recombinant human tissue inhibitor of metalloproteinases 3 (rHuTIMP-3) were obtained from Triple Point Biologics (Forest Grove, OR). All other chemicals and reagents were obtained from Sigma-Aldrich (Poole, UK).

Production, characterization, and specificity of monoclonal antibody (mAb) anti–TS-4N. The mAb anti–TS-4N recognizes a linear amino acid sequence (213FASLSRFV220) at the N-terminus of the catalytic domain of human ADAMTS-4. A synthetic peptide with the sequence FASLSRFVGGC representing F213–V220 of HuADAMTS-4 was coupled to carrier protein ovalbumin through the C-terminal cysteine, using established methods (16). Peptide synthesis, conjugation, immunization, cell fusion, and hybridoma selection were performed as previously described (16,17). A hybridoma clone designated anti–TS-4N, resulting from immunization of mouse with the ovalbumin-conjugated peptide, reacted strongly in an enzyme-linked immunosorbent assay with the immunizing peptide but showed no reactivity with unrelated peptide conjugates nor with the carrier protein.

To establish the specificity of anti–TS-4N, duplicate lanes of rHuADAMTS-4 (0.125 µg) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (10% slab gels) and transferred to nitrocellulose. One replicate was immunolocated with anti–TS-4N alone (1:200 ratio), and the second was immunolocated with anti–TS-4N (1:200 ratio) preincubated for 1 hour at 37°C with 50 µg synthetic peptide containing the sequence FASLSRFVGGC absorbed onto a strip of nitrocellulose membrane. Blots were developed using the Amersham ECL kit.

SDS-PAGE and Western blot analysis. ADAMTS-4 and ADAMTS-5 isoforms. Recombinant human ADAMTS-4 (0.125 µg protein per lane), truncated isoforms of rHuADAMTS-5 (10 µl per lane), detergent extracts partially purified via passage over Blue Sepharose 6 Fast Flow (30 µl per lane), and experimental media samples partially purified as bound eluate from heparin–agarose (50 µl per lane) were subjected to 10% SDS-PAGE.

TIMP-3. Recombinant human TIMP-3 (0.5 µg protein per lane), detergent extracts of agarose plugs (30 µl per lane), and experimental media samples (50 µl per lane) were subjected to SDS-PAGE (12% slab gels).

Gels were transferred to nitrocellulose membranes (16) and immunoblotted with appropriate antibodies, as follows: mAb anti–TS-4N (1:200 ratio) or polyclonal antibodies
RP1-ADAMTS-4 (1:500 ratio), recognizing the ADAMTS-4 C-terminal region; anti-TSSCAT, recognizing the ADAMTS-5 metalloproteinase domain; anti–ADAMTS-5 CT (1:500 ratio), recognizing the ADAMTS-5 C-terminal region; and RP2T3 (1:500 ratio), recognizing the TIMP-3 C-terminal region. Blots were incubated with an appropriate horseradish peroxidase–conjugated secondary antibody, and bands were visualized using the Amersham ECL kit according to the manufacturer’s protocol.

Culture of porcine chondrocytes in agarose. Chondrocytes were isolated from the metatarsophalangeal joints of 3–6-month-old pigs, using established procedures (18). Cells were filtered through a 40-μm nitex filter and washed, and then cell numbers were established. Cells were resuspended at 12 × 10⁶ cells/ml in DMEM, mixed (1:1 ratio) with a 2% (weight/volume) solution of agarose in DMEM (6 × 10⁶ cells/ml), and plated into 60-mm culture dishes precoated with 1% (w/v) agarose (2 ml/plate). Cultures were maintained in DMEM containing 10% (volume/volume) FCS, 25 m g/ml gentamicin (4 ml/dish) for 3 weeks. The medium was changed every 3–5 days.

IL-1α treatment of porcine chondrocytes cultured in agarose. Agarose cultures were washed 3 times for 10 minutes in DMEM containing 50 μg/ml gentamicin prior to the addition of experimental media, comprising DMEM (2 ml per dish) containing 50 μg/ml gentamicin and 25 μg/ml Phosphotin C in the absence (control media) or presence of human IL-1α (10 ng/ml). Each treatment was performed in triplicate, and cultures were maintained at 37°C in 5% CO₂ for 96 hours. Medium and agarose plugs were collected and stored at –80°C for later analyses.

Treatment of porcine chondrocyte–agarose cultures with cycloheximide. Agarose cultures were washed 3 times for 10 minutes before culture in control medium containing either 5 μg/ml cycloheximide in DMSO or DMSO alone, in the absence or presence of IL-1α (10 ng/ml), Each treatment was performed in triplicate, and cultures were maintained at 37°C in 5% CO₂ for 96 hours. Medium and agarose plugs were collected and stored at –80°C for later analyses.

Quantitation of sGAG content of experimental samples. The proteoglycan content of the medium was measured as sGAG by colorimetric assay, using dimethylmethylenen blue (DMMB) with chondroitin sulfate C from shark cartilage as a standard (19). Agarose plugs were extracted for 48 hours at 4°C using 10 volumes of 4M guanidinium chloride in 0.05M sodium acetate (pH 6.8) containing 0.01M EDTA, 0.1M 6-aminohexanoic acid, 0.005M benzamidine HCl, and 0.025M phenylmethylsulfonyl fluoride. The extract was then centrifuged at 4,800 revolutions per minute for 10 minutes and the supernatant dialyzed exhaustively against Milli-Q water (Millipore, Bedford MA). The remaining agarose was extracted with an equal volume of 1M NaOH by incubation at room temperature overnight. The samples were neutralized by dialysis against 0.1M acetic acid, then dialyzed exhaustively against Milli-Q water. The sGAG content of the 4M guanidinium chloride and the alkali-treated extracts was measured using the DMMB assay. The sum of the sGAG present in the extracts represented the total proteoglycan present in the agarose culture matrix.

Partial purification of ADAMTS isoforms from culture medium. Culture medium from control or IL-1α–treated cultures (0.5 ml) was incubated with 200 μl of heparin–agarose equilibrated in 100 mM Tris (pH 7.5), 50 mM NaCl, 0.05% (v/v) Brij-35, 1 mM CaCl₂, 1 mM MgCl₂ (equilibration buffer), for 10–30 minutes at 4°C in a microcentrifuge tube. The suspension was spun for 1–2 minutes at 12,000 rpm, and the supernatant was removed. The heparin–agarose was thoroughly washed with equilibration buffer prior to elution with 0.8M NaCl in equilibration buffer. These eluates were subjected to Western blot analysis.

Detergent extraction of ADAMTS-4 and ADAMTS-5 isoforms from agarose plugs. Agarose plugs were extracted in 2 ml of detergent extraction buffer (50 mM Tris HCl, 100 mM NaCl [pH 7] containing 0.5% [v/v] Nonidet P40) for 24 hours at 4°C (13). The extract was spun at 15,000 rpm for 30 minutes at 4°C, and the supernatant was removed. Detergent extracts of agarose plugs (200 μl) were incubated with Blue Sepharose 6 (500 μl) (GE Healthcare, Piscataway, NJ) equilibrated in equilibration buffer (see above), for 10–30 minutes at 4°C. The suspension was spun for 1–2 minutes at 12,000 rpm, and the supernatant was removed. Samples of unbound detergent extracts were subjected to Western blot analysis. Passage over Blue Sepharose 6 did not result in any loss of bands.

Inhibition of IGD–aggrecanase activity in heparin–agarose–purified culture medium using TIMP-3, N-TIMP-3 (a recombinant protein comprising the N-terminal region of TIMP-3), and mAb anti–TS-4N. At the 96-hour time point, heparin–agarose–bound eluate culture medium (300 μl; control and IL-1–treated) was added to rhHuTIMP-3 (0.04 nM), N-TIMP-3 (100 nM), anti–TS-4N hybridoma culture supernatant (500 μl), or a control antibody of the same isotype (mAb 70.6 [antidecorin] [20]). Aggrecan (porcine AD₈, 20 μg GAG equivalent) and an appropriate volume of 10× digest buffer (20 mM Tris, 100 mM NaCl [pH 7.5], 10 mM CaCl₂, containing 2.5% [v/v] Triton) were added. Digests were also established in the absence of inhibitors. Digests were incubated at 37°C for 24 hours prior to precipitation of the GAG-bearing aggrecan catabolites, using cetylpyridinium chloride (CPC). Digests were adjusted to 50 mM sodium sulfate, and a 10% (w/v) CPC solution was added dropwise. The precipitate was spun, the supernatant was discarded, and the pellets were washed twice in 0.05% (w/v) CPC. The pellets were resuspended in 80% (v/v) n-propanol in water (0.5 ml). One hundred microliters of saturated sodium acetate solution was added (1 drop of acetic acid and 3 ml of cold ethanol). The samples were left to precipitate overnight at 4°C, spun, the supernatant discarded, and the pellets dried under vacuum prior to resuspension in 0.1M Tris–acetate [pH 6.5], deglycosylation, and lysis in the presence of a Promega SpeedVac Savant. Samples were reconstituted in sample buffer containing 10% (v/v) β-mercaptoethanol, electrophoretically separated on 4–12% Tris–glycine gels, transferred onto nitrocellulose membrane, and analyzed by Western blotting using mAb BC-3 (21).

SDS-PAGE and Western blot analyses of aggrecan fragments in media samples. Aggrecan fragments in media samples were deglycosylated prior to separation by 4–12% gradient SDS-PAGE and transfer to nitrocellulose membranes (21). Immunoblotting of membranes and incubations with primary and secondary antibodies were performed as previously described (18), using the ProtoBlot Western Blot AP System (Promega) and mAb BC-3 (1:100 dilution) to detect aggrecanase-generated IGD–aggrecan catabolites.

Statistical analysis. All analyses were carried out using Minitab 1.3 software (Minitab, State College, PA). Triplicate
plates were treated in 3 experiments (total, n = 9). An Anderson-Darling test was used to show that the data were normally distributed. Paired t-tests were used to compare the amount of sGAG released to the medium of cultures treated in the absence or presence of IL-1 and those treated with or without IL-1 in the presence or absence of cycloheximide.

RESULTS

Validation of antibodies recognizing domain-specific epitopes in ADAMTS-4 and ADAMTS-5. Western blot analysis of rHuADAMTS-4 with mAb anti–TS-4N showed 4 bands, at 70, 55, 45, and 37 kd (Figure 1B). Analysis with a polyclonal antibody recognizing the ADAMTS-4 C-terminal spacer domain revealed the 2 higher molecular weight bands, at 70 kd and 55 kd (Figure 1C). The multiple bands recognized by these antibodies are consistent with the proposed sites of autocalysis within the rHuADAMTS-4 preparation (10).

Western blot analysis of truncated isoforms of rHuADAMTS-5 using anti–ADAMTS-5CAT, recognizing the ADAMTS-5 metalloproteinase domain in different molecular weight constructs, showed bands in the expected molecular weight ranges (Figure 1D): ADAMTS-5(5), a recombinant protein comprising the metalloproteinase and disintegrin-like domains of ADAMTS-5 (predicted molecular weight 35 kd), showed a doublet at 37 kd and 39 kd; ADAMTS-5(4), comprising the metalloproteinase to the first thrombospondin-1–like repeat of ADAMTS-5 (predicted molecular weight 40 kd), showed a band at 45 kd; and ADAMTS-5(2), comprising the metalloproteinase to the spacer domain of ADAMTS-5 (predicted molecular weight 68 kd), showed a doublet at 65 kd and 68 kd (Figure 1D). As expected, Western blot analysis using anti–ADAMTS-5CT, recognizing the ADAMTS-5 C-terminal region, did not reveal the isoforms ADAMTS-5(5) and ADAMTS-5(4), but did reveal a doublet of bands at 65 kd and 68 kd in ADAMTS-5(2) (Figure 1E).

Western blot analyses of ADAMTS-4 and ADAMTS-5 isoforms present in detergent extracts of chondrocyte–agarose plugs. Analysis of partially purified agarose detergent extracts at time 0 (prior to treatment in serum-free conditions) with antibodies recognizing ADAMTS-4 or ADAMTS-5 showed that a major immunopositive band of ~70 kd was present for both enzymes in the agarose matrix prior to treatment with IL-1 (Figure 2). This 70-kd band was recognized by both the catalytic and C-terminal–region antibodies for both enzymes, suggesting that in normal culture conditions both ADAMTS-4 and ADAMTS-5 are synthesized and sequestered in the matrix as large molecular weight isoforms, possibly bearing their prodomains. In addition, an immunopositive band of ~70 kd was detected, using antibodies to both ADAMTS-4 and ADAMTS-5 (Figure 2). This 70-kd band was recognized by both the catalytic and the C-terminal–region antibodies for both enzymes, indicating that furin-cleaved “active” forms of ADAMTS-4 and ADAMTS-5 were also present. Following serum-free treatment for 96 hours in the presence or absence of IL-1, a similar immunolocation profile was observed (data not shown). In addition, no lower molecular weight ADAMTS-4 or ADAMTS-5 isoforms were detected in the partially purified detergent extracts of agarose plugs.

Correlation of IL-1–induced GAG release from chondrocyte–agarose cultures with IGD–aggrecanase–generated aggrecan catabolites. Medium from agarose cultures in DMEM, in the absence (control) or presence of IL-1 for 96 hours, was analyzed for sGAG using the DMMB assay, and for IGD–aggrecanase–generated aggrecan catabolites using Western blot analysis with mAb...
BC-3 (Figure 3). As expected, the presence of IL-1 induced significantly increased release of sGAG into the culture medium ($P < 0.00001$) as compared with control cultures (Figure 3A). Seventy percent release of sGAG occurred in the first 24 hours of IL-1 exposure (Figure 3A), and over the 96-hour treatment period, 90% cumulative release of sGAG was observed in IL-1–treated cultures. Samples of culture media were analyzed for IGD–aggrecanase cleavage using mAb BC-3 (Figure 3B). BC-3–positive aggrecan catabolites were detected at 250–130 kd in IL-1–treated cultures but were absent in control cultures (Figure 3B).

Western blot analyses of ADAMTS-4 isoforms present in media. No zymogen (predicted molecular weight of HuADAMTS-4, 92.2 kd [22]) or furin-cleaved isoform of ADAMTS-4 (predicted molecular weight of HuADAMTS-4, 67.9 kd [22]) was detected in Western blots of heparin–agarose–bound media fractions (Figure 3C). Interestingly, co-migrating 37-kd isoforms of truncated ADAMTS-4 were detected by using antibodies to the metalloproteinase and spacer domains of ADAMTS-4 (Figure 3C). These ADAMTS-4 isoforms were detected at increased intensity in partially purified media samples from IL-1–treated agarose cultures when
compared with control cultures (Figure 3C, part 1). In addition, a ~55-kd band was detected by the antibody to the spacer domain (Figure 3C, part 2). This band stained at an equal intensity in both control and IL-1–treated cultures. The unbound media fractions from the heparin–agarose contained high molecular weight ADAMTS-4 and ADAMTS-5 isoforms, which were not up-regulated by IL-1 treatment (data not shown).

IGD–aggrecanase activity in heparin–agarose eluate (control and IL-1–treated). Heparin–agarose eluate from IL-1–treated cultures was used for 3 different aggrecan digests (Figure 4), as follows: after pretreatment in the presence or absence of N-TIMP-3 or TIMP-3 (Figure 4A), after pretreatment in the presence or absence of anti–TS-4N (Figure 4B), or after pretreatment in the presence or absence of a control mAb (anti–decorin). IGD–aggrecanase activity was detected only in heparin–agarose eluate from IL-1–treated culture media (Figure 4, lanes 2, 6, and 9) and not in control cultures (data not shown). These data provide evidence that the IGD–aggrecanase activity generated in response to IL-1 is able to bind heparin–agarose, and that this activity is not present in the absence of IL-1.

IGD–aggrecanase activity was greatly reduced in heparin–agarose eluates preincubated with rHuTIMP-3 or N-TIMP-3 (Figure 4A). Because TIMP-3 inhibits ADAMTS-4, ADAMTS-5, and ADAMTS-1 (23), this suggests that most of the IGD–aggrecanase activity in this culture system is attributable to ~1 of these enzymes. However, the majority of the IGD–aggrecanase activity was inhibited by preincubation with anti–TS-4N (Figure 4B). The specific inhibition of ADAMTS-4 by anti–TS-4N was demonstrated by showing minimal loss of staining for IGD–aggrecan catabolites in the presence of a control antibody (antidecorin) (Figure 4C).

Western blot analysis of endogenous TIMP-3 in detergent and experimental culture medium from control and IL-1–treated cultures. Because TIMP-3 has been postulated as being an endogenous inhibitor of aggrecanase activity (23,24), and we have shown that rHuTIMP-3 or N-TIMP-3 can inhibit the majority of the IGD–aggrecanase activity in media samples from IL-1–treated cultures, we sought to ascertain whether changes in TIMP-3 protein occurred as a result of IL-1 exposure. Figures 5A and B show Western blot analysis, under reducing and nonreducing conditions, of detergent extracts of agarose plugs (at time 0) as well as detergent extracts of agarose plugs and media samples from cultures treated for 96 hours in serum-free medium, with or without IL-1.

TIMP-3 was detected using a polyclonal antibody recognizing the C-terminal domain of the molecule. Under both native and reducing electrophoretic conditions, “free” TIMP-3 ran as a doublet at ~25 kd (Figures 5A, part 1 and B, part 1), with the upper band of the doublet possibly associated with increased glycosylation. In extracts of time 0 cultures, TIMP-3 was detected as a 25-kd doublet of “free” TIMP-3 (Figures 5A, part 2 and B, part 2). Higher molecular weight forms of TIMP-3 were also detected under both reducing and nonreducing conditions, possibly due to TIMP-3 associating with other matrix proteins. Experimental detergent extracts (Figures 5A, part 3 and B, part 3) showed banding patterns similar to those of the time 0 extracts. However, the proportion of the 25-kd TIMP-3 band detected in both media samples and detergent extracts of agarose plugs under nonreducing conditions was decreased in IL-1–treated cultures (Figure 5A, parts 3 and 4). Under reducing conditions, levels of TIMP-3 were identical, suggesting IL-1 does not up-regulate production of...
TIMP-3 (Figure 5B, parts 3 and 4). Intriguingly, under reducing conditions, increased levels of glycosylated TIMP-3 (upper band of the doublet) were detected in medium from IL-1–treated cultures (Figure 5B, part 4). However, there was no indication of this in detergent extracts from IL-1–treated cultures, suggesting a differential loss of glycosylated TIMP-3 into the medium in IL-1–treated cultures.

![Figure 5. Western blot analysis of samples electrophoresed under A, nonreducing conditions and B, reducing conditions. Recombinant human tissue inhibitor of metalloproteinases 3 (rhTIMP-3) served as the positive control (0.5 μg protein/lane). Samples were obtained from agarose cultures following treatment in the absence (control) or presence of 10 ng/ml interleukin-1α (IL-1α) for 96 hours; all samples were loaded at 50 μl per lane.](image)

TIMP-3 (Figure 5B, parts 3 and 4). Intriguingly, under reducing conditions, increased levels of glycosylated TIMP-3 (upper band of the doublet) were detected in medium from IL-1–treated cultures (Figure 5B, part 4).

![Figure 6. A, Percentage of total cumulative sulfated glycosaminoglycan (GAG) released into the medium from 3-week agarose cultures, following treatment with cycloheximide (CHX) or its carrier (DMSO) in the absence (control) and presence of interleukin-1α (IL-1α) for 96 hours. Bars show the mean ± SEM. B, Western blot analysis of aggreganase-generated aggregan metabolites containing the interglobulin domain neoepitope ARG (detected with monoclonal antibody BC-3) from 21-day agarose cultures. C, Western blot analyses of ADAMTS-4 isoforms bound to heparin–agarose and eluted in 0.8M NaCl (50 μl per lane) from media samples from 3-week agarose cultures probed with an antibody to the amino-terminus of the metalloproteinase domain of ADAMTS-4 (αTS-4N). Western blots of media samples were performed following treatment for 96 hours with control plus DMSO, IL-1α (10 ng/ml) plus DMSO, control plus cycloheximide, and IL-1α (10 ng/ml) plus cycloheximide.](image)
LOW MOLECULAR WEIGHT AGGREGANASE ISOFORMS IN CYTOKINE-INDUCED AGGREGAN PROTEOLYSIS 3017

Analysis of media samples from 3-week agarose cultures treated with or without IL-1 in the presence or absence of cycloheximide. The effects of cycloheximide on IGD–aggrecanase activity were investigated (Figure 6). As shown previously (Figure 3), IL-1–treated agarose cultures showed increased sGAG release to the medium compared with control cultures, and this increased release was statistically significant ($P = 0.02$) (Figure 6A). Western blot analysis of the medium with mAb BC-3 identified IGD–aggrecanase–generated aggrecan catabolites in media samples from IL-1–treated cultures (Figure 6B, part 2). In contrast, release of sGAG to the medium and the detection of corresponding IGD–aggrecanase–generated aggrecan catabolites was not seen in media samples from cultures treated with IL-1 plus cycloheximide (Figures 6A and B, part 4). In the presence of cycloheximide, no statistically significant difference in the percentage of total sGAG released was observed between control and IL-1–treated cultures ($P = 0.059$). Western blot analyses of heparin–agarose eluate of media from these cultures showed a significant reduction in detection (using anti–TS-4N) of the 37-kd isoform of ADAMTS-4 in media samples from cultures treated with both IL-1 and cycloheximide compared with cultures treated with IL-1 alone (Figure 6C, parts 2 and 4). These results indicate that de novo protein synthesis is required for IGD–aggrecanase activity and also for the generation of the 37-kd heparin–agarose–bound proteolytically active isoform of ADAMTS-4.

DISCUSSION

In the present study, multiple isoforms of ADAMTS-4 and ADAMTS-5 were synthesized, secreted, and sequestered during the development of an extracellular matrix in 3-week chondrocyte–agarose cultures. Western blot analysis of the extracts identified 90-kd isoforms of ADAMTS-4 and ADAMTS-5, containing catalytic and C-terminal regions of both enzymes. This molecular weight is suggestive of azymogen form of these enzymes. Treatment of these cultures in serum-free conditions, with or without IL-1, revealed very little change in any of the ADAMTS-4 and ADAMTS-5 isoforms detected from extracts of cultures in serum.

After exposure to IL-1, culture supernatants showed a series of co-migrating 37-kd isoforms of ADAMTS-4, which were detected at greater intensity in IL-1–treated cultures than in control cultures. These low molecular weight ADAMTS-4 isoforms have also been detected in media samples from IL-1–treated porcine articular cartilage explants (Powell AJ, et al: unpublished observations). Such ADAMTS-4 isoforms correlate with studies of chondrocyte monolayers treated with or without IL-1, showing ADAMTS-4 isoforms of 30 kd to 200 kd (25). Furthermore, 75 kd and 60 kd ADAMTS-4 isoforms have been reported in extracts of normal human tibial and femoral head cartilage (14). Experiments using rHuADAMTS-4 have suggested that lower molecular weight enzyme isoforms (55–30 kd) cleave at the IGD–aggrecanase site, while higher molecular weight isoforms cleave aggrecan within its C-terminal chondroitin sulfate attachment regions (13,15). The apparent absence of low molecular weight isoforms of ADAMTS-4 and ADAMTS-5 isolated from extracts of agarose plugs may result from masking of the epitopes through binding of the enzyme isoforms to aggrecan mediated through interaction with keratan sulfate (26). Following cleavage of the aggrecan substrate within the extracellular matrix and subsequent release of aggrecan catabolites to the culture media, dissociation of the low molecular weight ADAMTS-4 and ADAMTS-5 isoforms may occur.

In this porcine chondrocyte culture system, we have shown that IGD–aggrecanase activity in culture supernatants was attributable to ADAMTS-4 protein, because the majority of the IGD–aggrecanase activity was inhibited by preincubation with mAb anti–TS-4N. A soluble form of IGD–aggrecanase activity was previously reported in a matrix-free agarose culture system (18), and the current study suggests that this activity is attributable to ADAMTS-4. Immunodepletion experiments also suggested that ADAMTS-4 accounts for 75% of the aggrecanase activity in media from IL-1–treated bovine cartilage explants, while ADAMTS-5 accounted for only 15% (22). However, the possibility of other ADAMTS aggrecanases playing a lesser role has not been fully ruled out.

The addition of TIMP-3 to IL-1–treated explant cultures has been shown to inhibit GAG release (27). Therefore, the presence of endogenous TIMP-3 was examined, in order to determine the effect of IL-1 exposure on TIMP-3 metabolism. Numerous high molecular weight forms of TIMP-3 were detected in media and detergent extracts of agarose plugs, indicating that TIMP-3 is extensively associated with other matrix components (28,29). In addition, a 25-kd doublet (possibly unbound TIMP-3) was detected under nonreducing conditions in media samples and detergent extracts of agarose plugs, and the intensity of this band was reduced in extracts from IL-1–treated cultures. This suggests that IL-1 treatment decreases the amount of available unbound TIMP-3. Under reducing conditions, increased levels of glycosylated TIMP-3 were also detected in
medium from IL-1–treated cultures, suggesting differential loss of glycosylated TIMP-3 to the medium of IL-1–treated cultures.

In accordance with previous reports, IL-1–induced IGD–aggrecanase–generated aggrecan release was ablated by the presence of cycloheximide (30). In this study, we showed that de novo protein synthesis is required for the generation of the 37-kd isoform of ADAMTS-4, which is increased in IL-1–treated cultures. However, the higher molecular weight isoforms of ADAMTS-4 were unaffected by cycloheximide treatment, indicating that they were sequestered in the agarose culture matrix prior to treatment with cycloheximide. This observation suggests a number of possibilities, as follows: generation of IGD–aggrecan catabolites by ADAMTS-4 is a function of endogenous enzyme processed by autocatalytic mechanisms or other enzyme(s) in response to IL-1, as suggested by Gao et al (13,14); ADAMTS-4 is synthesized as a low molecular weight catalytically active isoform in response to IL-1 (alternative splicing of the ADAMTS-4 gene has recently been reported [31]); and/or ADAMTS-4 synthesized in response to IL-1 is preferentially processed to a low molecular weight catalytically active form. Extracellular processing has previously been proposed as a mechanism of ADAMTS-4 activation by IL-1 (25), and this processing has been suggested to be carried out by MT4-MMP (14).

Data presented in this study suggest that low molecular weight ADAMTS-4 isoforms are involved in IGD–aggrecan catabolic. This conclusion is supported by a recent study showing IGD–aggrecanase activity of ADAMTS-4 to be increased following removal of the spacer and TSP domains of the protein (15). Furthermore, rHuADAMTS-4 expressed in chondrosarcoma cells was secreted as 5 isoforms of 50–125 kd, with the 50-kd and 60-kd isoforms possessing the greatest IGD–aggrecanase activity (9).

The evidence presented here, that a 37-kd C-terminally truncated isoform of ADAMTS-4 is the major active soluble IGD–aggrecanase, is consistent with the results from bovine cartilage explant cultures (22) but contrasts with the results observed in transgenic mouse knockouts, in which ADAMTS-5 is the predominant enzyme responsible for aggrecan degradation with the onset of joint pathology (27,32). This difference in the source of IGD–aggrecanase, as it relates to disease development, could be attributable to species differences or culture methods used. Thus, defining the relative role of the different aggrecanases (i.e., ADAMTS-4 and ADAMTS-5) in the pathologic processes of human aggrecanolyis remains an important issue.

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AUTHOR CONTRIBUTIONS

Dr. Hughes had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Powell, Hughes.

Acquisition of data. Powell.

Analysis and interpretation of data. Powell, Little, Hughes.

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Antibody production. Little.

REFERENCES


Interleukin-1β Impairment of Transforming Growth Factor β1 Signaling by Down-Regulation of Transforming Growth Factor β Receptor Type II and Up-Regulation of Smad7 in Human Articular Chondrocytes

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Objective. Extracellular matrix deposition is tightly controlled by a network of regulatory cytokines. Among them, interleukin-1β (IL-1β) and transforming growth factor β1 (TGFβ1) have been shown to play antagonistic roles in tissue homeostasis. The purpose of this study was to determine the influence of IL-1β on TGFβ receptor type II (TGFβRII) regulation and TGFβ1 responsiveness in human articular chondrocytes.

Methods. TGFβ1-induced gene expression was analyzed through plasminogen activator inhibitor 1 and p3TP-Lux induction. Receptor-activated Smad (R-Smad) phosphorylation, TGFβ receptors, and Smad expression were determined by Western blotting and real-time reverse transcription–polymerase chain reaction techniques. Signaling pathways were investigated using specific inhibitors, messenger RNA (mRNA) silencing, and expression vectors.

Results. IL-1β down-regulated TGFβRII expression at both the protein and mRNA levels and led to inhibition of the TGFβ1-induced gene expression and Smad2/3 phosphorylation. Moreover, IL-1β strongly stimulated the expression of inhibitory Smad7. TGFβRII overexpression abolished the loss of TGFβ1 responsiveness induced by IL-1β. The decrease in TGFβRII required de novo protein synthesis and involved both the NF-κB and JNK pathways.

Conclusion. We demonstrate that IL-1β impairs TGFβ1 signaling through down-regulation of TGFβRII, which is mediated by the p65/NF-κB and activator protein 1/JNK pathways, and secondarily through the up-regulation of Smad7. These findings show that there is cross-talk in the signaling of 2 regulatory cytokines involved in inflammation.

Transforming growth factor β1 (TGFβ1) plays a key role in the homeostasis of several tissues, including connective tissues. It is one of the major cytokines that regulate the anabolic process of articular cartilage (1), where it enhances cell proliferation and extracellular matrix production, particularly that of specific markers, whereas it prevents their degradation (2). TGFβ1 molecules induce their signal through activation of a transmembrane heteromeric complex of serine/threonine kinases, e.g., type I (TGFβRI) and type II (TGFβRII) receptors (3). Although TGFβ1 directly interacts only with TGFβRII, both receptors are required for efficient response to the ligand. After binding, TGFβRI is recruited and thereafter phosphorylates its downstream target, receptor-activated Smad2 (R-Smad2) or R-Smad3 (4). Upon phosphorylation, the pathway-specific Smads form hetero-oligomeric complexes with common-mediator Smad4 (Co-Smad4) (5) and then transduce the signal to the nucleus by acting as a transcription factor (6,7). In contrast, inhibitory Smads (I-Smads), including Smad6 and Smad7, prevent the phosphorylation of R-Smads, resulting in inhibition of TGFβ1 signaling (8,9).
In healthy articular cartilage, the effects of TGFβ1 are counterbalanced by the action of interleukin-1β (IL-1β), providing a balance in tissue homeostasis (10,11). During osteoarthritis (OA), IL-1β is found in large amounts in synovial fluids and may actively participate in cartilage breakdown (12). It induces the activation of protein kinase cascades involving the MAPKs and/or the NF-κB pathway. The MAPK pathway consists of ERK, p38, and JNK. These MAPK subtypes are constitutively expressed in articular chondrocytes and transiently activated by numerous stimuli, including IL-1β and tumor necrosis factor α (TNFα) (13). Several studies suggest that MAPKs are also able to modulate the IL-1β-induced NF-κB pathway (14). NF-κB normally exists as an inactive cytoplasmic complex, the predominant form of which is a heterodimer composed of p50 and p65 (RelA) subunits, bound to inhibitory proteins of the IκB family. Its induction requires the activation of IκB/kinase complex that phosphorylates IκB molecules, triggering their degradation. Release of IκB results in the nuclear translocation of NF-κB and its binding to specific DNA sites, inducing the transcription of target genes (15).

The antagonistic effects between TGFβ1 and IL-1β with regard to tissue homeostasis are well documented. TGFβ1 antagonizes the main deleterious effects of IL-1β and decreases IL-1 receptor expression (10). Conversely, IL-1β can interfere with the TGFβ system (16), but little is known about the regulation of TGFβ signaling molecules by IL-1β. Modulation of their expression could play a key role in several diseases. In an animal model of OA, TGFβRII expression was shown to be dramatically decreased during advanced phases of the disease (17). In other studies, transgenic mice expressing a truncated TGFβRII in skeletal tissue were shown to develop cartilage degenerative symptoms similar to that of OA in humans (18).

We hypothesized that IL-1β may down-regulate TGFβRII expression, leading to a reduced responsiveness of cells to TGFβ1. Thus, in the present study, we examined IL-1β–related changes in TGFβRII expression, together with the potential alteration of chondrocyte responsiveness to TGFβ1. To evaluate the contribution of TGFβ1 signaling partners to the changes in TGFβ1 sensitivity, we also assessed TGFβRI and Smad expression/activation. In addition, we further investigated the mechanism whereby IL-1β modulates TGFβRII expression. We found that IL-1β strongly represses TGFβRII expression via the NF-κB and JNK pathways. Moreover, the cytokine is able to reduce TGFβ1-induced Smad2/3 phosphorylation and gene expression. This finding is consistent with the hypothesis that IL-1β impairs TGFβ1 signaling and therefore favors the catabolism of extracellular matrix components and tissue degradation.

**MATERIALS AND METHODS**

**Reagents.** Reagents were provided by Invitrogen (Cergy-Pontoise, France) unless indicated otherwise. Recombinant human IL-1β (Sigma-Aldrich, St. Quentin Fallavier, France) and TGFβ1 (R&D Systems, Lille, France) were resuspended in phosphate buffered saline (PBS)–bovine serum albumin (BSA) or PBS–HCl. Inhibitors were purchased from Sigma and dissolved in PBS (except for actinomycin D, which was dissolved in DMSO). MEK-1/2 inhibitor (U0126; Promega, Charbonnières, France), JNK inhibitor II (SP600125; Calbiochem, Strasbourg, France), and p38 kinase inhibitor (SB203580; Calbiochem) were dissolved in DMSO. Antibodies were obtained from Santa Cruz Biotechnology (through Tebu, Le Perray en Yvelines, France), except for mouse anti-phospho-MAPK and rabbit anti-MAPK antibodies (ERK-1/2), which were purchased from Upstate Direct/Chemicon International (Hampshire, UK). Rabbit anti–phospho-JNK and anti–total JNK, anti-p38 and anti–phospho-p38 were obtained from New England Biolabs (through Ozyme, St. Quentin, France). Oligonucleotides were supplied by Eurogentec (Angers, France).

**Cell culture and treatments.** OA cartilage was obtained from the femoral heads of 42 patients undergoing hip replacement surgery (ages 82–52 years; median 76 years), and normal femoral head cartilage was obtained postmortem from 10 subjects (ages 75–48 years; median 62 years). Chondrocytes were released by digestion with type XIV Pronase (2 mg/ml for 45 minutes; Sigma-Aldrich) and type I collagenase (2 mg/ml for 4 hours). The cells were then washed with PBS and seeded at 4 × 10^4 cells/cm² in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% fetal calf serum (FCS), 100 IU/ml of penicillin, 100 μg/ml of streptomycin, and 0.25 μg/ml of Fungizone, and then incubated at 37°C in a humidified atmosphere containing 5% CO₂ until they reached 80% confluency. Cells were used as primary cultures, except for the nucleofection assay, where they were passaged once. The medium was changed twice a week for a maximum of 12 days. Cells were maintained for 24 hours in medium containing 2% FCS before treatment with recombinant human IL-1β in the same medium. No differences in global effect of treatment with IL-1β or TGFβ1 were observed between specimens obtained postsurgery and those obtained postmortem. For nitric oxide assay, cells were incubated in DMEM plus 2% FCS without red phenol.

HaCaT, HT29 cells, synoviocytes, and human foreskin fibroblasts were maintained in 10% FCS–containing DMEM.

**Protein extraction and Western blotting.** Cells were rinsed and then scraped into RIPA lysis buffer supplemented with phosphatase and protease inhibitors. The extracts (30 μg protein) were subjected to fractionation by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes (Amersham Biosciences, Orsay, France), and reacted with rabbit TGFβRII (catalog no.
sc400, 1:2,500 dilution), Smad7 (catalog no. sc11392, 1:1,000 dilution), phospho-Smad2/3 (Ser433/435; catalog no. sc11769R, 1:500 dilution) polyclonal antibodies. Subsequently, membranes were incubated with anti-rabbit secondary peroxidase-conjugated antibody. Signals were revealed with SuperSignal West Pico Chemiluminescent Substrate (Pierce Perbio Science, Brébières, France) and exposure to x-ray film. The membranes were also reacted with anti-human β-actin (catalog no. sc62432) or lamin A (catalog no. sc6214) antibodies (1:500 dilution) to verify equal loading. Detection of ERK, JNK, and p38 was performed as previously described (19).

**RNA isolation and real-time reverse transcription-polymerase chain reaction (RT-PCR).** Total RNA was extracted with TRIzol reagent. Samples (2 μg) were treated with DNase 1 and reverse transcribed in a 25-μl volume reaction with random hexamers and Moloney murine leukemia virus reverse transcriptase. Products were diluted 1:100 and stored at −20°C until used for PCR.

The primers were designed with Primer Express software. Briefly, 5 μl of diluted complementary DNA was mixed with forward and reverse primers and 2× SYBR Green PCR Master Mix (Applied Biosystems, Courtaboeuf, France) in a 15-μl final volume. Thermal cycling parameters consisted of denaturation at 95°C for 10 seconds and annealing/extension at 60°C for 1 minute, for 40 cycles in an ABI Prism 7000 sequence detection system apparatus. Relative expression was calculated according to the 2^-ΔΔCt method (20).

**Nitric oxide assay.** Nitric oxide production was determined by quantifying nitrite in culture media, using the Greiss spectrophotometric method. Briefly, 100 μl of samples or sodium nitrite standard dilutions was mixed with 100 μl of Griess reagent (0.5% sulfanilamide, 0.05% naphthylethylene diamine dihydrochloride, 2.5% H₃PO₄) and incubated for 5 minutes. The absorption was recorded at 540 nm.

**Plasmids.** The p1670TβRII vector contains human promoter for TGFβRII (−1670/+36) upstream luciferase gene in basic pGL2 and was a gift from Dr. S.-J. Kim (National Institutes of Health, Bethesda, MD). The p3TP-Lux plasmid and pNF-κB-Lux vector contain responsive elements for TGFβ1 and NF-κB, respectively, upstream luciferase gene. These were kindly provided by Dr. J. Massagué (Memorial Sloan-Kettering Cancer Center, New York, NY) and Dr. B. de Crombrugge (M. D. Anderson Cancer Center, Houston, TX), respectively. The p65 expression vector pSG5-p65 was obtained from Dr. P. Jalilot (Ecole Normale Superieure de Lyon, Lyon, France). The mammalian expression vector, pSUPER (OligoEngine, Seattle, WA), was used for expression of short hairpin RNA (shRNA). A 19-nucleotide sequence (TTCTCCGAAAGTGTCAAGT) from p65 messenger RNA (mRNA) was separated by a 9-nucleotide noncomplementary spacer (TCTCTTGAAG) from the reverse complement of the same 19-nucleotide sequence. The insert was cloned into pSUPER and was named shRNA-p65 vector. A control vector (sh-Scramble) was constructed using a 19-nucleotide sequence (ACAGGTGTCAGAAAGGAGA) with no significant homology with any mammalian gene sequence; this was used as a control in the same experiments.

**Transfection experiments and luciferase assay.** Chondrocytes were transiently transfected using the calcium phosphate precipitation method. Reporter plasmid (pNF-κB-Lux or p3TP-Lux; 5 μg) was cotransfected with pSV40-β-galactosidase (pSV40-β-gal) expression vector (1 μg) as an internal control of transfection efficiency. It was previously verified that the transcriptional activity of this promoter was not affected by IL-1β treatment. After overnight transfection, the culture medium was replaced with DMEM containing 2% FCS, the chondrocyte cultures were incubated with IL-1β (1 ng/ml), and appropriate treatments were performed.

For p1670TβRII transfection, 1 × 10⁵ chondrocytes were nucleofected according to the optimized protocol with human chondrocyte Nucleofector solution (Amaxa, Cologne, Germany). Two different plasmids were introduced into the cells: p1670TβRII (or insertless basic pGL2; 4 μg) and pSV40-β-gal (1 μg). Upon nucleofection, cells were resuspended in media containing 20% FCS and placed in the incubator. After 8 hours, the chondrocyte cultures were then incubated for 24 hours in the presence or absence of IL-1β (1 ng/ml) in 10% FCS containing DMEM.

After the transfection experiments, the cells were washed with PBS and harvested in lysis buffer. Luciferase activity was assayed in total cell extracts (Luciferase Assay kit; Promega) in a luminometer, and β-galactosidase activity was measured by a colorimetric assay. Luciferase activities were normalized to the transfection efficiency, and transcription activities were expressed as relative luciferase units (mean ± SEM of triplicates).

**Synthesis and transfection of decoy oligonucleotides.** Oligonucleotide sequences for the NF-κB decoy were 5′-AGTTGAGGGGATTTCCCAAGG-3′ (forward) and 3′-GCCTGGGAATCCCCCTCACT-5′ (reverse) (consensus sequences are underlined). They were annealed overnight and transfected into cells using the Oligofectamine reagent according to the manufacturer’s instructions (Invitrogen). This decoy oligonucleotide was previously shown to bind the NF-κB transcription factor.

**Statistical analysis.** All experiments were repeated at least 3 times, each with different donors, and similar results were obtained. Only representative experiments are shown. Data are presented as the mean ± SEM of triplicate experiments. Statistical significance was determined by Student’s t-test. P values less than 0.05 were considered significant.

**RESULTS**

**Decreased expression of human TGFβRII after IL-1β treatment.** We investigated the effect of IL-1β on TGFβRII protein and mRNA expression in human articular chondrocytes in a time- and dose-dependent manner, using real-time RT-PCR and Western blotting (Figures 1A and B). IL-1β was found to significantly reduce both TGFβRII mRNA and protein steady-states, with maximal effect at doses of 0.1 ng/ml and 1 ng/ml, respectively. This marked inhibition effect (~70%) was seen as early as 1 hour of incubation and was maintained until at least 72 hours of incubation. The experiment was also performed in the presence of cycloheximide, which totally abolished the repressive effect of IL-1β that was observed in the presence of the vehicle (DMSO) (Figure
indicating that this down-regulation is dependent on de novo protein synthesis.

To determine whether the effect exerted by IL-1β was specific to chondrocytes, we repeated our analysis on different cell types (data not shown). We consistently observed a similar action of IL-1β on TGFβRII mRNA levels in all cell types tested: foreskin fibroblasts, synoviocytes, HaCaT cells, and HT29 cells. This suggests that the repressive effect of IL-1β on TGFβRII expression is a general mechanism, rather than a cell-specific phenomenon.

Further, human articular chondrocytes were transfected with p1670TβRII and the corresponding insertless vector basic pGL2. IL-1β treatment caused a marked decrease in luciferase activity in the TGFβRII promoter p1670TβRII, whereas it had no significant
effect on basic pGL2 alone (Figure 1D). Similar results were obtained in studies of synoviocytes (data not shown). Together, these data suggest that IL-1β down-regulation of TGFβRII occurs through a decrease in transcription activity of the promoter.

Enhancement of TGFβRII protein turnover by IL-1β treatment. Since the reduced expression of TGFβRII after treatment with IL-1β could be due to an increased rate of degradation and/or a reduced rate of synthesis, we determined the half-lives of both TGFβRII protein and mRNA. Human articular chondrocytes were incubated with actinomycin D (10 μg/ml) or cycloheximide (10 μg/ml) in addition to IL-1β. The half-life of TGFβRII mRNA was not significantly modified (Figure 2A). In contrast, inhibition of de novo translation clearly showed that IL-1β reduced the protein half-life by approximately one-third (Figure 2B). These data demonstrate that IL-1β not only down-regulates the expression of TGFβRII, but also enhances its degradation rate.

Inhibition of TGFβ1-induced gene expression by IL-1β treatment and abrogation of that inhibition by TGFβRII overexpression. We next investigated whether the decreased level of TGFβRII expression in IL-1β-treated chondrocytes was associated with a subsequent reduction in TGFβ1-induced gene expression. Thus, we analyzed the expression of plasminogen activator inhibitor 1 (PAI-1), a gene which is rapidly induced by TGFβ1. Human articular chondrocytes were incubated for 4 hours with TGFβ1 (2 ng/ml) following a 48-hour period of exposure to IL-1β 1 ng/ml. As shown in Figure 3A, IL-1β preincubation markedly decreased the TGFβ1-induced expression of PAI-1 mRNA. We also investigated the luciferase activities of the artificial TGFβ1-inducible reporter construct p3TP-Lux. Human
articular chondrocytes were cultured as above, preincubated with IL-1β, and then treated for 15 hours with TGFβ1. Preincubation with IL-1β induced a significant inhibition of the TGFβ1-induced luciferase activity of p3TP-Lux (Figure 3B).

To further investigate the consequences of TGFβRII down-regulation on cell response to TGFβ1, we repeated the above experiments, adding forced expression of TGFβRII. To this end, human articular chondrocytes were transfected with a TGFβRII expression vector (pCMV5-TβRII) or its corresponding insertless vector (pCMV5), then treated with IL-1β for 48 hours and further incubated with TGFβ1. In cells transfected with pCMV5 alone, TGFβ1 induced the expected increase in PAI-1 expression and p3TP-Lux activity. Conversely, when the cells were treated with IL-1β, TGFβ1 no longer produced its effects. Ectopic expression of TGFβRII was able to partially counteract the effects of IL-1β on the decrease in TGFβ1-induced stimulation of PAI-1 (Figure 3A) as well as totally abrogate the loss of activation of p3TP-Lux after TGFβ1 stimulation (Figure 3B). These data demonstrate that the TGFβRII expression level plays a crucial role in the alteration of TGFβ1-induced gene expression under IL-1β pretreatment.

**IL-1β–induced modulation of TGFβ1 signaling through inhibition of TGFβ1-induced Smad2/3 phosphorylation and up-regulation of Smad7 expression.** In addition to reduced TGFβRII expression, the reduction in TGFβ1 responsiveness could be caused by a down-regulation of R-Smad protein expression or by an increase in the expression of inhibitory Smads. Therefore, we examined the expression of the well-known TGFβ1 signaling molecules Smads 2 and 3 (R-Smad), Smad4 (Co-Smad), Smad7 (I-Smad), and TGFβRI at the mRNA level by real-time RT-PCR in human articular chondrocytes that had been treated with IL-1β (1 ng/ml). Our results indicated that IL-1β had little effect on the mRNA levels of Smads 2, 3, and 4 and TGFβRI (Figure 4A), but markedly increased the mRNA level of Smad7 (Figure 4B). The stimulatory effect of IL-1β on Smad7 expression was also observed at the protein level after 12 hours of incubation.

Since the reduction in TGFβ1-dependent gene induction by IL-1β could be due to impairment of the TGFβ1-Smad pathway, we investigated the effects of TGFβ1 on Smad2/3 phosphorylation after exposure to IL-1β. As expected, Western blot analysis showed that TGFβ1 induced a rapid phosphorylation of Smad2/3 (Figure 4C). Pretreatment with IL-1β led to a limited TGFβ1-induced phosphorylation of Smad2/3.

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Figure 3. Abrogation of the interleukin-1β (IL-1β)-induced loss of responsiveness of transforming growth factor β1 (TGFβ1) by overexpression of TGFβ receptor type II (TGFβRII). A, Human articular chondrocytes were transfected with the TGFβRII expression vector pCMV5-TβRII or with the insertless plasmid pCMV5 (5 μg). After 15 hours, the medium was removed and replaced with Dulbecco’s modified Eagle’s medium plus 2% fetal calf serum for 24 hours. Cells were then incubated with IL-1β (1 ng/ml) for 48 hours, prior to the addition of TGFβ1 (2 ng/ml) for 4 hours. Total RNA was isolated and analyzed by real-time reverse transcription–polymerase chain reaction, using plasminogen activator inhibitor 1 (PAI-1) and GAPDH primers. B, Human articular chondrocytes were cotransfected with the TGFβRII expression vector p3TP-Lux (5 μg) and with pSV40-β-gal (1 μg), pretreated as above, and then incubated for 18 hours with TGFβ1 (2 ng/ml). Cell layers were lysed, and luciferase activity was measured. The effects of IL-1β and TGFβ1 were expressed as the fold increase in relative luciferase units (RLU) as compared with untreated cells. Values are the mean and SEM of 3 independent experiments. *** = \( P < 0.001 \); ** = \( P < 0.01 \) versus controls.
These data clearly demonstrate that IL-1β alters the Smad pathway through inhibition of TGFβ1-induced Smad2/3 phosphorylation and up-regulation of Smad7. This interference with TGFβ1 signaling is likely to account for the impaired TGFβ1 response after IL-1β treatment.

Involvement of the NF-κB and JNK pathways in IL-1β regulation of TGFβRII. Since IL-1β is known to exert several of its effects through NF-κB, we investigated the putative involvement of NF-κB in the mechanism of inhibition of TGFβRII expression. To address this question, we used different approaches to the in vitro gain/loss of NF-κB function. First, overexpression of the p65 NF-κB subunit in human articular chondrocytes resulted in a dose-dependent decrease in TGFβRII expression, as compared with cells transfected with the insertless plasmid (Figure 5A). Furthermore, overexpression of the p65 subunit dramatically reduced the TGFβ1-induced PAI-1 expression that was seen in the studies described above (Figure 5A).

Figure 4. Impairment of transforming growth factor β1 (TGFβ1) signal transduction by interleukin-1β (IL-1β). A, Human articular chondrocytes were cultured as described in Figure 1A and then assayed by real-time reverse transcription–polymerase chain reaction (RT-PCR) for the expression of Smads 2, 3, and 4 and TGFβ receptor type 1 (TGFβRI [TβRI]) genes. Results are expressed as the percentage of controls after normalization against GAPDH. Values are the mean and SEM of triplicate experiments. B, Human articular chondrocytes were cultured as above and analyzed by real-time RT-PCR for Smad7 gene expression (top) and by Western blotting for Smad7 protein expression (bottom). For Western blotting, cells were incubated for 12 hours with IL-1β, in parallel with lamin A as a control for equal loading. Values are the mean and SEM of triplicate experiments. ** = P < 0.01; *** = P < 0.001 versus controls. C, Human articular chondrocytes were cultured as described in Figure 1A and were then either not preincubated or preincubated for 48 hours with IL-1β (1 ng/ml), prior to the addition of TGFβ1 (2 ng/ml) for 30 minutes (top) or for 1 hour (bottom). Phosphorylated Smad2/3 and β-actin levels were determined by Western blotting.
We also attempted to inhibit NF-κB in several ways. Pyrrolidine dithiocarbamate (PDTC), a widely used inhibitor of the NF-κB pathway, was able to block the IL-1β induction of the reporter plasmid pNF-κB-Lux, which validates its efficiency in our system (data not shown). Real-time RT-PCR and Western blot experi-
ments showed that PDTC significantly abrogated the IL-1β–induced down-regulation of TGFβRII at both the mRNA and protein levels (Figure 5B).

In another study, we prevented the action of NF-κB by a decoy experiment. Here again, addition of a NF-κB decoy oligonucleotide neutralized the inhibition of TGFβRII expression induced by IL-1β (Figure 5C). Moreover, in order to target p65 mRNA silencing, transient transfection of human articular chondrocytes with shRNA-p65 induced an important decrease in p65 expression that seemed to be sufficient to induce the increase in TGFβRII expression (Figure 5D). Taken together, these data strongly support a key role of the NF-κB pathway in IL-1β–induced down-regulation of TGFβRII.

IL-1β is known to enhance the expression and activity of inducible nitric oxide synthase, with subsequent release of nitric oxide, which acts as a mediator of the deleterious effects of the cytokine. Therefore, we investigated its putative involvement in TGFβRII regulation. When nitric oxide synthesis was blocked by Nω-monomethyl-L-arginine (L-NMMA), the inhibitory
effect of IL-1β still occurred, suggesting a nitric oxide-independent pathway (Figure 6A).

MAPK subgroup pathways are also known to be implicated in IL-1β signaling. Specific inhibition of MEK-1/2 with U0126 abolished the phosphorylation of ERK-1/2 induced by IL-1β at both the protein and mRNA levels, without altering its effects on TGFβRII (Figure 6B). Moreover, SB203580 limited the phosphorylation of p38 and decreased the expression of TGFβRII (Figure 6C), suggesting that the effects of IL-1β on TGFβRII expression are not mediated though this pathway. In contrast, inhibition of JNK with SP600125 totally abrogated the inhibitory effect of IL-1β on TGFβRII at the protein and mRNA levels (Figure 6D). These data strongly suggest the direct involvement of JNK and probably of the activator protein 1 (AP-1) transcription factor in the regulation of TGFβRII by IL-1β.

**DISCUSSION**

Several studies have shown that inactivation of TGFβ receptors induces TGFβ resistance and contributes to disease pathogenesis (21,22). In OA, dysfunction of TGFβ1 signaling may contribute to an insensitivity to TGFβ1 and lead to the degradation of articular cartilage. IL-1β plays a major role in cartilage catabolism and could account for such a deterioration in cell responsiveness. In the present study, while IL-1β did not alter the expression of TGFβRI, it caused an early and durable reduction in TGFβRII expression through neosynthesis of an as-yet-unknown protein. This down-regulation was observed in the transcription activity of the human TGFβRII promoter and in the mRNA and protein steady-state levels. Several cytokines and growth factors are known to regulate TGFβ receptors, and discordant regulation of TGFβRI and TGFβRII has previously been reported (23,24). Ultraviolet irradiation has also been shown to modulate the expression of TGFβRII without affecting the expression of TGFβRI (25). Moreover, we found that IL-1β clearly diminished the half-life of TGFβRII proteins, indicating that the cytokine not only reduces the transcription of TGFβRII, but it also enhances its degradation. Therefore, modification of the expression of TGFβRII should alter the cell surface binding capacity for TGFβ1 and subsequent signaling.

We also demonstrated that IL-1β impairs TGFβ1 responsiveness, since the cytokine prevented the transcription of TGFβ1 target genes, such as PAI-1. It also inhibits the TGFβ1 signaling pathways, as reflected by the reduced induction of Smad2/3 phosphorylation, with no effect on R-Smad expression. In parallel studies, IL-1β pretreatment prevented the induction of the TGFβ1-sensitive reporter construct p3TP-Lux. Interestingly, ectopic overexpression of TGFβRII completely overcame the IL-1β-induced inhibition of TGFβ1-induced p3TP-Lux promoter activity and PAI-1 expression. This indicates that TGFβRII inhibition is an essential mechanism by which IL-1β impairs the TGFβ1 response. Therefore, IL-1β inhibition of TGFβRII gene expression appears to be the primary mechanism of deterioration in the TGFβ1 signal in several cell types, suggesting that TGFβRII down-regulation is a fundamental response to IL-1β, rather than a cell type-specific response. Interestingly, as shown for chondrocytes, the down-regulation observed in synoviocytes was also due to inhibition of transcription.

In addition, attenuation of the TGFβ1 response may also be due to a diminished level of intracellular TGFβ1 signaling molecules (R-Smad and/or Co-Smad) or an up-regulation of intracellular inhibitors (I-Smad) (26,27). While the levels of mRNA for Smads 2, 3, and 4 were not affected by IL-1β, the levels of Smad7 mRNA and protein were increased. These data are consistent with those of other studies showing elevated expression of Smad7 mRNA (28). Therefore, Smad7 induction might represent an additional mechanism whereby IL-1β antagonizes TGFβ1 signaling, that is, by reducing the phosphorylation of Smads 2 and 3 and their subsequent translocation into the nucleus.

In addition, we investigated the mechanisms whereby IL-1β exerts its effects. First, we examined the NF-κB pathway, since it plays a pivotal role in IL-1β responses. Given that the p65 NF-κB is able to regulate the expression of Smad7 (29) and TGFβ1 (30,31), we investigated its role in regulating TGFβRII gene expression. We first ascertained that IL-1β induced a rapid and durable increase in p65 expression (data not shown). Thereafter, we found that p65 overexpression led to decreased TGFβRII mRNA levels (as well as TGFβRII promoter activity [data not shown]) and to a decreased TGFβ1-induced response. In contrast, we showed that PDTC, which acts by blocking the degradation of IκBα and the subsequent translocation of NF-κB to the nucleus, abolished the repression of TGFβRII. Competitive inhibition of NF-κB binding by decoy experiments totally overcame the TGFβRII down-regulation. Moreover, p65 gene silencing induced an increase in TGFβRII expression, confirming the essential function of this NF-κB subunit in TGFβRII transcription activity.

The fact that NF-κB was involved in counteracting the action of TGFβ1 is consistent with the report that RelA/NF-κB stimulated the expression of the inhib-
ity Smad7 and stabilized its association with activated TGFβRII (29), leading to diminished effects of TGFβ1 in the presence of TNFα. However, our data suggest a direct role of NF-κB in the regulation of TGFβRII. Computational analysis (TFBind software) of the human TGFβRII promoter revealed a putative NF-κB site in the positive regulatory element 2 region of the first exon. However, no evidence exists for its ability to bind the factor. In addition, despite the fact that NF-κB is commonly described as an activator transcription factor, our results strongly support its involvement in the inhibition of TGFβRII expression. That NF-κB could down-regulate transcription is also consistent with studies reporting its inhibitory action through direct interaction with DNA at Sp1–like sites (32) or by sequestration of CBP/p300 transcription coactivators (33,34). Therefore, we cannot exclude a similar mechanism in the IL-1β regulation of TGFβRII.

Downstream of NF-κB, nitric oxide, which is produced in high amounts by inducible nitric oxide synthase, is one of the major mediators of IL-1β in cartilage (35). Hence, we speculated that the unknown neosynthesized protein could be inducible nitric oxide synthase and that nitric oxide could mediate the IL-1β-induced repression of TGFβRII expression. However, L-NMMA, an inhibitor of nitric oxide synthesis, was unable to modulate TGFβRII expression in the presence of IL-1β, suggesting a nitric oxide–independent mechanism.

IL-1β also triggers several signaling cascades, including sequential activation of various protein kinases, such as MAPK (36). Several studies have suggested that ERK is able to modulate IL-1β enhancement of TGFβ1 expression, since the MEK-1 inhibitor PD98059 can reverse the stimulation of TGFβ1 by IL-1β (37). In our studies, using the more specific MEK inhibitor U0126, no effect of ERK-1/2 phosphorylation blockade was observed on the levels of TGFβRII mRNA or protein, which suggests that another pathway is involved. Similar results have been previously observed in studies using the human pancreatic carcinoma–derived cell line Panc1 (38). Moreover, SB203580, an inhibitor of the p38 pathway, decreased the expression of TGFβRII to levels similar to those following IL-1β treatment. Thus, insofar as IL-1β induces p38 phosphorylation, these data suggest that this cytokine down-regulates TGFβRII through another signaling pathway.

In arthritis, signaling via JNK is one of the major mechanisms elicited by IL-1β. Indeed, by dimerization, c-Jun participates in the formation of AP-1, which, in turn, activates the transcription of many IL-1β–regulated genes. When we used SP600125 to block JNK and c-Jun phosphorylation, it totally abolished IL-1β inhibition of TGFβRII expression at both the mRNA and protein levels, suggesting that the effect of IL-1β is mediated through c-Jun phosphorylation and AP-1 activation. Previous studies have shown a role of AP-1 in the regulation of TGFβ1 signaling and responsiveness. For example, dominant-negative forms of MEKK-1, JNK-1, and MKK-4 (an upstream activator of JNK) were shown to inhibit TGFβ1-induced gene expression (39–41). The mechanism involves, at least, Smad7 and Smad3. Indeed, it has been shown that c-Jun/AP-1 is able to physically interact with Smad3 and thereby suppress Smad3-driven gene transactivation (42). On the other hand, AP-1, in cooperation with NF-κB, is also able to induce Smad7 gene expression (43). Since it is well established that AP-1 and NF-κB activation can be induced by IL-1β, a similar mechanism might be suggested here.

The functional cooperation between AP-1 and NF-κB is well documented. They cooperate to regulate the expression of the TGFβ1 gene upon IL-1β stimulation (31). Moreover, it has been reported that Jun or Fos can interact with the p65 subunit of NF-κB (44). In addition, JNK is able to regulate the transcription activity of NF-κB independently of IκB degradation and NF-κB nuclear translocation, and the inhibitor SP600125 decreases the activation of AP-1 as well as the DNA binding of NF-κB (45). Together, these data suggest that in our system, JNK might act indirectly on TGFβRII expression via a modulation of NF-κB activation. Therefore, although putative sites for AP-1 have been described in the human TGFβRII promoter (46), AP-1 proteins could act through other sites. For example, AP-1 protein has previously been shown to cooperate individually with RelA proteins at the NF-κB site of the promoter of specific cellular genes (47,48). However, it is not clear whether p65 and AP-1 were recruited to the native TGFβRII promoter. Studies are in progress to determine whether they directly bind DNA to affect TGFβRII transcription upon IL-1β stimulation.

In summary, our study is the first to demonstrate that IL-1β impairs the TGFβ1/Smad pathway as a result of down-regulation of TGFβRII in human chondrocytes. Since the binding of TGFβ1 to TGFβRII is the first critical step in the TGFβ1 signaling cascade, this reduced expression of TGFβRII causes reduced responsiveness of TGFβ1 and thereby down-regulates TGFβ1 target genes. In addition, our results show that this effect is dependent on the NF-κB and JNK pathways. This impairment might induce an imbalance in the homeosta-
sis of several tissues and, therefore, favor the development of degenerative and inflammatory diseases.

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AUTHOR CONTRIBUTIONS

Drs. Bauge and Boumédiene had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Bauge, Pujol, Galéra, Boumédiene.

Acquisition of data. Bauge, Legendre.

Analysis and interpretation of data. Bauge, Galéra, Boumédiene.


Statistical analysis. Leclercq, Elissalde.

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tor-β receptor II expression in the rabbit experimental osteo-


Enhanced Integrative Repair of the Porcine Meniscus In Vitro by Inhibition of Interleukin-1 or Tumor Necrosis Factor α

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Objective. To examine the hypotheses that increasing concentrations of interleukin-1 (IL-1) or tumor necrosis factor α (TNFα) inhibit the integrative repair of the knee meniscus in an in vitro model system, and that inhibitors of these cytokines will enhance repair.

Methods. Explants (8 mm in diameter) were harvested from porcine medial menisci. To simulate a full-thickness defect, a 4-mm–diameter core was removed and reinserted. Explants were cultured for 14, 28, or 42 days in the presence of 0–1,000 pg/ml of IL-1 or TNFα. Explants were also cultured in the presence of IL-1 or TNFα with IL-1 receptor antagonist (IL-1Ra) or TNF monoclonal antibody (mAb). At the end of the culture period, biomechanical testing, cell viability, and histologic analyses were performed to quantify the extent of repair.

Results. Mechanical testing revealed increased repair strength, cell accumulation, and tissue formation at the interface over time under control conditions. Pathophysiologic concentrations of both IL-1 and TNFα significantly decreased repair strength, cell migration, and tissue formation at the interface. The addition of IL-1Ra or TNF mAb to explants prevented the effects of IL-1 or TNFα, respectively.

Conclusion. Our findings document that physiologically relevant concentrations of IL-1 and TNFα inhibit meniscal repair in vitro and therefore may also inhibit meniscal repair during arthritis or following joint injury. The finding that IL-1Ra and TNF mAb promoted integrative meniscal repair in an inflammatory microenvironment suggests that intraarticular delivery of IL-1Ra and/or TNF mAb may be useful clinically to promote meniscal healing following injury.

Menisci are C-shaped fibrocartilaginous tissues situated between the femoral condyles and the tibial plateau of the knee. These structures are essential for the normal biomechanical function of the knee, including load bearing, shock absorption, joint congruity, and joint stability (1–3). The meniscus contains 60–70% water and 70% collagen by dry weight (4), as well as smaller amounts of proteoglycans, noncollagenous proteins, lipids, and cells (5). The extracellular matrix is maintained by meniscal fibrochondrocytes that exhibit phenotype characteristics of both fibroblasts and chondrocytes (6). The meniscus is an inhomogeneous structure that shows significant differences in composition, cellularity, and vascularity, depending on distance from the peripheral edge. The outer one-third of the meniscus (sometimes called the “red-red zone” because of its vascular supply) is composed of type I collagen (4,7,8). The cells in this region are fibroblast-like (6,9), and the tissue is vascularized by vessels that penetrate into the tissue from the perimeniscal capillary plexus (10). In contrast, the inner region of the meniscus (called the “white-white zone”) contains fibrochondrocyte-like cells (6,9) as well as both type I and type II collagen (7,8), has a higher aggrecan content than the outer region, and is avascular (10).

Tears in the meniscus are a common knee injury, and nearly 15% of all athletic injuries of the knee involve the meniscus (11). In addition to the pain and loss of function associated with the initial meniscal injury, damage or loss of meniscal tissue is associated with degenerative changes in the joint that ultimately lead to osteoarthritis (OA) (12–14). Studies of an ovine model of meniscal repair have shown that un repaired and failed repair of the meniscus leads to the development of

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tibiofemoral and patellofemoral OA within 6 months of a radial meniscal tear (15). In addition, both partial meniscectomy and partially healed meniscal incisions in a canine model were shown to result in significant increases in cartilage chondropathy and decreased cartilage tensile properties (12). Furthermore, studies in patients with symptomatic knee OA have revealed that both meniscal damage and malposition are associated with an increased risk of cartilage loss (16). In patients with medial meniscal tears and extrusion, there is also a loss of medial cartilage volume and rapid disease progression in the medial compartment of the joint (17).

Current clinical therapies for meniscal injury seek to preserve and repair the damaged tissue. Repair of meniscal lesions in the outer one-third of the tissue is generally favorable, with healing by fibrovascular scar formation, but repair of lesions in the avascular region is negligible (18). This difference in repair potential between the inner and outer regions of the meniscus is related to the vascular supply. Furthermore, explants from both the inner and outer zones of the meniscus have the same intrinsic repair capacity in vitro (19), suggesting that meniscal lesions could be repaired if provided with the appropriate intraarticular conditions.

Inflammatory cytokines, particularly interleukin-1 (IL-1) and tumor necrosis factor α (TNFα), are up-regulated in injured and degenerated joints. The concentrations of these cytokines in synovial fluid have recently been reported in patients undergoing arthroscopy following a meniscal tear (20). IL-1α concentrations were between 25 pg/ml and 160 pg/ml, while TNFα concentrations were between 10 pg/ml and 75 pg/ml in knees with grade I or grade II changes (according to the International Cartilage Repair Society classification system). Inflammatory cytokine concentrations were elevated in patients whose knees had grade III or grade IV changes, with levels of IL-1α between 40 pg/ml and 175 pg/ml and TNFα levels between 10 pg/ml and 150 pg/ml. Furthermore, full-thickness meniscal tears in rabbits show elevated staining for IL-1α for at least 14 days (21), suggesting that IL-1α may be produced for several weeks after the initial injury.

Many degradative and proinflammatory pathways are induced by the up-regulation of IL-1 and TNFα in the joint (22–25). IL-1 increases the production of the inflammatory mediators nitric oxide (26–30) and prostaglandin E2 (26,29,31) in a dose-dependent manner. Furthermore, IL-1 up-regulates matrix metalloproteinases (MMPs) (28,29,32), increases proteoglycan release (27,29), suppresses collagen synthesis (29), and up-regulates TNFα expression (28) in meniscal cells and explants. Similarly, TNFα increases the production of nitric oxide (29) and inhibits the synthesis of proteoglycans in meniscal cells and explants (26,33). These catabolic and anabiotic effects of IL-1 and TNFα have been shown to inhibit the repair of meniscal lesions when present at very high concentrations (34). However, the long-term effects of lower, physiologically relevant concentrations of these cytokines on meniscal repair are not known.

One potential approach to promoting meniscal repair in a proinflammatory microenvironment is to inhibit the activity of the inflammatory cytokines IL-1 and TNFα. IL-1 receptor antagonist (IL-1Ra) prevents IL-1 signaling by competing with IL-1 for binding to receptors. IL-1Ra blocks the binding of the IL-1 receptor accessory protein to the IL-1 receptor and thus prevents downstream signaling (35). A recombinant human IL-1Ra, anakinra (Kineret; Amgen, Thousand Oaks, CA), is currently approved by the Food and Drug Administration (FDA) for the treatment of patients with rheumatoid arthritis (RA) (36). In contrast, TNFα activity can be blocked by the anti-TNF monoclonal antibody (mAb) infliximab (Remicade; Centocor, Malvern, PA), which binds TNFα and neutralizes its biologic activity, or by the soluble TNFα receptor etanercept (Enbrel; Immunex, Seattle, WA). Etanercept and infliximab are currently approved by the FDA for the treatment of patients with RA (37).

The goal of this study was to investigate the in vitro effects of IL-1 and TNFα and inhibitors of these proinflammatory cytokines on the integration of juxtaposed meniscal tissue from the outer, vascularized region. We hypothesized that inflammatory cytokines prevent the repair of meniscal lesions in a dose-dependent manner, and that inhibition of these cytokines will enhance meniscal repair. We used an in vitro model of meniscal repair (19) to examine the effects of a range of physiologic concentrations of IL-1 and TNFα on the healing of explants from the outer region of the meniscus. Explants were cultured for 14, 28, or 42 days, and meniscus healing was investigated using mechanical testing to determine the shear strength, histologic features, and fluorescence confocal microscopic characteristics of the tissue interface. In addition, the effects of inhibiting IL-1 and TNFα on the repair of meniscal lesions were determined.

**MATERIALS AND METHODS**

**Meniscal repair model system.** We used an explant model system described previously (19,34) to study integrative repair of the meniscus. Medial menisci were harvested from...
the knees of 2–3-year-old skeletally mature female pigs; tissues were obtained from a local abattoir. Cylindrical explants were harvested from the femoral surface of the outer zone of the meniscus, which has previously been shown to be vascularized (38), using an 8-mm biopsy punch (Miltex, York, PA) that was oriented perpendicular to the meniscal surface. To simulate a full-thickness meniscal tear, a central core of 4 mm was punched completely through each explant, using a biopsy punch (Miltex) and then immediately reinserted in the same orientation. Explants were then cut from the meniscus at a thickness of 3 mm, using a scalpel blade oriented parallel to the meniscal surface.

Explants were incubated for 1 hour at 37°C in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Carlsbad, CA) containing 1,000 units/ml of penicillin/streptomycin (Invitrogen). Medium was removed, and explants were then cultured at 37°C in an atmosphere of 5% CO₂ in DMEM containing 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT), 0.1 mM nonessential amino acids (Invitrogen), 10 mM HEPES buffer solution (Invitrogen), 100 units/ml of penicillin/streptomycin, and 37.5 μg/ml of L-ascorbic acid 2-phosphate (Sigma-Aldrich, St. Louis, MO). Media were replaced every 3 days.

Explants (≥10 per treatment group, each from different porcine menisci) were cultured for 14, 28, and 42 days and treated with 10 pg/ml, 100 pg/ml, and 1,000 pg/ml of either recombinant porcine IL-1α or recombinant porcine TNFα (both from R&D Systems). While there are 2 isoforms of IL-1 (IL-1α and IL-1β), both have the same potency. IL-1α appears to be the more prominent factor early in the inflammatory process, whereas IL-1β is the more dominant isoform in advanced inflammation (24). Thus, our study focused on the effects of IL-1α as the early-acting proinflammatory isoform.

To test the effects of inhibitors of IL-1 and TNFα activity over 14 days, explants were incubated with either 150 ng/ml of recombinant porcine IL-1 receptor antagonist (IL-1Ra) or 2.4 μg/ml of anti-porcine TNFα mAb (both from R&D Systems) in the presence or absence of 100 pg/ml of IL-1 or 1,000 pg/ml of TNFα, based on the results of initial studies showing significant inhibition of repair at these concentrations. The concentrations of IL-1Ra (median effective dose for IL-1Ra in the presence of 75 pg/ml of IL-1 is 20–60 ng/ml) and TNFα mAb (median neutralization dose for TNFα mAb in the presence of 50 pg/ml of TNFα is 0.015–0.06 μg/ml) were selected to inhibit 100% of the activity of 100 pg/ml of IL-1 or 1,000 pg/ml of TNFα, respectively. Both the cytokines and the inhibitors were added simultaneously to the culture medium.

**Biomechanical testing to determine the interfacial shear strength of repair.** The interfacial shear strength of repair between the inner core and outer ring was determined using a push-out test as described previously (19). Cultured specimens (8-mm outer ring with integrated 4-mm inner core) were placed into a custom-built testing fixture that consisted of a suspended cup with a central 5-mm hole in the base. Using a materials testing system (ElectroForce 3200; Bose-EnduraTEC, Eden Prairie, MN), a 3-mm–diameter rod was displaced at a constant rate of 0.0833 mm/second until the inner core was pushed fully through the outer ring. Force measurements were acquired throughout the duration of the test by a load cell (model 31/6775-06; Sensotec, Columbus, OH). After testing, the sample thickness was measured using a custom-built vision system consisting of a black/white digital video camera (Sony Electronics, Park Ridge, NJ) and a 44-mm video lens (Infinity, Boulder, CO). Images were acquired and analyzed using LabVIEW Vision Builder AI for Windows (National Instruments, Austin, TX). The peak force at failure was normalized by the area of the interface between the outer ring and inner core to determine the interfacial shear strength.

**Live/dead staining of the meniscal repair model explants.** To assess cell viability and accumulation in the interface, explants were stained with the Live/Dead Viability/Cytoxicity kit (Invitrogen). Briefly, samples were washed for 5 minutes in phosphate buffered saline (PBS; Mediatech, Herndon, VA) and then labeled for 30 minutes at room temperature with 4 μM ethidium homodimer 1 and 4 μM Calcein AM in PBS. Explants were then washed 3 times for 5 minutes each in PBS. Samples were visualized and imaged using a confocal laser scanning microscope (LSM 510; Carl Zeiss Instruments, Thornwood, NY).

**Histologic staining of meniscal repair model explants.** Meniscal explants were fixed overnight at 4°C in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) and 100 mM sodium cacodylate trihydrate (Electron Microscopy Sciences), pH 7.4. Samples were dehydrated in ethanol, infiltrated with xylene, and embedded in paraffin. Sections (10 μm) were stained with Harris’ hematoxylin with glacial acetic acid (Poly Scientific, Bay Shore, NY), 0.02% aqueous fast green (Sigma-Aldrich), and Accustain Safranin O solution (Sigma-Aldrich). Sections were viewed with an Axiosvert 5100 microscope (Carl Zeiss Instruments) and photographed using a Coolpix 990 digital camera (Nikon, Melville, NY).
Statistical analysis. Statistical analyses of the shear strength data were performed using Statistica 7.0 software (StatSoft, Tulsa, OK). A two-factor analysis of variance (ANOVA) and the Newman-Keuls post hoc test were performed to determine significant differences (α = 0.05) and the interactive effect of inflammatory cytokine concentration and time in culture. All other analyses were performed using a one-way ANOVA and the Newman-Keuls post hoc test to identify significant differences between treatments.

RESULTS

Decreased interfacial shear strength of repair after IL-1 and TNFα treatment. In control explants, the shear strength of repair in the interface increased over the 42-day culture period (P < 0.05 for day 14 compared with day 28 and day 42) (Figure 1). IL-1 treatment significantly decreased the shear strength at each time point (P < 0.05 for all IL-1–treated samples compared with controls) (Figure 1A). This inhibitory effect was present even at the lowest concentration of IL-1 (10 pg/ml), which significantly decreased the strength of repair at all time points (P < 0.05). There was a dose response to IL-1 treatment, such that increasing the concentration of IL-1 resulted in decreased interfacial shear strength. In addition, there was an interactive effect of IL-1 concentration and time on the meniscal interfacial shear strength of repair (P < 0.005).

Treatment with TNFα also resulted in a dose-dependent decrease in the shear strength of repair (Figure 1B). In particular, at all time points, the highest dose of TNFα (1,000 pg/ml) significantly decreased the
interfacial shear strength of repair ($P < 0.05$). After 42 days in culture, the interfacial shear strength was decreased as compared with control explants at all TNFα concentrations tested ($P < 0.05$). There was also an interactive effect of the TNFα concentration and time on the meniscal interfacial shear strength of repair ($P < 0.05$).

**Inhibition of cell accumulation at the repair site after IL-1 and TNFα treatment.** Over time in culture, increased cell accumulation was observed at the interface of meniscal repair model explants under control conditions (Figures 2B, F, and J and Figures 3A, E, and I). Cell death was observed in the interface immediately after meniscal repair model punches were harvested from the meniscus on day 0 (Figure 2A), but this region was repopulated with live cells over time in culture. Over time, no additional cell death was observed in samples cultured under control conditions or in samples treated with IL-1. Increasing concentrations of IL-1 diminished cell accumulation in the interface, particularly at the highest concentration of IL-1 and with longer time in culture (Figures 2I and M).

In samples cultured with TNFα for 14 and 28 days (Figures 3B, C, D, F, G, and H), cell accumulation in the meniscal repair interface was similar to that in samples cultured in the absence of TNFα (Figures 3A and E). However, after 42 days in culture, there was an apparent decrease in fibrochondrocyte accumulation in the interface in the presence of TNFα (Figures 3J and K), particularly in explants treated with 1,000 pg/ml of TNFα (Figure 3L), as compared with control conditions (Figure 3I). There was no apparent cell death observed in explants cultured in the presence of TNFα.

**Inhibition of tissue repair in the interface of meniscal repair model explants after IL-1 and TNFα treatment.** Histologic analyses revealed healing of the meniscal defect at the interface over time in meniscal explants that were cultured under control conditions (Figures 4A, B, E, and H). On day 0, the interface was clearly visible between the inner core and the outer ring.
of the meniscal repair explants (Figure 4A). In addition, the freshly isolated explants stained positively for Safranin O, indicating the presence of proteoglycans. This proteoglycan staining was lost over time in culture. Over time, the gap in the interface was filled with an extracellular matrix that stained strongly with fast green, revealing the presence of collagen fibers (Figures 4A, B, E, and H). In contrast, no visible tissue repair was detected in explants that were treated with either IL-1 (Figures 4C, F, and I) or TNFα (Figures 4D, G, and J) at all time points evaluated.

Prevention of the IL-1-mediated decrease in the interfacial shear strength of repair with IL-1Ra treatment. Treatment of meniscal repair model explants with IL-1 for 14 days decreased the shear strength of repair \((P < 0.005)\) (Figure 5A). However, the addition of IL-1Ra to explants treated with IL-1 prevented this decrease in the interfacial shear strength \((P < 0.05)\) for explants treated with IL-1 compared with explants treated with IL-1 and IL-1Ra). In contrast, the inclusion of TNF mAb in IL-1–treated samples did not increase the shear strength of repair above that measured in explants treated with IL-1 alone. Culture of meniscal repair explants with IL-1Ra alone did not alter the shear strength of repair, as compared with explants cultured under control conditions (Figure 5C).

Prevention of the TNFα-mediated decrease in the interfacial shear strength of repair with TNF mAb treatment. Treatment of meniscal explants with TNFα for 14 days decreased the shear strength of repair, as compared with control explants \((P < 0.05)\) (Figure 5B). However, inclusion of TNF mAb in the presence of TNFα prevented this decrease in the interfacial shear strength of repair \((P < 0.05)\) for TNFα-treated explants compared with TNFα- and TNF mAb–treated samples). Addition of IL-1Ra in the presence of IL-1 decreased the interfacial shear strength \((P < 0.05)\) (Figure 5C).

Prevention of the TNFα-mediated decrease in the interfacial shear strength of repair with TNF mAb treatment. Treatment of meniscal repair model explants for 14 days with 100 pg/ml of IL-1, 100 pg/ml of IL-1 plus 150 ng/ml of IL-1Ra, and 100 pg/ml of IL-1 plus 2.4 μg/ml of TNF mAb \((n = 8–14\) explants per treatment group) or B, 1,000 pg/ml of TNFα; 1,000 pg/ml of TNFα plus 150 ng/ml of IL-1Ra; and 1,000 pg/ml of TNFα plus 2.4 μg/ml of TNF mAb \((n = 8–12\) explants per treatment group). C, No alteration of the interfacial shear strength following treatment with IL-1Ra and TNF mAb in the absence of exogenous proinflammatory cytokines. The interfacial shear strength of repair was measured after treatment of meniscal repair model explants for 14 days with A, 100 pg/ml of IL-1, 100 pg/ml of IL-1 plus 150 ng/ml of IL-1Ra, and 100 pg/ml of IL-1 plus 2.4 μg/ml of TNF mAb \((n = 8–14\) explants per treatment group) or B, 1,000 pg/ml of TNFα; 1,000 pg/ml of TNFα plus 150 ng/ml of IL-1Ra; and 1,000 pg/ml of TNFα plus 2.4 μg/ml of TNF mAb \((n = 8–12\) explants per treatment group). C, No alteration of the interfacial shear strength following treatment with IL-1Ra and TNF mAb in the absence of exogenous proinflammatory cytokines. The interfacial shear strength of repair was measured after treatment of meniscal repair model explants for 14 days with 150 ng/ml of IL-1Ra and with 2.4 μg/ml of TNF mAb. In all studies, the controls received no treatment. Values are the mean and SEM. a = \(P < 0.05\) versus control; b = \(P < 0.05\) versus treatment with IL-1 plus IL-1Ra; c = \(P < 0.05\) versus treatment with TNFα plus TNF mAb.

Figure 4. Inhibition of tissue repair at the interface of meniscal repair explants following treatment with interleukin-1 (IL-1) and tumor necrosis factor α (TNFα). Paraffin-embedded sections of meniscal repair model explants were stained with hematoxylin to identify the cell nuclei, fast green (green staining) to identify the collagen fibers, and Safranin O (red staining) to identify the proteoglycans. Arrow indicates the interface of the inner core and the outer ring on all images. Shown are representative samples from each treatment group, as follows: control samples obtained on day 0 (A), day 14 (B), day 28 (E), and day 42 (H); samples treated with 1,000 pg/ml of IL-1 obtained on day 14 (C), day 28 (F), and day 42 (I); and samples treated with 1,000 pg/ml of TNFα obtained on day 14 (D), day 28 (G), and day 42 (J). Bars = 50 μm.

Figure 5. A and B, Prevention of the interleukin-1 (IL-1)–mediated and tumor necrosis factor α (TNFα)–mediated decrease in the interfacial shear strength of repair following treatment with IL-1 receptor antagonist (IL-1Ra) and TNF monoclonal antibody (mAb). The interfacial shear strength of repair was measured after treatment of meniscal repair model explants for 14 days with A, 100 pg/ml of IL-1, 100 pg/ml of IL-1 plus 150 ng/ml of IL-1Ra, and 100 pg/ml of IL-1 plus 2.4 μg/ml of TNF mAb \((n = 8–14\) explants per treatment group) or B, 1,000 pg/ml of TNFα; 1,000 pg/ml of TNFα plus 150 ng/ml of IL-1Ra; and 1,000 pg/ml of TNFα plus 2.4 μg/ml of TNF mAb \((n = 8–12\) explants per treatment group). C, No alteration of the interfacial shear strength following treatment with IL-1Ra and TNF mAb in the absence of exogenous proinflammatory cytokines. The interfacial shear strength of repair was measured after treatment of meniscal repair model explants for 14 days with 150 ng/ml of IL-1Ra and with 2.4 μg/ml of TNF mAb. In all studies, the controls received no treatment. Values are the mean and SEM. a = \(P < 0.05\) versus control; b = \(P < 0.05\) versus treatment with IL-1 plus IL-1Ra; c = \(P < 0.05\) versus treatment with TNFα plus TNF mAb.
of TNFα did not alter the shear strength of repair, as compared with samples treated with TNFα alone. Furthermore, treatment of explants with TNF mAb alone did not affect the interfacial shear strength of repair, as compared with explants under control conditions (Figure 5C).

**Restoration of cell accumulation and tissue formation in the meniscal repair interface in the presence of IL-1 and TNFα after IL-1Ra and TNF mAb treatment.** Over the 14-day culture period under control conditions, fibrochondrocytes accumulated in the meniscal repair interface (Figure 6A) and formed new tissue to repair the interface (Figure 6D). As shown in Figures 2 and 4, IL-1 potently inhibited these processes. However, the addition of IL-1Ra in the presence of IL-1 was able to overcome this inhibition and promote cell accumulation (Figure 6B) and tissue formation (Figure 6E) in the repair model interface. Likewise, TNFα treatment alone suppressed cell accumulation and tissue formation in the interface (Figures 3 and 4), but the inclusion of TNF mAb in the presence of TNFα enhanced cell accumulation (Figure 6C) and tissue repair (Figure 6F) in the meniscal repair interface.
DISCUSSION

Our results clearly demonstrate that meniscal tissue exhibits an intrinsic repair capacity in vitro, with increasing repair strength over time and increased cell accumulation and tissue formation in the injury interface under control conditions. The presence of either IL-1 or TNFα at pathophysiologic concentrations (20) inhibited integrative repair, cell accumulation, and tissue formation in a dose-dependent manner. These findings support the hypothesis that meniscal repair may be inhibited or delayed in vivo due to local or systemic increases in IL-1 and TNFα, as well as potentially other proinflammatory cytokines. Importantly, we found that inhibition of IL-1 or TNFα restored the capacity for integrative repair, although little interaction was observed in the activities of these 2 cytokines. Taken together, our findings suggest that the inhibition of IL-1 and/or TNFα activity is a potential strategy for promoting meniscal repair in the inflammatory microenvironment of an injured joint.

The capacity for intrinsic repair of the meniscus is consistent with that of other cartilaginous tissues, in particular, articular cartilage, which exhibits integrative tissue repair in a similar manner in vitro (39–41). The strength values observed in our study of meniscal repair using adult porcine explants were comparable to the values obtained in studies of cartilage repair using adult bovine explants cultured in apposition and tested for adhesive strength (8 kPa versus 21 kPa, respectively, at 14 days) (39). These studies have shown that the repair strength of cartilage is dependent on such factors as collagen metabolism and maturation, suggesting that factors that influence matrix synthesis or degradation may have a significant effect on integrative repair.

Of note was the finding that both IL-1 and TNFα significantly inhibited the intrinsic repair of the meniscus in a dose-dependent manner. These effects were observed in the quantitative measure of repair (shear strength) as well as the qualitative measures (cell accumulation and histologic features). There were no differences between treatment groups in the outer diameter of the inner core or the inner diameter of the outer ring (data not shown), suggesting that the differences in shear strength were likely due to interfacial repair, and not simply to altered interference fit. At equivalent concentrations in our porcine meniscal repair model system, IL-1 inhibited the integrative meniscal repair more potently than did TNFα. As little as 10 pg/ml of IL-1 significantly decreased the repair strength by 44% at 14 days, while 10 pg/ml of TNFα suppressed the repair of meniscal repair only 25% at 42 days in culture. Importantly, these concentrations of IL-1 and TNFα are in the range of values measured in the synovial fluid of patients following a meniscal tear (20), which suggests that physiologically relevant concentrations of these cytokines could have short-term and long-term effects on meniscal repair. Furthermore, the range of IL-1 concentrations evaluated in this study fall within the range of IL-1β concentrations measured in patients with RA (115–193 pg/ml) and OA (21–146 pg/ml) (42,43). Average synovial fluid TNFα concentrations in patients with RA (170 pg/ml) and OA (80 pg/ml) (42) also fall within the spectrum of concentrations evaluated in this study. Therefore, our results indicate that repair of meniscal lesions in RA and OA patients may also be inhibited due to the presence of IL-1 and TNFα in the synovial fluid.

The mechanisms by which IL-1 and TNFα inhibit repair remain to be elucidated, but appear to involve increased degradation of the extracellular matrix through the up-regulation of MMPs and aggrecanases that prevent new matrix formation in the gap, and thus appear to inhibit cell accumulation. Recent studies have shown that IL-1 increases MMP activity of meniscal cells and that inhibition of MMP activity decreases the release of glycosaminoglycans and increases the mechanical function of IL-1–treated meniscal explants (44). However, inhibition of aggrecanases fails to prevent the IL-1–mediated loss of glycosaminoglycans and the decrease in mechanical properties, suggesting that these effects of IL-1 are mediated by MMPs, and not aggrecanases. IL-1 and TNFα may also suppress matrix macromolecule biosynthesis (26,28,29,32,33).

In addition to decreasing the interfacial shear strength of repair, IL-1 and TNFα also decreased cell accumulation in the interface region of meniscal repair explants. Cell accumulation, presumably due to a combination of migration and proliferation, appears to be an important component of meniscal repair in vivo, and previous studies have shown complete repopulation of meniscal allografts (45) or a devitalized meniscal plug that was reinserted into the meniscus in an animal model (46). In the present study, cell accumulation at the interface was associated with increased tissue formation and interfacial shear strength of repair. In contrast to our study, other researchers have found that isolated outer zone bovine meniscal cells exhibit increased cell migration with short-term (4-hour) exposure to 1,000 pg/ml of IL-1 (47). The differences in our results are likely explained by the differences in our culture methods (explants versus isolated cells in monolayer) and/or the incubation time with IL-1 (2–6 weeks versus 4...
hours). It is important to note that in our study, cell accumulation appeared to be suppressed in the presence of proinflammatory cytokines, while cell viability was not altered. Therefore, decreases in shear strength were not simply due to increases in cell death in the presence of IL-1 or TNFα.

Treatment of meniscal explants with IL-1Ra inhibited the actions of IL-1 on interfacial shear strength, cell accumulation, and tissue repair. However, IL-1Ra did not alter the repair of meniscal explants treated with TNFα. Likewise, TNF mAb inhibited the activity of TNFα on the meniscal repair model explants but did not alter the activity of IL-1 on the meniscal repair explants. This lack of cross-inhibition suggests that the effects of IL-1 on the repair process was not through the up-regulation of TNFα production by the meniscal explants, and similarly, the effects of TNFα were not due to up-regulation of IL-1 production by the explants. In addition, in the absence of exogenous inflammatory cytokines, IL-1Ra or TNF mAb did not increase the interfacial shear strength of repair, suggesting that factors associated with isolation of the meniscal explants and creation of the defect model were not sufficient to cause the production of IL-1 and TNFα by the meniscus to an extent that influences integrative repair.

Many studies have shown the beneficial effects of IL-1Ra and TNF mAb in the treatment of RA and OA (35,37,48). The treatment of transgenic mice expressing human TNFα with IL-1Ra and TNF mAb resulted in nearly complete suppression of synovial inflammation, bone erosion, and cartilage destruction (49). In a separate study using mice that constitutively express human TNFα, treatment with TNF mAb prevented further cartilage damage in older mice and actually promoted cartilage repair in younger mice (7–8 weeks of age) (50). Clinical trials have also shown the promise of IL-1Ra gene therapy for the treatment of arthritis (51). In our study, we provide evidence that IL-1Ra and TNF mAb may have beneficial effects on the meniscus, specifically for integrative meniscal repair in an inflammatory microenvironment.

Thus, we postulate that intraarticular delivery of IL-1Ra and/or TNF mAb will be useful clinically to promote meniscal healing following a meniscal tear. In addition, it may be possible to enhance meniscal repair by intraarticular injection of synoviocytes or stem cells expressing IL-1Ra complementary DNA. Other cytokines, such as IL-17, may provide additional targets for anticytokine therapies (26), or alternatively, proanabolic growth factors may provide a means of enhancing matrix biosynthesis (52–56) to accelerate meniscal repair. The in vitro model presented in this study provides a novel and quantitative means of studying the influence of specific pharmacologic inhibitors and their mechanisms of action on integrative repair of the meniscus.

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AUTHOR CONTRIBUTIONS

Dr. Guilak had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. McNulty, Weinberg, Guilak.

Acquisition of data. McNulty, Moutos.

Analysis and interpretation of data. McNulty, Moutos, Weinberg, Guilak.


Statistical analysis. McNulty.

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Clinical images: Bamboo spine in a patient with ankylosing spondylitis

The patient, a 42-year-old man, was diagnosed at our clinic as having ankylosing spondylitis. He had been receiving alternative treatments such as acupuncture and herbal medicines. Three-dimensional computed tomography showed a vivid example of spinal ankylosis, or “bamboo spine” (A and B). Of interest, 3-dimensional computed tomography also revealed a few areas where ankylosis was not yet complete.

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Delayed Memory B Cell Recovery in Peripheral Blood and Lymphoid Tissue in Systemic Lupus Erythematosus After B Cell Depletion Therapy

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Objective. Recent data suggest that the reconstituting peripheral B cell compartment after B cell depletion therapy may be functionally immature, with a preponderance of transitional B cells and a paucity of memory B cells. This study was undertaken to determine the magnitude, duration, and cause of these defects in rituximab-treated systemic lupus erythematosus (SLE) patients.

Methods. Fifteen patients with SLE previously treated with rituximab as part of a phase I/II dose-escalation study were evaluated during a long-term followup (mean followup period 41 months). B cells from peripheral blood and tonsils were assessed using multicolor flow cytometry, and their developmental pathway was classified based on the expression of defined surface markers.

Results. Reconstitution of peripheral blood CD27+ memory B cells was delayed for several years after B cell depletion therapy in a subset of patients with prolonged clinical responses and autoantibody normalization. This delay correlated with the degree of expansion of B cells of a transitional phenotype during the B cell reconstitution phase \( (P = 0.005) \) and the absence of baseline autoantibodies directed against extractable nuclear antigens (RNP, Sm, Ro antigen, La antigen). Despite the paucity of peripheral blood memory cells and the prolonged expansion of functionally immature transitional B cells, tonsil biopsy tissues revealed active germinal center (GC) reactions, but with decreased Fc receptor homolog 4–positive memory B cells.

Conclusion. These results suggest heterogeneity in the B cell depletion and reconstitution process that impacts clinical and immunologic outcomes in SLE. The presence of GC reactions, but with altered memory B cell subpopulations in tonsils, suggests that peripheral blood memory cell reconstitution lags behind a slow secondary lymphoid tissue recovery, with important implications for immunologic competence and tolerance.

Rituximab is a chimeric mouse/human monoclonal antibody directed against the B cell–specific antigen CD20, which depletes B lymphocytes in vivo from the pre–B cell stage in bone marrow, when CD20 is first expressed, to the mature B cell stage. Due to its efficacy in the depletion of both normal and malignant B cells, rituximab represents an effective treatment for B cell lymphomas and has emerged as a promising treatment for multiple autoimmune diseases, including systemic lupus erythematosus (SLE), as we and other investigators have previously described (1–4).

However, the long-term immunologic effects of rituximab, the mechanism(s) whereby B cell depletion

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induces amelioration of autoimmunity, and the immunologic predictors of treatment response remain elusive. In particular, understanding B cell repopulation is critical since regeneration of a humoral immune system, which offers protective immunity as well as censoring of autoactivity, will require the development of a variety of mature naive and memory B cells that have been appropriately selected (5). Of note, the potential consequences of B cell depletion on the immunologic environment that contributes to B cell development and maturation, including distortion of lymphoid architecture, diminished T cell assistance, and reduction of dendritic cell (DC) function, are largely unexplored in humans (6–10).

Emerging data on B cell subsets in patients whose B cells are reconstituting after B cell depletion therapy suggest that B cell maturation may be delayed. Thus, we have recently reported an expansion of functionally immature transitional B cells and decreased memory B cells in lymphoma patients recovering from rituximab-mediated B cell depletion (11). Similarly, during the preparation of this article, 2 reports were published that describe the repopulation of the peripheral blood of rituximab-treated rheumatoid arthritis (RA) patients with immature transitional B cells and a slow and delayed recovery of CD27+ memory B cells; levels of memory B cells remained reduced for >2 years in one study (12), and reductions were associated with longer clinical response in the other study (13).

We have also reported, as part of a phase I/II dose-escalation study of rituximab in SLE, that B cell depletion has the potential to reverse preexisting abnormalities in peripheral B cell homeostasis and tolerance, including memory B cell expansion, the presence of circulating plasmablasts, and expansion of autoreactive 9G4 memory B cell populations (2,14). In this study, we further characterize the emerging B cell populations during early immunologic reconstitution and the recovery of memory B cells, both in peripheral blood and lymphoid tissue, in these patients during long-term followup. Notably, immunologic reconstitution was variable, with patients experiencing different degrees and durations of CD24high,CD38high transitional B cell expansion, which correlated with delayed memory recovery and clinical response.

The subset of patients with clinical responses lasting >5 years had a prolonged predominance of transitional B cells beyond that previously recognized, as revealed by the analysis of rhodamine extrusion, and a severe delay in peripheral blood memory B cell recovery. Peripheral lymphoid tissue from patients with delayed peripheral blood memory B cell recovery during long-term followup revealed a relative paucity of memory B cell subpopulations with an absence of the Fc receptor homolog 4 (FcRH-4)–positive memory subset, despite the presence of active germinal center (GC) reactions and follicular dendritic cell (FDC) networks. Thus, the lymphoid tissue is slow to recover after B cell depletion therapy, and peripheral blood memory B cell reconstitution may lag even further behind lymphoid tissue recovery, with important implications for both immune competence and tolerance.

**PATIENTS AND METHODS**

**Study patients.** Selected SLE patients (n = 15) were included in the present study if they were available to provide samples for detailed serial flow cytometry. For ease of comparison, the patient numbers were the same as those used in our previous articles (2,14) (Table 1). Eligible patients were those older than 18 years who met the American College of Rheumatology criteria for the classification of SLE (15) and had clinically active disease, as defined by a Systemic Lupus Activity Measure (SLAM) score >5 (16). (See Table 1 and ref. 2 for complete patient characteristics.)

Rituximab was provided by Genentech (San Francisco, CA) and Biogen Idec (San Diego, CA). Patients were treated as part of a dose-escalation protocol added to current treatment, as follows: low dose = 1 infusion of 100 mg/m², intermediate dose = 1 infusion of 375 mg/m², or high dose = 4 weekly infusions of 375 mg/m², as previously reported (2). Effective B cell depletion (defined as the depletion of B cells to <1% of the total peripheral blood lymphocytes [PBLs] or an absolute cell count of <5 cells/μl) was previously observed in 10 of 17 patients in this cohort, which correlated with clinical improvement (2). Clinical response duration was defined as the length of time that this improvement was maintained before the SLAM score or the SLE Disease Activity Index score increased by 3 from the lowest value achieved, or before an increase in medication was required (17,18). Serum autoantibodies (IgG and IgM) were measured using commercial enzyme-linked immunoassorbent assays, using a Kallestad anti–double-stranded DNA kit (Bio-Rad, Hercules, CA) and a Quanta Lite Microwell assay kit (Inova Diagnostics, San Diego, CA).

**Sample procurement and cell isolation.** Detailed written informed consent was obtained from all patients and healthy controls, in accordance with protocols approved by the Human Subjects Institutional Review Board of the University of Rochester Medical Center. PBLs from SLE patients and healthy controls were isolated from heparinized blood by Ficoll-Hypaque density-gradient centrifugation (Pharmacia Biotech, Uppsala, Sweden). Tonsil samples were obtained from consenting patients by triangular adenoid forceps biopsy. Surgical tonsillectomy specimens were used as controls for comparison. Samples from age-matched subjects were used for immunohistochemical analysis. Subjects whose samples were used for flow cytometry ranged from 4 to 48 years of age, since B cell subsets were found to be independent of age (data not shown).
Table 1. Characteristics of the systemic lupus erythematosus patients*

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age/sex/race</th>
<th>Rituximab dose</th>
<th>Reconstitution†</th>
<th>Clinical response‡</th>
<th>Other concurrent treatment</th>
<th>Baseline transitional cells, %§</th>
<th>Reconstitution transitional cells, %</th>
<th>Peak absolute transitional cells, µl</th>
<th>Memory cells, %¶</th>
<th>Anti-dsDNA, IU/ml#</th>
<th>ENAs at baseline</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>37/F/C</td>
<td>Low</td>
<td>2 months</td>
<td>9 months</td>
<td>1.5</td>
<td>23</td>
<td>7</td>
<td>8</td>
<td>105/111</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>45/M/C</td>
<td>Low</td>
<td>12 months</td>
<td>Ongoing</td>
<td>AZA</td>
<td>5</td>
<td>44</td>
<td>35</td>
<td>5.8</td>
<td>150/10</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>44/F/C</td>
<td>Low</td>
<td>NA</td>
<td>Nonresponder</td>
<td>–</td>
<td>4</td>
<td>25</td>
<td>13</td>
<td>4.6</td>
<td>154/109</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>41/F/C</td>
<td>Low</td>
<td>9 months</td>
<td>6 months</td>
<td>HCQ, AZA</td>
<td>6</td>
<td>2.6</td>
<td>0.3</td>
<td>82</td>
<td>193/185</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>29/F/A</td>
<td>Low</td>
<td>12 months</td>
<td>Ongoing</td>
<td>HCQ, AZA</td>
<td>0.1</td>
<td>31</td>
<td>50</td>
<td>5.8</td>
<td>381/74</td>
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<tr>
<td>6</td>
<td>39/F/AA</td>
<td>Low</td>
<td>NA</td>
<td>Nonresponder</td>
<td>HCO</td>
<td>1.2</td>
<td>3.6</td>
<td>0.9</td>
<td>56</td>
<td>1,020/1,997</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>39/F/C</td>
<td>Medium</td>
<td>NA</td>
<td>Nonresponder</td>
<td>AZA</td>
<td>2.6</td>
<td>2.4</td>
<td>0.8</td>
<td>67</td>
<td>92/122</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>30/F/AA</td>
<td>Medium</td>
<td>NA</td>
<td>Nonresponder</td>
<td>MYC</td>
<td>35</td>
<td>11</td>
<td>20</td>
<td>7</td>
<td>1,065/ND</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>22/F/H</td>
<td>Medium</td>
<td>3 months</td>
<td>12 months</td>
<td>HCQ, AZA</td>
<td>5.7</td>
<td>37</td>
<td>17</td>
<td>57.3</td>
<td>1,783/1,054</td>
<td>+</td>
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<tr>
<td>10</td>
<td>47/F/C</td>
<td>Medium</td>
<td>6 months</td>
<td>6 months</td>
<td>HCQ</td>
<td>3.5</td>
<td>23.7</td>
<td>4</td>
<td>ND</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>43/F/C</td>
<td>Medium</td>
<td>12 months</td>
<td>12 months</td>
<td>AZA</td>
<td>3.5</td>
<td>15.4</td>
<td>0.9</td>
<td>51</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>41/F/C</td>
<td>Medium</td>
<td>9 months</td>
<td>6 months</td>
<td>HCQ</td>
<td>8</td>
<td>32</td>
<td>13</td>
<td>82</td>
<td>810/1,640</td>
<td>–</td>
</tr>
<tr>
<td>13</td>
<td>30/F/C</td>
<td>High</td>
<td>12 months</td>
<td>Ongoing</td>
<td>AZA</td>
<td>0.1</td>
<td>34</td>
<td>190</td>
<td>7.4</td>
<td>536/10</td>
<td>–</td>
</tr>
<tr>
<td>14</td>
<td>31/F/C</td>
<td>High</td>
<td>18 months</td>
<td>3 months</td>
<td>AZA</td>
<td>1.5</td>
<td>4.4</td>
<td>0.3</td>
<td>48.3</td>
<td>110/240</td>
<td>+</td>
</tr>
<tr>
<td>15</td>
<td>29/F/AA</td>
<td>Medium</td>
<td>NA</td>
<td>Nonresponder</td>
<td>HCQ</td>
<td>0.5</td>
<td>0.5</td>
<td>0.03</td>
<td>69</td>
<td>1,129/ND</td>
<td>+</td>
</tr>
</tbody>
</table>

* anti-dsDNA = anti–double-stranded DNA; ENAs = extractable nuclear antigens; C = Caucasian; AZA = azathioprine; HCQ = hydroxychloroquine; A = Asian; AA = African American; MYC = mycophenolate; ND = not determined (because patient was receiving high-dose steroids with or without cyclophosphamide for nonresponsive disease).
† Time point at which B cells began to be detectable at >5 cells/µl. NA = not applicable (because only partial B cell depletion was achieved).
‡ Time point at which disease activity indexes began to increase (Systemic Lupus Activity Measure score and/or SLE Disease Activity Index score by 3 points and/or necessity to increase steroid dosage), indicating that a relapse had occurred. “Ongoing” indicates that a relapse end point had not yet been reached (long-term responder).
§ Transitional cell percentage as a fraction of total peripheral blood B cells, determined based on CD24 and CD38 expression.
¶ Memory cell percentage as a fraction of the total peripheral blood B cells at long-term followup (3–5 years). Patients 3 and 8 had low memory cell fractions at baseline and were not included in the overall analysis of memory B cells. The percentage of memory cells in long-term responders (mean ± SD 63 ± 0.9%) was significantly different from that in short-term responders and nonresponders (51.1 ± 23.2%) (P = 0.009) and normal controls (30.5 ± 6.9%) (P = 0.001).
# At baseline/long-term followup (3–5 years).
were washed in PBS/BSA and, in some cases, were incubated with streptavidin–peridinin chlorophyll A protein. Cells were then washed and fixed in PBS/1% paraformaldehyde before analysis using a FACSCalibur or an LSR II flow cytometer (Becton Dickinson, Mountain View, CA). B cells were identified based on CD19 expression, and B cell numbers were calculated based on the white blood cell count, the percentage of lymphocytes, and the percentage of CD19 cells identified by flow cytometry. The normal range for peripheral blood CD19+ B cells is 50–375 cells/µL. B cell reconstitution was defined as the time when B cells were again detectable in peripheral blood at >5 cells/µL.

B cells in peripheral blood were additionally classified by developmental stage using multiparameter flow cytometry based on the expression of defined surface markers as follows: immature (CD38high, CD24high, IgD−), transitional (CD38high, CD24high, IgD+), naive (CD38intermediate, CD24low, IgD+, CD27−), pre-GC (CD38high, CD24low, IgD+), non–isotype-switched memory (CD27+, IgD+), and switched memory (CD27+, IgD−) (14,19–22). Memory B cells were also defined according to their mature B cell 5 (Bm5) phenotype within the Bm1–Bm5 classification (Bm1: IgD+, CD38−; Bm2: IgD+, CD38low; Bm2′: IgD+, CD38high; Bm3–Bm4 [GC in the tonsils]: IgD−, CD38high; early Bm5: IgD−, CD38low; Bm5: IgD−, CD38−) (19). In contrast to peripheral blood, CD27 expression in tonsils does not strictly correlate with memory because a variable fraction of the Bm5 memory compartment does not express CD27, and the majority of GC cells also express CD27. Tissue memory B cells were additionally examined for the recently described FcRH-4 (23,24). Measurements of peripheral blood transitional cells were obtained from patients at multiple time points when B cells were detectable. Time points included in the original study were baseline and 1, 2, 3, 6, 9, and 12 months; analysis was conducted post hoc for transitional cells. Measurements were also obtained from all patients at additional time points beyond 12 months. Tonsil biopsy samples were obtained at a single reconstitution time point.

**Flow cytometry.** Immunofluorescence staining for flow cytometric analysis was performed by incubating PBLs with excess monoclonal antibodies in phosphate buffered saline (PBS)/1% bovine serum albumin (BSA) on ice for 20 minutes after blocking with 10 μg of human IgG for 20 minutes. Cells were washed in PBS/BSA and, in some cases, were incubated with streptavidin–peridinin chlorophyll A protein. Cells were then washed and fixed in PBS/1% paraformaldehyde before analysis using a FACSCalibur or an LSR II flow cytometer (Becton Dickinson, Mountain View, CA). B cells were identified based on CD19 expression, and B cell numbers were calculated based on the white blood cell count, the percentage of lymphocytes, and the percentage of CD19 cells identified by flow cytometry. The normal range for peripheral blood CD19+ B cells is 50–375 cells/µL. B cell reconstitution was defined as the time when B cells were again detectable in peripheral blood at >5 cells/µL.

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**ATP-binding cassette B1 (ABC1B1) transporter activity revealed by rhodamine extrusion.** Naïve B cells were distinguished from transitional cells and memory B cells by the expression of ABC1B1 transporter activity and rhodamine 123 dye extrusion, as previously described (25). Briefly, cells were stained in culture medium at 37°C with rhodamine 123 at 6 μM for 10 minutes and chased for 3 hours prior to flow cytometry analysis (25). Dose-ranging studies were performed to determine the optimal loading concentrations and timing for extrusion (data not shown). B cells were gated as described above, and rhodamine 123 extrusion was examined. Specifically, gated memory B cells (CD27+) do not express the ABC1B1 transporter and thus fully retain rhodamine 123. In contrast, naïve B cells (CD38intermediate, CD24low, IgD+, CD27−) effectively extruded rhodamine 123, with a significant difference in the mean fluorescence intensity (MFI) of rhodamine 123 on normal naïve B cells (n = 6 donors) as compared with the MFI of rhodamine 123 on normal memory B cells (P = 3.0 × 10−5). In order to control for slight variations in rhodamine 123 loading, naïve MFIs were corrected relative to the nonextruding memory population. Gated B cell populations with an MFI >2 SD above the mean in normal naïve cells were considered inefficient extruders of rhodamine 123.
Immunohistochemical analysis of tonsillar tissue. Serial tonsil sections were stained using an LSAB2 system according to the manufacturer’s instructions (Dako, Carpinteria, CA). Briefly, tonsil tissue was flash frozen in OCT compound, and 4-μm-thick acetone-fixed cryostat sections were incubated at room temperature for 60 minutes with primary antibodies (anti-CD19 no. 555415, anti-CD4 no. 347327, and anti-IgD no. 555779 were obtained from BD Biosciences, San Jose, CA; anti-CD23 M4M6, Ki-67 Ki-S5, and anti-FDC CNA.42 were obtained from Dako; goat anti–FcRH-4 was obtained from R&D Systems, Minneapolis, MN), followed by detection with biotinylated anti-mouse or anti-goat antibody, streptavidin–peroxidase reagent (Dako), and aminoethylcarbazole solution. These steps were repeated for the second antibody, with the exception of the chromogens used, fuchsin and dianisobenzidine. The tissue was mounted using Permount medium and a coverslip.

Primary and secondary (GC) follicles were carefully enumerated by morphometric analysis of the FDC area (number/mm² tissue) using ImageJ software (NIH Image, National Institutes of Health, Bethesda, MD; online at: http://rsb.info.nih.gov/ij/). Both primary and secondary follicles were defined by FDC staining, with secondary follicles also distinguished by the presence of a dark zone (Ki-67⁺), a light zone (CD23⁺), and asymmetric IgD staining. Serial sections were obtained from at least 5 different levels of the tissue blocks, and results of the analysis were averaged. Sections imaged on a Leica microscope (Leica, Nussloch, Germany) were imported using ImageJ software and analyzed. Using a tool to discriminate based on shade differences, FDC network areas (identified as CNA.42⁺) were calculated, as were total tissue areas, IgD⁺ tissue areas, and FcRH-4 intensity. This program used a threshold tool to set a gray level that differentiated the stained cells from background. The program measured the area of the signal above the threshold (in pixels). The FDC area divided by the total lymphoid area of the section was defined as the FDC fraction (FDC%). Data were also expressed as FDC area relative to IgD⁺ area (FDC/IgD⁺).

These measures represent normalized global assessments of the size of the FDC compartment and provide a basis for comparison of lymphoid samples that differ in overall size and in number and size of the primary and secondary follicles in the sample. GC areas were also calculated based on the total secondary follicle FDC area divided by the total lymphoid area. This morphometric analysis has been previously documented to distinguish functionally relevant changes in FDC networks and immunologic activity (26).

Statistical analysis. Data were expressed as the mean ± SD. Statistical comparisons of mean values were performed using a nonparametric Mann-Whitney U test with XLSTAT Excel software (Addinsoft, New York, NY). P values less than 0.05 were considered significant.

RESULTS

Delayed peripheral blood memory B cell reconstitution after rituximab treatment. Two predominant populations of B cells have been identified in healthy adult peripheral blood: naive and memory subsets. Naive B cells are IgD⁺ and CD27⁻, and circulating memory B cells are CD27⁺ (27) (Figure 1A). Based on the relative expression of IgD and IgM, memory B cells can be further subdivided into isotype-switched memory cells (IgM⁺,IgD⁻) and nonswitched memory cells (largely IgM⁺,IgD⁺). During initial ontogeny, memory B cells accumulate at a significant rate and typically represent 10–20% of all peripheral blood B cells by year 2 before nearly reaching adult levels (30%) by year 5 (28). Hence, it was striking to find very low levels of memory B cells in a subset of our rituximab-treated SLE patients for long periods (up to 5 years posttreatment) (Figure 1A and Table 1). On average, for the subset of patients in clinical remission at 5 years posttreatment (n = 3), the mean ± SD percentage of total memory B cells was 6.3 ± 0.9% (normal 30.5 ± 6.9%) (P = 0.001) (Figure 1B). Decreased memory fractions were found for both switched memory cells (3.6 ± 0.5% versus 18.3 ± 5.8%; P = 0.001) and nonswitched memory cells (2.7 ± 0.4% versus 12.2 ± 4%; P = 0.001). Despite this, total serum IgG and IgM levels were maintained in the normal range (data not shown), consistent with the notion that serum Ig levels are maintained by long-lived plasma cell populations.

Interestingly, the subset of SLE patients with shorter-term or no clinical responses (Table 1) had a faster memory B cell recovery compared with the long-term responders (at 3–5 years posttreatment, post-switched memory cells 42.8 ± 18.1% versus 3.6 ± 0.5% [P = 0.036]; total memory cells 51.1 ± 23.2% versus 6.3 ± 0.9% [P = 0.009]) (Figure 1B). In contrast, nonswitched memory B cell fractions were not significantly different between short-term and long-term responders (7.4 ± 9.3% versus 2.7 ± 0.4%). Thus, long-term clinical response is associated with a prolonged and global delay in peripheral blood memory B cell recovery, whereas short-term clinical response is associated with a rapid and selective expansion of postswitched memory B cells.

Expansion of peripheral blood transitional B cells during B cell reconstitution posttreatment. Human transitional B cells, those cells in developmental transition between immature B cells in the bone marrow and mature naïve B cells in the periphery, have recently been defined based on the high expression of CD38 and CD24 (21,22). Of note, upon examination of CD27 and IgD alone, these cells could not be distinguished from the IgD⁺,CD27⁻ naïve compartment. Using the CD38⁻,CD24 classification scheme to define early transitional 1 (T1) and later transitional 2 (T2) cells (Figure
Figure 2. Correlation of the expansion of functionally immature transitional B cells during immunologic reconstitution with delayed memory B cell recovery. A, CD19+ gated peripheral blood memory B cells from 2 systemic lupus erythematosus (SLE) patients were examined by flow cytometry for the expression of CD24 and CD38 after B cell depletion therapy. The B cells of SLE patient 14 (a long-term clinical responder) reconstituted at 12 months and those of SLE patient 4 (a short-term clinical responder) reconstituted at 9 months. The transitional B cell populations are circled, with the T2 and T1 subsets gated based on incremental CD24 and CD38 expression in the normal controls and reference to lymphoid tissue with variable predominance of these populations. B, Correlation between transitional B cell expansion at reconstitution and delayed memory B cell recovery at long-term followup (f/u). Transitional B cell fractions were defined as in A, and memory B cell fractions were defined as in Figure 1A. C, Rhodamine 123 (R123) dye extrusion in the defined peripheral blood B cell populations. A representative normal control (of 6) displayed 2 major B cell populations, naive (N) B cells (red = extruders), which were low in rhodamine 123, and memory (M) B cells (CD27+), which were high in rhodamine 123 (green = nonextruders) (the mean fluorescence intensity [MFI] in the populations was significantly different \( P = 3.0 \times 10^{-5} \)). The T1/T2 populations (blue) represent a comparatively small subset and were inefficient extruders of rhodamine 123, similar to memory B cells. Early during B cell reconstitution (15 months postrituximab treatment [p rituximab]), a patient displayed enriched R123high and R123intermediate cells, with a large T1/T2 population but very few memory B cells. The CD24low,CD28intermediate,CD27+ gated naive population displayed relatively inefficient rhodamine 123 extrusion compared with the normal naive population, suggesting that most of these cells were actually intermediate in phenotype between T2 and mature naive cells. The MFI of the gated naive populations (normalized relative to memory MFI) is shown to provide a quantitative assessment of rhodamine 123 extrusion. These late transitional (T) B cells (CD24high,CD28high) were still present at time points long after B cell depletion (28 months) but were gradually replaced by naive B cells.
2A), we found a significantly higher frequency of transitional B cells in the peripheral blood of SLE patients whose B cells were reconstituting (19.3 ± 14.6%) compared with normal control peripheral blood (4.4 ± 2.4%) (P = 0.016) and with the SLE cohort at baseline (5.2 ± 8.6%) (P = 0.009) (Table 1). Although overall, SLE patients who underwent reconstitution displayed an increase in transitional B cells, we noted heterogeneity in the degree of expansion. This heterogeneity is summarized in Table 1 and depicted in Figure 2A by the
behavior of these cells and to define the immunologic consequences of a prolonged expansion of transitional B cells. Hence, we analyzed the function of emerging B cells by examining their ability to extrude the vital dye rhodamine 123, which depends on the activity of ABCB1, which is up-regulated during the final differentiation from transitional B cells to mature naive cells and is irreversibly lost on memory cell differentiation (25). Thus, normal peripheral blood naive B cells (CD38int,CD24int,IgD+).CD27−) are effective rhodamine 123 extruders, in contrast to memory and transitional B cells (Figure 2C).

Soon after B cell depletion therapy began, there was an expansion of CD24high,CD38high transitional B cells, with inefficient rhodamine 123 extrusion. Notably, B cells that based on surface markers (CD38int,CD24int,IgD+,CD27−) appear to be naive actually contain a large fraction of inefficient rhodamine 123 extruders, consistent with their status as late transitional B cells that have down-regulated CD24 and CD38. Strikingly, this predominance of immature transitional B cells persists even at late time points after B cell depletion, as revealed in the analysis of an SLE patient at 28 months posttreatment (Figure 2C). We have also recently documented the functional immaturity of reconstituting transitional B cells based on reduced proliferative responses and increased in vitro apoptosis as compared with mature naive B cells (11).

Table 2. Frequency of tonsil B cell subsets (%) and morphometric quantitation of GC activity

<table>
<thead>
<tr>
<th>Subset</th>
<th>Healthy subjects (n = 13)</th>
<th>Untreated SLE patients (n = 6)</th>
<th>SLE patients after rituximab treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Long-term responders (n = 2)</td>
</tr>
<tr>
<td>Naive (Bm1/2)</td>
<td>36.5 ± 10.5</td>
<td>31.4 ± 16.6</td>
<td>51.5 ± 4.9†</td>
</tr>
<tr>
<td>GC (Bm3/4)</td>
<td>31.9 ± 9.4</td>
<td>20.4 ± 8.3</td>
<td>19 ± 1.4</td>
</tr>
<tr>
<td>Memory (Bm5)</td>
<td>23 ± 7</td>
<td>38.6 ± 18.4</td>
<td>22.5 ± 9.2</td>
</tr>
<tr>
<td>CD27+</td>
<td>51.3 ± 8.3</td>
<td>58.3 ± 15</td>
<td>31.5 ± 4.9§</td>
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<tr>
<td>CD27+ in PB</td>
<td>ND</td>
<td>23.7 ± 19.1</td>
<td>4.0 ± 2.8§</td>
</tr>
<tr>
<td>FDC%</td>
<td>11.8 ± 5.4</td>
<td>9.1 ± 3.0</td>
<td>11.5 ± 0.6</td>
</tr>
<tr>
<td>FDC/IgD</td>
<td>0.54 ± 0.25</td>
<td>0.44 ± 0.01</td>
<td>0.68 ± 0.11</td>
</tr>
<tr>
<td>GC%</td>
<td>8.9 ± 4.2</td>
<td>6.7 ± 2.9</td>
<td>6.3 ± 2.9</td>
</tr>
</tbody>
</table>

* Values are the mean ± SD relative representation of B cell subsets, as determined by flow cytometry. All values were determined in tonsil tissue, except for CD27+ cells in peripheral blood (PB), which provides a comparison of the memory cell fraction in PB. The follicular dendritic cell (FDC) network area, indicative of ongoing immune reactions, was quantitated as a percentage of the total tissue area occupied by germinal center FDC networks (GC%) was also quantitated. Bm = mature B cells; ND = not determined.
† P = 0.02 versus healthy subjects.
‡ P < 0.0001 versus systemic lupus erythematosus (SLE) long-term responders, untreated SLE patients, and healthy subjects.
§ P = 0.01 versus healthy subjects.
¶ P < 0.0001 versus healthy subjects and untreated SLE patients.

comparison of a patient in whom reconstitution was characterized by striking transitional B cell expansion and another patient whose numbers of peripheral transitional cells were comparable to those of healthy subjects.

Patients with prolonged clinical responses had higher frequencies of transitional B cells at reconstitution (36 ± 6.8% versus 4.4 ± 2.4% in healthy controls [P < 0.0001] versus 15.0 ± 12.8% in short-term responders [P = 0.031]), as well as higher absolute transitional B cell numbers (92 ± 85 cells/μL versus 6.4 ± 7.3 cells/μL in short-term responders) (P < 0.0001) (Table 1). There was a significant negative correlation between the transitional cell fraction at reconstitution and the long-term memory cell recovery (R² = 0.5028, P = 0.005), suggesting a link between a reconstitution dominated by transitional B cells and a long-term deficiency in memory B cells (Figure 2B). Patients with prolonged remissions were also characterized by a more limited autoantibody repertoire at baseline, with positive anti–double-stranded DNA but negative extractable nuclear antigens (ENAs) (e.g., Ro antigen, La antigen, U1 RNP, Sm) as compared with the other patients who had at least 1 ENA at baseline (P = 0.001) (Table 1).

Functional immaturity of B cells posttreatment.

To the best of our knowledge, transitional B cells in autoimmune diseases have only been characterized by surface phenotype, and not by function. Such characterization, however, is important in order to understand the behavior of these cells and to define the immunologic consequences of a prolonged expansion of transitional B cells.
the peripheral blood repertoire by transitional B cells and the deficiency of memory B cells might be due to dissolution of the normal lymphoid architecture, which is critical for B cell maturation. Tonsil biopsies were performed on SLE patients with delayed peripheral blood memory B cell reconstitution 5 years posttreatment (n = 2) and were compared with SLE short-term responders posttreatment (n = 2) and controls (6 untreated SLE patients, 13 age-matched healthy subjects). When analyzed by flow cytometry, tonsil B cells contained GC phenotype cells (CD38<sup>high</sup>,IgD<sup>−</sup>) (Figure 3A and Table 2) at similar levels in long-term responders (19 ± 1.4%) as in untreated SLE patients (20.4 ± 8.3%) but at lower levels than in healthy controls (31.9 ± 9.4%) (P = 0.0005).

When defined as the Bm5 subset, memory B cells were present in the lymphoid tissue (22.5 ± 9.2%) at levels that were not significantly different from those in untreated SLE patients (38.6 ± 18.4%; P = 0.2) and healthy controls (23 ± 7%; P = 0.9). However, CD27<sup>+</sup> B cells in the tonsil, which comprise both GC and memory B cells, were reduced in the long-term responders posttreatment (31.5 ± 4.9%) as compared with untreated SLE patients (58.3 ± 15%) and healthy controls (51.3 ± 8.3%) (P < 0.0001). Additionally, naive B cells in the tonsil Bm1/2 compartment were correspondingly significantly increased in the long-term responders posttreatment (51.5 ± 4.9%) as compared with untreated SLE patients (31.4 ± 16.6%) and healthy controls (36.5 ± 10.5%) (P = 0.02). Thus, B cell subsets are altered in the reconstituting lymphoid compartment. In contrast, short-term responders posttreatment had a very low fraction of tonsil naive Bm1/2 cells (18.5 ± 2.1%), high fractions of memory Bm5 cells (74 ± 5.7%), and high fractions of CD27<sup>+</sup> B cells (76 ± 11.3%) (Table 2 and Figure 3A).

There was a correlation between the tissue (Bm5) and peripheral blood (CD27<sup>+</sup>) memory B cell fractions at identical time points (R<sup>2</sup> = 0.4, P = 0.037; n = 11). Indeed, it is notable how closely the lymphoid compartment reflects the peripheral blood B cell results. For example, the untreated SLE patient depicted in Figure 3 had a large CD27<sup>−</sup>,IgD<sup>−</sup> subset in both peripheral blood and tissue, and the SLE posttreatment short-term responder (patient 4) had a preponderance of memory B cells in both peripheral blood and tissue. However, tissue memory cell fractions overall were greater than peripheral blood memory B cell fractions, a contrast particularly evident in the 2 long-term responders posttreatment, where there was a significant discordance between tonsil memory cell and peripheral blood memory cell compartments (Figure 3 and Table 2). This suggests a lag in peripheral blood memory B cell recovery behind tissue memory recovery in the long-term responders posttreatment.

As an additional evaluation of the lymphoid memory compartment, we examined the expression of FcRH-4, an interesting inhibitory Fc receptor homolog that has been described as being present in a subset of tonsil CD27<sup>−</sup> memory cells (23). FcRH-4 is expressed on one-third of Bm5 memory B cells in normal tonsil tissue and appears to define a functionally distinct subpopulation of mucosal-associated memory cells that secrete high levels of Ig in response to T cell cytokines but fail to proliferate in response to B cell receptor

Figure 4. Occurrence of GC reactions, but with an altered memory B cell compartment, after B cell depletion and reconstitution. A, Immunohistochemical staining of tonsil tissue obtained from a control tonsillectomy sample. B cell zones were identified with CD19 and further divided into the follicular mantle (labeled with anti-IgD), follicular dendritic cells (FDCs; labeled with FDC-specific antibody CNA.42), GC dark zone (labeled with Ki-67 [blue]), and GC light zone (labeled with anti-CD23). T cells (CD4<sup>+</sup>) are found in the follicular T cell zones and sparsely within the GCs. FcRH-4<sup>+</sup> memory B cells are concentrated in the interfollicular T cell zones and adjacent to T cells within the GCs. B, GCs in tonsil tissue from SLE patient 14 during immunologic reconstitution. CD38<sup>+</sup> plasma cells are present in the interfollicular areas (also staining GC B cells), but FcRH-4<sup>+</sup> memory B cells are dramatically decreased as compared with findings in control samples. Panels surrounded by boxes show highermagnification views of the boxed areas in the adjacent panels. (Original magnification × 4; × 10 in panels surrounded by boxes.) See Figure 3 for other definitions.
ligation (23). The developmental regulation of this subset has not been entirely defined, although its abundance in normal tonsillectomy samples was independent of age over an interval of 4–48 years (data not shown) (23). Of note, the subset of memory cells that expressed FcRH-4 was greatly decreased in the Bm5 compartment in long-term responders undergoing reconstitution as compared with healthy controls (Figure 3A). Given that FcRH-4+ cells represent mostly CD27− memory cells, this finding is consistent with the fact that virtually all Bm5 cells in these patients express CD27 (data not shown).

Consistent with the flow data, GC reactions were evident on histologic analysis of long-term responders, although less well-organized, as revealed by FDC staining with the specific antibody CNA.42, Ki-67 antigen staining for the GC dark zone, and CD23 staining for the GC light zone (Figure 4). Morphometric analysis revealed similar immunologic activity compared with healthy controls and untreated SLE patients, as quantitated by FDC networks (FDC% and FDC/IgD ratio) and the lymphoid area occupied by GCs (GC%) (Table 2). Moreover, CD38+ plasma cells were present in the extrafollicular areas, suggestive of ongoing immune reactions. Also consistent with the flow analysis, tissue staining for FcRH-4+ memory B cells demonstrated a dramatic decrease of this subset in the long-term responders (Figure 4B) as compared with healthy controls (Figure 4A), untreated SLE patients, and short-term responders (P < 0.0001 for the difference in FcRH-4 staining). This suggests alterations in memory B cell generation in the subset of patients with a paucity of circulating memory B cells.

Combined with the flow cytometry data, these results suggest a peripheral lymphoid compartment still in recovery after B cell depletion in the long-term responders. Interestingly, tonsils of short-term responders had reduced FDC and GC fractions histologically, corresponding to a significant reduction in GC phenotype cells, by flow cytometry (Table 2). Combined with the dramatic expansion of memory B cells in these tonsils, this finding leads us to speculate that tissue depletion of memory B cells was incomplete, leading to the occupation of immunologic niches by residual memory B cells and the inhibition of new GC reactions.

DISCUSSION

This study identifies a subset of SLE patients with prolonged clinical and immunologic remission after B cell depletion therapy in which the peripheral compartment is remarkable for a longstanding scarcity of memory B cells and prolonged dominance of transitional B cells. Given that lymphotoxin α−bearing B cells are critical for the organization of B cell follicles, the T cell zone, and the marginal zone (29), we reasoned that disruption of these locales (critical for the selection and development of B cells) could account for these observations. However, GCs and FDC networks in the tonsils were intact in patients with prolonged peripheral blood memory B cell depletion. On the other hand, even several years after B cell depletion, the peripheral lymphoid tissue was disturbed, with a relative increase in naive B cells compared with antigen-activated subsets and a loss of the recently described tissue-based FcRH-4−bearing memory B cells. The discrepancy between the very low memory B cell fractions in the peripheral blood and the presence of memory B cells in the lymphoid tissue suggests that peripheral blood memory B cell reconstitution lags behind tissue recovery.

Our studies provide insight into the heterogeneity of human memory B cell populations, their potential origin, and their dynamic generation in peripheral lymphoid tissue. Naive B cells are activated within secondary lymphoid tissues by antigen stimulation with T cell help to form GCs, the site of B cell proliferation, somatic hypermutation, and Ig class switching. Within the GC, B cells may undergo differentiation to Ig-secreting plasma cells or memory B cells. It is worth bearing in mind, however, that human memory B cells are a heterogeneous population composed of IgD−isotype-switched (either IgG+ or IgA+), nonswitched, and the recently described subset of memory B cells that lacks the classic CD27 marker (23). The latter population expresses the unique Fc receptor homolog, FcRH-4, and appears poised to undergo plasma cell differentiation in response to T cell–dependent stimulation. Our observation that this memory cell subpopulation is strikingly decreased during recovery from B cell depletion therapy is consistent with the idea that it represents a different sublineage of memory B cells. Similarly, the nonswitched IgM memory B cell population in peripheral blood likely represents a distinct memory cell lineage related to the splenic marginal zone B cell and is important for T cell–independent immune responses against encapsulated bacteria, such as pneumococcus (28,30).

Interestingly, SLE patients with shorter clinical responses posttreatment experienced faster and more pronounced peripheral blood expansion of post-GC memory B cells than of marginal zone memory B cells, also consistent with the distinct origin of these cell
populations. We suspect that tissue depletion of post-GC memory cells may be incomplete in these patients, providing an opportunity for the selective expansion of this subset in the lymphopenic environment. On the other hand, rituximab-treated patients with prolonged clinical remissions had a prolonged delay in both switched and nonswitched memory B cell reconstitution. These patients may experience more complete tissue B cell depletion that erases preexisting memory, setting into motion a feedback mechanism of exuberant bone marrow–derived repopulation with the subsequent slow development of a new B cell memory compartment as the lymphoid tissue recovers. Based on our findings, we suggest that a reconstitution profile dominated by memory B cells, as opposed to transitional B cells, may represent an immunologically relevant marker by which to guide decisions regarding treatment with rituximab.

Our findings are consistent with posttransplantation data that suggest prolonged B cell dysfunction, including the absence of somatic hypermutation (31), delayed class switching (32), and disrupted GC reactions (33). Consistent with these findings and our own, recent evidence indicates that the recovery of CD27+ memory B cells after hematopoietic stem cell transplantation is delayed (34), as is peripheral blood memory B cell recovery after targeted B cell depletion in transplant recipients (35), RA patients (12,13), and lymphoma patients (11). In contrast to reconstitution after stem cell transplantation, where the immaturity of other immune cells and the disruption of lymphoid architecture by conditioning regimens are likely to contribute to slow B cell development and maturation, pure B cell depletion would not be expected to result in such a delayed immunologic reconstitution unless human B cells play fundamental roles in the development and organization of the lymphoid architecture and in the regulation of T cells and DCs (36). Indeed, our data do suggest that targeted B cell depletion may alter the generation of memory B cells in the peripheral lymphoid tissue for a prolonged period, likely due in part to changes in lymphoid organization. The latter will need to be further defined through examination of lymphoid biopsies at earlier time points after B cell depletion therapy.

Another unique aspect of our study is the finding that B cell reconstitution varies between patients in a way that impacts the ultimate clinical and immunologic outcome. The fact that in a subset of SLE patients, reconstituted B cells are predominantly memory B cells is in contradistinction to RA and lymphoma patients treated with rituximab, where transitional and naive B cells universally predominate early (11,13). It will be important to confirm this finding in larger numbers of SLE patients treated with full-dose rituximab. Although it is possible that some of the heterogeneity in reconstitution observed here is related to the dose-escalating nature of our study, we believe this is unlikely to be a major contributor, given that patients with memory versus transitional B cell predominance were found in both low- and full-dose treatment groups.

The observation that rapid outgrowth of memory B cells in SLE is associated with antibodies against ENAs raises the intriguing possibility of a causal relationship. Since autoantibodies against ENAs and other RNA binding proteins may be particularly potent inducers of interferon-α (37), it is tempting to speculate that a memory B cell–predominant reconstitution may be related to the effects of interferon-α on B cell population dynamics.

Regardless of the underlying explanation, our findings have important implications for the selection of patients who might be particularly responsive to B cell depletion therapy. The prolonged clinical remissions attained in the subset of SLE patients whose reconstituted B cells were predominantly transitional B cells raises the possibility that a new B cell compartment may develop with proper censoring of autoreactivity after B cell depletion therapy. A definitive answer to this critical question will have to await larger studies with formal testing of both emerging B cell autoreactivity and memory B cell responses (both for exogenous and self antigens). Moreover, it will be essential to understand the mechanisms that underlie this effect. One scenario is that during B cell repopulation, stochastic events and environmental hits fail to converge and reproduce the breakdown of tolerance characteristic of the initial disease. Alternatively, the absence of B cells could create profound changes in other immune cells, including T cell subsets (altered T helper cell polarization, defective migration of CXCR5+ follicular T helper cells, or T regulatory cell expansion) (7,8,38–40) and DCs (9), as has been suggested in recent studies (41,42). Finally, a reconstitution dominated by immature and virgin B cells may lead preferentially to T cell anergy (10).

Our results demonstrate that B cell recovery following rituximab treatment in SLE is associated with a delay in peripheral blood memory B cell reconstitution that correlates with a reconstitution dominated by an expansion of immature transitional B cells and more prolonged clinical responses. Disruption of the normal peripheral lymphoid generation of B cell memory may be one mechanism underlying this observation. Although the broader applicability of these findings should
be confirmed in a larger group of patients, the possibility is raised that profound B cell depletion and subsequent de novo reconstitution may provide the autoimmune system a chance to re-form. It is important to bear in mind, however, that this may be at the expense of a treatment-induced immunodeficiency. Continued study of B cell–depleted patients should greatly enhance our ability to rationally tailor therapy, contribute to our understanding of basic aspects of B cell biology and homeostasis, and shed invaluable light on the influence of B cells on other arms of the immune system.

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AUTHOR CONTRIBUTIONS

Dr. Anolik had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Anolik, Looney, Sanz.

Acquisition of data. Anolik, Barnard, Owen, Zheng.

Analysis and interpretation of data. Anolik, Barnard, Owen, Sanz.


Statistical analysis. Anolik, Looney.

Patient recruitment. Kemshetti.

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CD19 Hyperexpression Augments Sle1-Induced Humoral Autoimmunity but Not Clinical Nephritis

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Objective. B cell hyperactivity is a common denominator in murine and human systemic lupus erythematosus. Some susceptibility genes in lupus are associated with B cell hyperactivity, but others are clearly not. While the Sle1 lupus susceptibility locus of NZM2410/NZW origin leads to chromatin-focused autoimmunity, genetically engineered overexpression of CD19 leads to “generalized” B cell hyperactivity. We undertook this study to determine the degree to which generalized B cell hyperactivity can amplify lupus pathogenesis.

Methods. To elucidate the impact of generalized B cell hyperactivity on Sle1-triggered autoimmunity, B6 mice bearing the human CD19 transgene were rendered congenic for the Sle1 genetic locus and phenotyped for serologic, cellular, and pathologic evidence of lupus.

Results. As expected, B6.Sle1.hCD19Tg/Tg mice, homozygous at Sle1 and bearing the hCD19 transgene, exhibited high levels of IgM and IgG anti-DNA/antiglomerular autoantibodies, skewed B cell subsets, and profoundly activated B and T cells. Despite exhibiting glomerular IgM, IgG, and complement deposits, these mice did not exhibit accelerated mortality or any clinical evidence of renal dysfunction.

Conclusion. Generalized B cell hyperactivity may augment humoral autoimmunity, but this may not suffice to engender end-organ disease in lupus. These findings allude to the presence of an additional distal checkpoint that dissociates pathogenic autoantibody formation and renal immunoglobulin deposition from the progression to clinical nephritis in lupus.

“Generalized B cell hyperactivity” has been documented in several murine models of lupus (1,2). This phenomenon is evidenced by the activated surface phenotype of B cells, heightened spontaneous secretion of antibodies by cultured B cells, and the expanded population of B1 cells seen in most murine models of lupus (3–8). Transfer studies involving allotype-marked bone marrow (as well as pre–B cells) and tetraparental chimera experiments have also indicated that these phenotypes are genetically encoded in a B cell–intrinsic manner (9–13). Generalized B cell hyperactivity has also been documented in patients with lupus (14–16). The advent of microsatellite-based linkage analysis has shed light on the genetic basis of B cell hyperactivity in lupus. Linkage analysis in several murine models has uncovered multiple lupus susceptibility loci (for review, see refs. 17–20). In particular, a couple of loci on mouse chromosome 4 have been identified by several investigators to be linked to autoantibody production or other features of B cell hyperactivity (21–28). When 1 of these loci, Sle2 of NZM2410/NZW origin, was introgressed onto the normal C57BL/6 (B6) background, it precipitated multiple lupus susceptibility loci (for review, see refs. 17–20). In particular, a couple of loci on mouse chromosome 4 have been identified by several investigators to be linked to autoantibody production or other features of B cell hyperactivity (21–28). When 1 of these loci, Sle2 of NZM2410/NZW origin, was introgressed onto the normal C57BL/6 (B6) background, it precipitated several B cell phenotypes including polyclonal hypergammaglobulinemia and elevated B1 cell formation (29–31).

In addition to the above lessons gleaned from mapping studies in lupus, additional molecular insights have also arisen from “engineered” mouse models that exhibit lupus-like disease. For example, aberrant expression of molecules such as CD19, CD21, CD22, Lyn, src homology 2 (SH2) domain–containing phosphatase 1 (SHP-1), Btk, and Vav can all lead to generalized B cell...
hyperactivity and to a varying degree of autoreactivity (for review, see refs. 32–34). Importantly, it appears that several of these molecules may be acting in concert to determine the stringency of B cell tolerance and the degree of B cell hyperactivity (35,36). Using a wide spectrum of transgenic/knockout models, the prediction that the strength of B cell signaling can greatly influence the degree of B cell hyperactivity (as gauged by serum IgM levels and/or the extent of B1 cell expansion) has been directly verified (37,38). Of relevance to this communication is the CD19 molecule, the cell surface density of which is carefully regulated during B cell differentiation (36,39,40). Based on the observation that human CD19 and murine CD19 function equivalently (41), transgenic mice expressing varying levels of human CD19 have been generated and analyzed. In these mice, the hypoppression of CD19 on B cells leads to increased serum antibodies, activated B2 cells, and elevated B1 cell development; in contrast, the loss of CD19 reverses these same phenotypes (41–47).

Although it is quite clear that B cell hyperactivity is a common denominator in murine lupus, the precise contribution of B cell hyperactivity toward the pathogenesis of lupus remains to be elucidated. In addition, although B1 cell expansion and elevated levels of antinuclear antibodies (ANAs) and prominent in most reverse- and forward-genetic models of lupus, end-organ disease and clinical nephritis are not concordantly seen in every model. We entertained several alternative explanations for this apparent discordance. First, it was possible that the serum antibodies elicited by generalized B cell hyperactivity in some of these models might not be sufficient in quantity or quality for the induction of pathology. For example, one could propose that the phenomenon of B cell hyperactivity might simply be promoting the hypoppression of “natural autoantibodies,” which might not be pathogenic. Alternatively, it remained possible that the observed discordance might have arisen from the potential impact of the different molecules (that modulate B cell hyperactivity) on cell types other than B cells. For instance, the effects of SHP-1 and Lyn on neutrophil biology and of Lyn/Fyn on intrinsic renal processes are well documented (48–50).

To investigate the role played by generalized B cell hyperactivity in the pathogenesis of lupus, we elected to study a B6-based congenic model in which tolerance to chromatin is breached by the expression of a lupus susceptibility locus, Sle1, of NZM2410/NZW origin. In this study, B6 mice rendered congenic for the NZM2410/NZW-derived “z” allele of Sle1 are referred to as B6.Sle1 mice (51,52). B6.Sle1-congenic mice, unlike the B6 controls, exhibit high titers of IgG antihistone–DNA ANAs due to a B cell–intrinsic loss of tolerance to chromatin (51,52). Despite a high penetrance of IgG antichromatin antibodies, the penetrance of IgG anti–double-stranded DNA (anti-dsDNA) ANAs and nephrophilic autoantibodies and the incidence of glomerulonephritis (GN) are relatively low in this strain. In addition, these mice do not exhibit any expansion of B1 cells or polyclonal hypergammaglobulinemia, the hallmarks of generalized B cell hyperactivity. Thus, although Sle1 initiates the cascade of events leading to lupus (i.e., breach of tolerance to chromatin), this model lacks the additional genetic players that appear to be required for the generation of pathogenic ANAs and clinical GN (53–58). To specifically ascertain whether “generalized B cell hyperactivity” has the potential to transition Sle1-driven incipient autoimmunity to full-blown lupus, we have generated and studied B6.Sle1.hCD19Tg/Tg mice, which are homozygous at Sle1 and bear the hCD19 transgene (hCD19Tg), a transgene that dictates generalized B cell hyperactivity with little impact on other cell types.

MATERIALS AND METHODS

Animal models. B6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and subsequently bred in our animal colony. B6.Sle1NZM2410/NZM2410 mice (referred to as B6.Sle1 mice) are congenic homozygotes for the 95% confidence interval flanking Sle1, derived from NZM2410, with termini at D1Mit101 and D1Mit155 on chromosome 1. The immunologic phenotypes of this strain have previously been reported (51). Likewise, B6.hCD19-transgenic mice (B6.hCD19Tg/Tg mice) bearing the hCD19 transgene have previously been demonstrated to develop a significant degree of B cell hyperactivity (42,44). B6.Sle1 mice were bred with B6.hCD19Tg/Tg mice to derive F1 mice. These F1 mice were brother-sister mated to derive progeny; from these progeny, mice that were double “homozygotes” at both loci were selected. Mice expressing Sle1 and hCD19Tg both homozygously, are referred to as B6.Sle1.hCD19Tg/Tg mice. Sle1 was identified using microsatellite primers, and zygosity of the hCD19 transgene was ascertained by flow cytometry using an antibody to human CD19, as described earlier (42,44). In addition, mice that were hemizygous for the hCD19 transgene (but homozygous for Sle1) were also studied; these are referred to as B6.Sle1.hCD19Tg/Tg mice. All mice used in this study were bred and housed in a specific pathogen–free colony at the University of Texas Southwestern Animal Resources Center. Both male and female mice were used, since no significant sex differences were noted in any of the phenotypes studied.

Cellular and serologic phenotyping. Splenocytes, peritoneal cavity cells, and inguinal lymph nodes (LNs) were obtained and prepared for flow cytometry as described elsewhere (53–55). Flow cytometric analysis was performed as
described previously (53,55). The following dye- or biotin-coupled antibodies were obtained from BD PharMingen (San Diego, CA): anti-CD4 (RM4-5), anti-CD5 (53-7.3), anti-CD8 (Ly-2), anti-CD21 (7G6), anti-CD23 (B3B4), anti-CD24 (M1/69), anti-CD25 (7D4), anti-CD43 (S7), anti-CD44 (IM7), anti-CD45R/B220 (RA3-6B2), anti-CD62L (MEL14), anti-CD69 (H1.2F3), anti-CD80/B7-1 (16-10A1), anti-CD86/B7-2 (GL1), and IgM (R.6.60.2). Dead cells were excluded on the basis of scatter characteristics, and 10,000 events were acquired per sample. The mean linear units on the forward scatter channel were used as indicators of cell size. For the in vitro ANA production assays, red cell–depleted splenocytes (with or without flow sorting based on surface expression of B220, CD21, and CD23) were cultured at 10^6/ml at 37°C with or without lipopolysaccharide (20 μg/ml; Sigma, St. Louis, MO). Supernatants were harvested 5 days postculture, diluted 1:2, and assayed for IgG ANAs by enzyme-linked immunosorbent assay (ELISA). Total serum IgM and IgG antibody levels as well as the levels of anti-dsDNA, antihistone, and antihistone–DNA antibodies in sera or supernatant were assayed as described previously (53,55). The glomerular-binding ELISA has also been described previously (53,55).

In this report, B220+/CD23low,CD5− B cells are broadly labeled as “B1b” cells, in keeping with the use of this terminology in earlier reports. However, additional surface markers and functional assays were used to further classify or identify these cells as marginal-zone B cells, preplasmablasts, or as described previously (42,44), B6. B cells are prominently activated in these mice (Table 1). A greater fraction of CD69+ activated/memory CD4 T cells that were CD69+ were noted in these mice (Table 1). A greater fraction of CD69+ activated/memory CD4 T cells that were CD69+ were noted in these mice (Table 1).

### Results

#### Phenotypes in the parental strains. As expected from earlier studies (51), B6.Sle1-congenic mice revealed modest splenomegaly with several features of lymphocyte activation, as summarized in Table 1. Thus, for instance, compared with those of B6 controls, B6.Sle1 mouse spleens were larger (P < 0.009) and had a greater fraction of CD69+ CD4 T cells (P < 0.02), CD69+ CD8 T cells (P < 0.007), and B7-2–positive B cells (P < 0.0003). Likewise, consistent with earlier reports (42,44), B6.hCD19Tg/Tg mice exhibited several interesting B cell phenotypes (Table 1). These included an expansion of B1a cells in the spleen (P < 0.0004) and in the peritoneal cavity (P < 0.008) compared with the B6 controls (Table 1). In addition, B2 cells of B6.hCD19Tg/Tg mice expressed increased surface levels of B7-1/CD80 (P < 0.0005); B7-2/CD86 (P < 2 × 10^-6); B haplotype of class II major histocompatibility complex gene, I-A (P < 0.005); and CD44 (P < 3 × 10^-5) compared with B6 mouse–derived B2 cells. Compared with B6 control mouse spleens, B6.hCD19Tg/Tg mouse spleens also exhibited increased CD4:CD8 ratios (P < 0.002) and significantly expanded populations of activated/memory CD4 T cells that were CD69+ (P < 0.035), CD25+ (P < 0.04), CD62Llow (P < 0.03), and CD45RBlow (P < 5 × 10^-6). Splenic CD8 T cells were also prominently activated in these mice (Table 1). A similar degree of CD4 T cell activation was also noted in the LNs of B6.hCD19Tg/Tg mice (Table 1).
Epistatic impact of Sle1/CD19 on T cell phenotypes. To determine whether generalized B cell hyperactivity as dictated by the hCD19 transgene could amplify any of the Sle1-induced immunophenotypes, we next examined B6.Sle1.hCD19<sup>Tg</sup><sup>Tg</sup> mice. Clearly, this strain did not differ from B6.Sle1 mice in spleen size (P > 0.4), LN size (P > 0.1; data not shown), total spleen cell numbers (P > 0.9), or total LN cell numbers (P > 0.76), as detailed in Table 1. However, all lymphocyte subsets from B6.Sle1.hCD19<sup>Tg</sup><sup>Tg</sup> mice were far more activated in phenotype than those from B6.Sle1 controls. Thus, B6.Sle1.hCD19<sup>Tg</sup><sup>Tg</sup> mouse splenocytes exhibited elevated CD4:CD8 ratios (P < 0.0005) and increased levels of CD4 activated/memory T cells, as evidenced by the percentage of CD4 T cells that were CD69<sup>+</sup> (P < 0.0002), CD62L<sup>low</sup> (P < 0.064), and CD45RB<sup>low</sup> (P < 0.045), compared with B6.Sle1 mouse splenic T cells. Likewise, these mice also exhibited significantly increased levels of activated B cells relative to B6 and B6.Sle1 controls (Table 1). Similar differences in T cell activation were also seen in the LNs of these mice (Table 1).

It was interesting to note, however, that the lymphocyte activation status of B6.Sle1.hCD19<sup>Tg</sup><sup>Tg</sup> mice paralleled that observed in B6.hCD19<sup>Tg</sup><sup>Tg</sup> mice. Indeed, the CD4:CD8 ratios, the activation patterns of CD4 and
CD8 T cells, and B cells of these 2 strains did not differ significantly from each other (Table 1). Although B6.Sle1.hCD19Tg/Tg mice resembled the B6.Sle1 “parents” in exhibiting modest lymphopoenomegaly and the B6.hCD19Tg/Tg “parents” in terms of their lymphocyte activation status, they clearly exhibited a couple of epistatic phenotypes that were not prominent in either “parental” strain. One of these differences was the significant expansion (in terms of absolute numbers) of activated CD4+ T cells compared with controls (Table 1).

Epistatic impact of Sle1/CD19 on B cell phenotypes. Perhaps more important, B6.Sle1.hCD19Tg/Tg mouse spleens revealed a respectable expansion of B220+CD23low,CD5- B cells (broadly labeled as “B1b” cells) relative to follicular B2 cells (B220+,CD23+,CD5-) or B1a cells (B220intermediate,CD23low,CD5+). Thus, in age-matched B6 mouse spleens, follicular B2 cells, “B1b” cells, and B1a cells constituted 55.8%, 10.2%, and 2.0%, respectively, of all live cells (Table 1), with a similar distribution being noted in B6.Sle1 mouse LNs also displayed a similar expansion of activated CD4 T cells compared with controls (Table 1).

From the above cellular and serologic studies, it appeared that the hCD19 transgene–encoded B cell hyperactivity was significantly boosting the degree of lymphocytic activation and humoral autoimmunity in the context of Sle1. Previous work has elegantly delineated how the B cell phenotypes dictated by CD19 overexpression function as a quantitative trait (44,46). In order to assess whether hemizygosity for the hCD19 transgene
had an intermediate effect on Sle1-triggered autoimmunity, we next examined B6.Sle1.hCD19Tg/H11002 mice that were hemizygous for the hCD19 transgene, but homozygous at Sle1. As shown in Figure 2, transgene-hemizygous mice exhibited intermediate levels of “B1b” and B2 cells, CD4:CD8 ratios, B cell activation (as evidenced by surface levels of CD44), and serum ANAs. Thus, in the context of a lupus-prone genome, generalised B cell hyperactivity (as dictated by the hCD19 transgene) appears to regulate autoimmunity in a quantitative, gene dose–dependent manner, consistent with previous reports (44,46).

**Epistatic impact of Sle1/CD19 on renal disease.**

Given the significant impact of the hCD19 transgene on Sle1-triggered ANA formation, we predicted that B6.Sle1.hCD19Tg/− mice would have significant renal disease. The different strains were therefore monitored for urinary protein excretion and BUN levels. The levels of urinary protein excreted by 12-month-old B6.Sle1 mice did not differ significantly from the levels excreted by age-matched B6 controls (Figure 3A). Interestingly, a couple of the B6.hCD19Tg/− mice exhibited increased proteinuria (Figure 3A). Surprisingly, despite the significant degree of humoral autoimmunity, elevated proteinuria was not a prominent feature of B6.Sle1.hCD19Tg/− mice. The low frequency of significant proteinuria (defined as >1.2 mg/24 hours) in B6.Sle1.hCD19Tg/− mice did not differ significantly from that seen in B6.Sle1 and B6.hCD19Tg/− controls (Figure 3A). Likewise, none of these strains exhibited elevated levels of BUN (>30 mg/dl) (Figure 3B) or accelerated mortality (data not shown).

To gain a more accurate assessment of renal function, we next examined the glomerular filtration rates in these mice. As shown in Figure 3C, B6.Sle1.hCD19Tg/− and age-matched B6 mice had similar inulin clearance rates. Hence, the above findings deviate quite sharply from the observations in several
other polygenic models of lupus in which Sle1 was also a common denominator. The epistatic interplay of Sle3, Faslpr, and Yaa with Sle1 has been observed to trigger nephrophilic IgG ANA formation and renal disease (53,55,58). For instance, unlike the B6.Sle1 and B6.Faslpr controls, B6.Sle1.Faslpr mice have been noted to exhibit significantly greater levels of proteinuria (1.7 ± 0.2 mg/24 hours) and BUN (41.5 ± 9.7 mg/dl) as early as age 3–6 months, accompanied by accelerated mortality (55).

Figure 2. Gene-dose effect of the hCD19 transgene (CD19Tg) in the context of Sle1. Twelve-month-old B6.Sle1.hCD19Tg−/− hemizygous (Tg−/−) mice were compared with age-matched B6.Sle1 and B6.Sle1.hCD19Tg+/− (Tg+/−) mice with respect to the levels of splenic “B1b” cells (B220−,CD23−,CD5− B cells) (n = 5–6 per group) (A), splenic B2 cells (n = 5–6 per group) (B), mean fluorescence intensity (MFI) of CD44 on splenic B2 cells (n = 5–6 per group) (C), splenic CD4:CD8 ratios (n = 5–6 per group) (D), serum levels of IgG anti-dsDNA antibodies (n = 12 per group) (E), and serum levels of IgG anti-ssDNA antibodies (n = 12 per group) (F). Each symbol represents a single mouse. Horizontal bars represent mean values. P values are versus B6.Sle1.hCD19Tg+/− hemizygous mice, by Student’s t-test. See Figure 1 for other definitions.

Figure 3. Minimal evidence of clinical renal disease in B6.Sle1.hCD19Tg+/− mice. Twenty-four-hour urinary protein levels (A), blood urea nitrogen (BUN) levels (B), and inulin clearance rate (as a measure of the glomerular filtration rate) (C) were assayed in 12-month-old C57BL/6 (B6) mice, B6.Sle1 mice, B6.hCD19+/− (B6.CD19) mice, and B6.Sle1.hCD19+/− (B6.Sle1.CD19) mice. Each symbol represents a single mouse. Horizontal bars represent mean values. P values are versus B6.Sle1.hCD19+/− mice, by Student’s t-test. NS = not significant.
Likewise, clinical disease is prominent in B6.\textit{Sle1}.\textit{Yaa} and B6.\textit{Sle1}.hCD19\textsuperscript{Tg/Tg} polycongenic models (58).

We entertained several possible reasons that potentially account for the apparent discordance between the expected clinical phenotype based on the serologic findings and the actual renal status observed in B6.\textit{Sle1}.hCD19\textsuperscript{Tg/Tg} mice. First, although B6.\textit{Sle1}.hCD19\textsuperscript{Tg/Tg} mice were seropositive for IgG anti-dsDNA ANAs, the titers of these antibodies may not have been sufficiently high to cause disease. Second, it was possible that the anti-dsDNA antibodies seen in these mice might have been of low avidity (for dsDNA) compared with those present in the other seropositive, polycongenic strains that do go on to develop disease. Third, it remained possible that end-organ pathology was contingent upon some nephrophilic specificity other

\begin{figure}
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\caption{Comparison of autoantibodies in B6.\textit{Sle1}.hCD19\textsuperscript{Tg/Tg} and B6.\textit{Sle1}.\textit{Fas}\textsuperscript{fr} mice. A, Serum IgG anti-dsDNA, IgG antihistone–DNA, and IgG antiglomerular antibody levels in 12-month-old B6.\textit{Sle1} mice (n = 12), 5–6-month-old B6.\textit{Sle1}.\textit{Fas}\textsuperscript{fr} mice (n = 12), and 12-month-old B6.\textit{Sle1}.hCD19\textsuperscript{Tg/Tg} mice (n = 12). Younger B6.\textit{Sle1}.\textit{Fas}\textsuperscript{fr} mice were not available, since this strain exhibits a 6-month mortality rate >80\% (55). Each symbol represents a single mouse. Horizontal bars represent mean values. Dotted lines indicate mean levels of the corresponding antibodies in 12 sera. P values are for autoantibody levels in B6.\textit{Sle1}.\textit{Fas}\textsuperscript{fr} mice versus those in B6.\textit{Sle1}.hCD19\textsuperscript{Tg/Tg} mice, by Student’s \textit{t}-test. B and C, Sera from 5–6-month-old B6.\textit{Sle1}.\textit{Fas}\textsuperscript{fr} mice (n = 4) (B) and 12-month-old B6.\textit{Sle1}.hCD19\textsuperscript{Tg/Tg} mice (n = 4) (C) were tested for dsDNA binding by enzyme-linked immunosorbent assay, in the presence of increasing concentrations of NaCl. Each line represents data pertaining to serum from an individual mouse. The mean inhibitory concentration of NaCl required for dampening DNA binding by 50\% (IC\textsubscript{50}, dotted line) was recorded for each serum sample tested as an indicator of its avidity for dsDNA. The IC\textsubscript{50} values for dsDNA binding did not differ significantly between the 2 groups of sera (P > 0.05). See Figure 1 for other definitions.}
\end{figure}
than anti-dsDNA antibodies. Finally, one could posit that these different strains varied in their degrees of end-organ disease, not because of differences in the quantity or fine specificity of their respective serum antibodies, but because of intrinsic differences in renal susceptibility to immune-mediated damage.

To test the above possibilities, we next compared the serologic phenotypes of B6.Sle1.hCD19Tg/Tg and B6.Sle1.FasΔpr mice (55). As depicted in Figure 4A, B6.Sle1.hCD19Tg/Tg mouse sera exhibited higher levels of IgG anti-dsDNA antibodies ($P < 0.004$) and IgG anti-histone–DNA antibodies ($P < 0.07$) compared with B6.Sle1.FasΔpr mouse sera. In addition, B6.Sle1.hCD19Tg/Tg mice also harbored significantly elevated levels of serum IgG antiglomerular antibodies ($P < 0.02$) (Figure 4A). Thus, the increased end-organ pathology seen in B6.Sle1.FasΔpr mice was not simply the result of elevated serum levels of anti-dsDNA antibodies or any differences in the nephrophilic potential of sera from either of these strains. Next, increasing NaCl concentrations were used to gauge the avidity of serum antibodies for dsDNA in these 2 strains. As illustrated in Figures 4B and C, B6.Sle1.FasΔpr mouse sera exhibited anti-dsDNA 50% inhibition concentration (IC$_{50}$) values (mean $\pm$ SEM) of 0.23 $\pm$ 0.05 M, while B6.Sle1.hCD19Tg/Tg mouse sera exhibited anti-dsDNA IC$_{50}$ values of 0.17 $\pm$ 0.001 M ($P > 0.1$), indicating that the sera from these 2 strains did not differ significantly in their avidity for dsDNA, as gauged by the ease with which anti-dsDNA antibodies were dissociated from the target DNA.

Given that B6.Sle1.hCD19Tg/Tg mice possessed significantly higher amounts of nephrophilic IgG anti-dsDNA ANAs, we wondered whether these antibodies 1) targeted the kidneys for deposition and 2) induced any pathologic changes in the kidneys. As depicted in Figures 5A–D, B6.Sle1.hCD19Tg/Tg mouse kidneys revealed prominent IgG, IgM, and C3 deposits in their

![Figure 5](image-url)
glomeruli, in some cases exceeding the level of deposits observed in B6.Sle1.Fas<sup>pr</sup> mice. Moreover, 6 of the 6 B6.Sle1.hCD19<sup>Tg/Tg</sup> mouse kidneys examined exhibited severe proliferative GN (grades 3 and 4, with an average grade of 3.7) (Figure 5E), with prominent perivascular and periglomerular infiltrates. In addition, B6.Sle1.hCD19<sup>Tg/Tg</sup> mouse kidneys exhibited more severe tubulointerstitial disease compared with control mouse kidneys (Figure 5F). Thus, despite the exuberant levels of serum anti-dsDNA and antiglomerular antibodies, the uniform presence of renal Ig and C3 deposits, and pathologic evidence of renal damage, B6.Sle1.hCD19<sup>Tg/Tg</sup> mice remained remarkably healthy clinically. These findings lend credence to the existence of an additional checkpoint that dictates whether mice with renal Ig deposits and histologic nephritis progress to developing clinical nephritis with compromised renal function.

**DISCUSSION**

Generalized B cell hyperactivity is a well-studied trait that has been documented in all mouse models of lupus studied to date. Indeed, this appears to be an important underlying trait in human lupus as well, as recently observed (15). Moreover, the B cell phenotypes observed in mice bearing induced mutations or transgenic overexpression of several signaling molecules such as CD19, CD22, Btk, Lyn, SHP-1, and Vav further bolster the notion that generalized B cell hyperactivity can potentially contribute to systemic autoimmunity (for review, see refs. 32–34). Nevertheless, the genetic origins and precise pathogenic contributions of B cell hyperactivity to spontaneously arising lupus have remained unclear. To delineate the specific contribution of generalized B cell hyperactivity to the development of lupus, we have utilized 2 unique mouse strains, both on the “normal” B6 background. While the B6.hCD19<sup>Tg/Tg</sup> mouse is a well-established model for generalized B cell hyperactivity, B6.Sle1 mice exhibit a B cell–intrinsic loss of tolerance to chromatin, but not generalized B cell hyperactivity, due to genetic aberrations in SLAM/ Ly108, Cr2, and possibly other genes (59–61). Importantly, neither model exhibits high titers of nephrophilic IgG anti-dsDNA autoantibodies or clinical disease. By breeding and analyzing mice bearing both these genetic components, we aimed to define the precise contribution of generalized B cell hyperactivity to the development of lupus.

Several of the cellular phenotypes noted in B6.Sle1.hCD19<sup>Tg/Tg</sup> mice resembled those seen in 1 of the 2 parental strains. Thus, while these mice mirrored the B6.Sle1 mice in terms of the extent of lymphoproliferation, they resembled the B6.hCD19<sup>Tg/Tg</sup> mice with respect to their lymphocyte activation status. In contrast, 2 cellular phenotypes appeared to be epistatic in nature. Most interestingly, B6.Sle1.hCD19<sup>Tg/Tg</sup> mice exhibited prominent expansion of B220<sup>+</sup> B cells lacking CD23, CD21, and CD5, constituting >75% of all splenic B cells. Clearly, they did not resemble anergic B cells, based on their surface phenotype and their ability to produce ANAs. In addition, they did not appear to represent immature or transitional B cells. Based on their syndecan expression and their ability to produce IgG ANAs, they are likely to represent preplasmablasts; importantly, similar B cells have also been reported in related murine models (62). In addition, they may also harbor chronically activated memory B cells similar to those described in other murine studies (63). We believe these potentially autoreactive B cells may be the end product of 2 genetic cues. First, the presence of Sle1 is likely to have breached tolerance leading to the persistence of anti-self (e.g., antichromatin) B cells (60). Second, these potentially self-reactive B cells may also be programmed to receive excessive intracellular signaling following B cell receptor crosslinking by self antigens, owing to the presence of the hCD19 transgene. Curiously, B cells with this peculiar phenotype were not prominent in either of the parental strains. One could therefore speculate that excessive and persistent triggering by self antigens may be a critical factor required for the development of such B cells.

A second interesting epistatic cellular trait exhibited by B6.Sle1.hCD19<sup>Tg/Tg</sup> mice was the massive expansion of activated CD4<sup>+</sup> T cells. Indeed, a similar phenotype of a lesser degree was seen in B6.hCD19<sup>Tg/Tg</sup> controls. Since the hCD19 transgene is expressed only in B cells, it appears likely that the T cell phenotype is a consequence of the intrinsically hyperactive B cells. It is becoming increasingly clear that the peripheral B cell pool plays an important role in shaping the development, differentiation, activation, and function of T cells (64–67). Since Sle1 also impacts B cells directly (52), it appears likely that the prominent expansion of activated T cells in B6.Sle1.hCD19<sup>Tg/Tg</sup> mice may be the direct consequence of a “superactivated” B cell compartment. Although the mechanistic basis for this phenomenon is unclear, the increased levels of costimulatory molecules on hyperactive B cells may be contributing in part. Thus, the heightened expression of B7 molecules on
B6.Sle1.hCD19Tg/Tg mouse B cells (as detailed in Table 1) may very well play an important role in mediating the T cell phenotypes seen in this strain. A non–mutually exclusive possibility is that the T cell phenotypes may be the result of the transgene being integrated into loci that influence T cell function. Clearly, additional independent transgene founders ought to be examined to test this possibility.

B6.Sle1.hCD19Tg/Tg mice constitute one of several polycongenic models of lupus on the B6 background that have recently been characterized. The NZM2410-derived “Sle2” interval was the first locus associated with B cell hyperactivity that was bred to B6.Sle1-congenic mice (58). Surprisingly, however, Sle2-associated B cell hyperactivity had only a modest impact on the phenotypes associated with Sle1 (58). The more pronounced phenotypes noted in B6.Sle1.hCD19Tg/Tg mice compared with B6.Sle1.Sle2-bicomgenic mice may be the direct consequence of the more profound degree of B cell hyperactivity associated with the CD19 transgene, or with additional modifier genes that may be present within the Sle2 interval. B6.Sle1.Sle3, B6.Sle1.FasFpr, B6.Sle1.Sle2.Sle3, and B6.Sle1.Yaa mice are previously reported polycongenic strains that develop lupus nephritis, although with differing degrees of severity (53,55,58). Compared with the above strains, the serologic and histopathologic phenotypes seen in B6.Sle1.hCD19Tg/Tg mice in the present study constitute the most severe that we have noted thus far. Despite these prominent serologic and pathologic phenotypes, clinical disease (i.e., elevated proteinuria, BUN levels, or mortality) was not prominent in these mice. This contrasts sharply with the renal phenotypes and mortality seen in the other B6.Sle1-based polycongenic lupus models alluded to above.

There is an increasing body of reports underlining the observation that the presence of anti-DNA antibodies may not always be concordant with renal disease. Chan and colleagues generated a very interesting MRL-based mouse strain that lacked circulating antibodies; despite lacking antibodies, this strain still exhibited several aspects of renal pathology and disease (68). Likewise, Waters and coworkers documented a locus on murine chromosome 4 that was important for the development of renal disease in the absence of ANAs (69). The present report documents an example of the reverse scenario, in which high levels of IgG antinuclear and antiglomerular antibodies (and glomerular deposits) are present, but not renal disease. Similar uncoupling of autoantibody deposition from renal disease has also been noted in Fc receptor–deficient mice (70).

Indeed, discordance between serum ANAs and renal disease has also been documented in human lupus (71,72).

The Ig deposits and pathologic changes seen in the kidneys of B6.Sle1.hCD19Tg/Tg mice were apparently insufficient to compromise renal function. One possibility is that the excessive levels of IgM deposits in the kidneys of B6.Sle1.hCD19Tg/Tg mice (as plotted in Figure 5D) may be playing a protective role in ameliorating the effects of local inflammatory cascades that may otherwise be triggered by IgG deposits. Indeed, IgM autoantibodies in patients with systemic lupus erythematosus (SLE) have been noted to be negatively associated with nephritis (73,74). Clinically occult proliferative lupus nephritis has also been documented in subsets of SLE patients (75,76), although the relevance of excessive glomerular IgM deposits in these patients remains to be established. It is conceivable that additional pathologic events, including extensive crescent formation and sclerotic loss of filtration units, may be necessary before renal function becomes evidently compromised in these mice (and patients). It is also possible that the end-organ disease seen in the other B6.Sle1-based polycongenic models may reflect the potential impact of the other loci (e.g., Sle3, FasFpr, etc.) on other cells (e.g., renal-intrinsic cells) that may play key roles in the pathogenesis of nephritis. Experiments are in progress to test these alternative hypotheses.

In conclusion, generalized B cell hyperactivity clearly has the potential to precipitate a wide spectrum of autoantibody specificities, depending on the genetic background. Thus, on the B6 background, it may only lead to anti–single-stranded DNA and antihistone–DNA specificities, lacking glomerular reactivity. Alternatively, on a genetic background in which self tolerance to nuclear antigens is presumably breached (e.g., in the context of Sle1), generalized B cell hyperactivity may have the capacity to give rise to a potentially pathogenic Ig repertoire, consisting of high levels of glomeruli-targeting, dsDNA-reactive autoantibodies accompanied by the accumulation of activated “B1b”-like B cells and activated CD4 T cells. Despite their potential to affect serum ANA levels, generalized B cell hyperactivity and antinuclear/antiglomerular autoantibodies may not be sufficient for the eventual development of fatal renal disease, alluding to the existence of additional checkpoint(s) that dictate whether renal function is compromised. Unraveling the molecular mechanisms that potentially operate to compromise renal function (in the face of an immunologic insult) will greatly augment our understanding of this disease.
AUTHOR CONTRIBUTIONS

Dr. Mohan had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Shi, Xie, Tedder, Mohan.

Acquisition of data. Shi, Xie, Chang, Zhou.

Analysis and interpretation of data. Shi, Xie, Zhou, Mohan.

Manuscript preparation. Shi, Xie, Tedder, Mohan.

Statistical analysis. Shi, Xie, Mohan.

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Menopause Hormonal Therapy in Women With Systemic Lupus Erythematosus

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Objective. To evaluate the effects of menopause hormonal therapy on disease activity in women with systemic lupus erythematosus (SLE).

Methods. We conducted a double-blind, randomized clinical trial involving 106 women with SLE who were in the menopausal transition or in early or late postmenopause. Patients received a continuous-sequential estrogen-progestogen regimen (n = 52) or placebo (n = 54). Disease activity was assessed at baseline and at 1, 2, 3, 6, 9, 12, 15, 18, 21, and 24 months, according to the SLE Disease Activity Index (SLEDAI). The primary outcome measure was global disease activity, estimated by measuring the area under the SLEDAI curve. Secondary outcome measures included maximum SLEDAI score, change in SLEDAI score, incidence of lupus flares, median time to flare, medication use, and adverse events. Results were studied using intent-to-treat analysis.

Results. At baseline, demographic and disease characteristics were similar in both groups. Mean ± SD SLEDAI scores were 3.5 ± 3.3 and 3.1 ± 3.4 in the menopause hormonal therapy and placebo groups, respectively (P = 0.57). Disease activity remained mild and stable in both groups throughout the trial. There were no significant differences between the groups in global or maximum disease activity, incidence or probability of flares, or medication use. Median time to flare was 3 months in both groups. Thromboses occurred in 3 patients who received menopause hormonal therapy and in 1 patient who received placebo. One patient in each group died during the trial due to sepsis.

Conclusion. Menopause hormonal therapy did not alter disease activity during 2 years of treatment. However, an apparently increased risk of thrombosis seems to be a real threat in women with SLE who receive menopausal hormone therapy.

Systemic lupus erythematosus (SLE) is an autoimmune disease that primarily affects young women. Nonetheless, an increasing number of women with lupus now reach the postmenopausal stage because of their susceptibility to development of premature menopause, and because of the extraordinary improvement in the prognosis and survival rate (1,2).

Menopause entails the risk of developing vasomotor and other symptoms, as well as chronic conditions, such as osteoporosis. Although menopause hormonal therapy is the most effective treatment for vasomotor and urogenital symptoms, the results of the Heart and Estrogen/Progestin Replacement Study (HERS) and the Women's Health Initiative (WHI) trials, which showed that the risks outweigh the benefits (3,4), dramatically altered the medical practices of such therapy (5). Despite a striking decrease in the use of menopause hormonal therapy, many women remain eligible for it. Current guidelines recommend menopause hormonal therapy at the lowest effective dose and for the shortest time necessary (6).

Several studies have established the relevance of sex hormones in SLE. Overall, these data suggest that estrogens favor the development and/or exacerbation of the disease, while androgens seem to be protective (7–17).

Although a high rate of SLE flares in women...
taking combined oral contraceptives has been reported (18), recently we and the investigators in the Safety of Estrogens in Lupus Erythematosus: National Assessment (SELENA) group published the results of 2 randomized clinical trials, which showed that estrogen-containing oral contraceptives did not increase the risk of SLE disease activity exacerbation (19,20). Menopause hormonal therapy was safe, well tolerated, and did not increase the risk of lupus flares in observational studies (21–23); however, the SELENA group detected a slight increase in the risk of developing mild or moderate, but not severe, flares (24).

We have sought to evaluate the effects of menopause hormonal therapy on disease activity, menopause symptoms, bone mineral density, lipid profile, and mammographic breast density in women with SLE who were in the menopausal transition or in early or late postmenopause. The results described herein focused on the question of whether there is a clinically significant difference in lupus activity (25), as measured by the SLE Disease Activity Index (SLEDAI) (26), in women receiving menopause hormonal therapy in comparison with women taking placebo. The study followup was completed prior to the publication of the WHI study, and data about other outcomes will be presented in future studies.

PATIENTS AND METHODS

Study population. We conducted a randomized, double-blind, placebo-controlled, 24-month clinical trial in women with SLE who attended the outpatient clinic at our hospital. From November 1997 through November 1999, 1,981 women with SLE as defined by the American College of Rheumatology 1982 criteria (27) were assessed for eligibility. Eligible women were those who met any 2 of the following criteria: amenorrhea of ≥6 months (except for women who had undergone a hysterectomy), serum follicle-stimulating hormone levels of ≥30 IU/liter, menopause symptoms (e.g., hot flashes, night sweats, or vaginal dryness), and age of ≥48 years. Exclusion criteria included an age of >65 years, severe lupus activity at baseline (SLEDAI score >30), use of estrogens within 3 months of the screening visit, serum creatinine level of ≥2.0 mg/dl, hypertriglyceridemia (≥500 mg/dl), metabolic bone diseases, liver disease, untreated hyperthyroidism, thrombosis within the past 6 months, malignancy, endometrial hyperplasia, undiagnosed uterine bleeding, or cervical dysplasia.

Menopause status was assigned according to the Stages of Reproductive Aging Workshop definitions (28): women who had presented with endometrial bleeding within the 12 months prior to the study initiation were considered to be in the menopausal transition, whereas those with amenorrhea of at least 12 months’ duration were postmenopausal. Early postmenopausal women included those with ≥5 years of amenorrhea, whereas late postmenopausal women were those with >5 years of amenorrhea. Premature menopause was diagnosed in those women who presented with natural or medically induced menopause at age 40 or younger (6).

Study protocol. A computer-generated randomization list was used to assign the women to either menopause hormonal therapy (a continuous-sequential estrogen-progestogen regimen of conjugated equine estrogens 0.625 mg/day plus 5 mg/day of medroxyprogesterone acetate by mouth for the first 10 days per month) (Premarin and Cyclic, respectively; Wyeth Pharmaceuticals, NaucaLpan, Mexico) or a biologically inert placebo identical in appearance and packaging size to the medications in the active treatment regimen. All women received a daily supplement of 1,200 mg of calcium carbonate and 400 IU of vitamin D. The study coordinator assigned the next available number to each patient upon her entry into the trial, and pharmacy personnel dispensed the study medications according to the randomization list. Only the study coordinator (PL-R) could access the allocation list.

To maintain blinding, the rheumatologists (JS-G, MG-P) were instructed not to provide care for gynecologic or menopause symptoms, and the women were asked to avoid discussing any related issue with them. All women were told during the first visit that they might experience uterine bleeding independently of the treatment assigned. The randomization code was broken at the end of the study.

Clinical assessments. At baseline, data on sociodemographic and clinical characteristics were collected by the investigators. Information about the course of SLE, such as the date of diagnosis (i.e., the date on which 4 of the 11 criteria were met), duration of disease, number of criteria met, and damage accrual according to the Systemic Lupus International Collaborating Clinics/American College of Rheumatology Damage Index (29), was extracted from each patient’s medical chart.

SLE activity was assessed at baseline and at 1, 2, 3, 6, 9, 12, 15, 18, 21, and 24 months. Two rheumatologists (JS-G, MG-P) performed all assessments. To reduce the variability in these evaluations, a training session and a calibration exercise in the application of the SLEDAI were held before the study began and each rheumatologist examined the same patients. Treatment of the disease was administered according to the judgment of each participating provider and was recorded at each evaluation. An independent examination at each study visit was performed by the study gynecologists (MD-C, LJ-S), who were unblinded to treatment assignments. They were not allowed to either modify the treatment randomly assigned or prescribe additional treatments for osteoporosis or menopause symptoms. Adherence to treatment was assessed by self-reported medication intake, and by measurement of serum levels of estradiol at baseline and at 1, 2, 3, 6, and 15 months by specific radioimmunoassay, using reagents and protocols provided by the World Health Organization Matched Reagent Programme (Geneva, Switzerland). Study treatment was discontinued in women who had severe lupus activity, thrombosis, any other severe complications, or who needed prolonged immobilization.

The study was approved by the Institutional Committee of Biomedical Research at the Instituto de Ciencias Medicas. All patients provided written informed consent.

Outcome measures. The primary outcome measure was global disease activity throughout the followup period,
estimated as the area under the SLEDAI curve (AUC) (30).
The secondary outcome measures were the incidence of lupus
flares, the time to the first flare, changes in SLEDAI values
from baseline noted at each followup visit, and maximum
disease activity. Lupus flares and severe flares were defined as
increases in the SLEDAI score of ≥3 points or ≥12 points,
respectively, from the previous visit (31).
Data were also analyzed by SLEDAI score strata
(0, 1–5, 6–11) among the patients with active disease (SLEDAI
score of ≥1) at baseline, using the SLEDAI 2000 (SLEDAI-
2K) (32) and a modified SLEDAI (M-SLEDAI) that excludes
microhematuria and pyuria because they may be associated
with the treatment. We also recorded data on SLE treatment,
hospitalizations, thromboses, and deaths.

Statistical analysis. Data were expressed as the
mean ± SD. Between-group comparisons of lupus activity
were measured by the SLEDAI AUC, maximum SLEDAI
score, and change in SLEDAI score from baseline at each
followup visit. Continuous variables were compared using
Student's t-test, and categorical variables were examined using
the chi-square test or Fisher's exact test. Within-group com-
parisons were calculated using Wilcoxon's signed rank test.
Analysis of the incidence of flares was based on
incidence-density rates, with patient-years of followup as the
denominator, and with relative risk (RR) and 95% confidence
interval (95% CI) as the measures of association. For each
patient, we calculated the time from baseline until the first
flare, withdrawal from the study, end of followup, or death
(whichever came first). The probability of flares throughout
the study was calculated using life-table analyses and the log
rank test. All reported P values are 2-sided.

On the assumption of a mean ± SD baseline SLEDAI
value of 5.43 ± 5.04 (31), we estimated that the planned
sample size would provide an 80% chance of detecting a
difference in the SLEDAI score of ≥3 points (on a scale of
0–105, with higher scores indicating greater severity), at a
significance level of 0.05. With allowance for a 20% loss to
followup, the planned sample size was 54 patients per group.
All analyses were conducted by the intent-to-treat method
using SPSS software, version 11.5 (SPSS, Chicago, IL).

RESULTS

Characteristics of the study population. Of the
1,981 patients who underwent screening, 1,875 were
excluded because they did not meet the inclusion criteria
(n = 1,832) or declined to participate (n = 43), leaving
106 patients to be randomly assigned to either meno-
pause hormonal therapy (n = 52) or placebo (n = 54)
(Figure 1). Ninety-five menopausal women did not
qualify according to the study participation criteria, but
none was excluded from participation because of severe
disease activity (SLEDAI score >30). The eligibility,
enrollment, and recruitment fractions were 7.5%, 71%,
and 5%, respectively, and the number of women who
had to be screened in order to identify 1 patient to be
enrolled in the study was 18.7. Women who declined to
participate in the study and those who underwent ran-
domization did not differ in most demographic charac-
teristics, including previous use of menopause hormonal
therapy (P = 0.64), and the features of their disease
were similar. Nevertheless, those who underwent ran-
domization were younger (mean ± SD 48.8 ± 7.6 years
versus 52.7 ± 6.6 years; P = 0.004), developed meno-
pause at an earlier age (mean ± SD 41.5 ± 7.7 years
versus 44.2 ± 7.0 years; P = 0.04), had a higher body
mass index (BMI) (mean ± SD 26.9 ± 5.2 kg/m² versus
24.1 ± 3.8 kg/m²; P = 0.002), had a history of smoking
(P = 0.01), and consumed alcohol (P = 0.06) more than
women who declined to participate.

The baseline demographic features and manifes-
HORMONE THERAPY AND DISEASE ACTIVITY IN SLE

Table 1. Selected baseline characteristics of the 106 women, by treatment group

<table>
<thead>
<tr>
<th></th>
<th>Menopause hormonal therapy (n = 52)</th>
<th>Placebo (n = 54)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Demographic features</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, mean ± SD (range)</td>
<td>47.5 ± 7.4 (28–65)</td>
<td>50.0 ± 7.7 (28–63)</td>
</tr>
<tr>
<td>Education, mean ± SD</td>
<td>10.5 ± 4.9</td>
<td>10.3 ± 4.7</td>
</tr>
<tr>
<td>BMI, mean ± SD kg/m²</td>
<td>27.4 ± 6.1</td>
<td>26.3 ± 4.3</td>
</tr>
<tr>
<td>Current smoker</td>
<td>10 (19.2)</td>
<td>12 (22.2)</td>
</tr>
<tr>
<td>Menopause characteristics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age at menopause, mean ± SD years†</td>
<td>40.0 ± 7.5</td>
<td>42.9 ± 7.8</td>
</tr>
<tr>
<td>Years since menopause, mean ± SD†</td>
<td>8.1 ± 5.7</td>
<td>8.3 ± 6.7</td>
</tr>
<tr>
<td>Menopause-related stages‡</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Menopausal transition</td>
<td>12 (23)</td>
<td>11 (20)</td>
</tr>
<tr>
<td>Early postmenopause</td>
<td>13 (25)</td>
<td>20 (37)</td>
</tr>
<tr>
<td>Late postmenopause</td>
<td>27 (52)</td>
<td>23 (43)</td>
</tr>
<tr>
<td>Premature menopause</td>
<td>17 (33)</td>
<td>13 (24)</td>
</tr>
<tr>
<td>FSH level, IU/liter§</td>
<td>83.7 ± 48.0</td>
<td>79.0 ± 51.6</td>
</tr>
<tr>
<td>Previous use of hormone therapy</td>
<td>18 (35)</td>
<td>22 (41)</td>
</tr>
<tr>
<td>Disease characteristics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Disease duration, mean ± SD years</td>
<td>9.6 ± 8.9</td>
<td>8.9 ± 7.2</td>
</tr>
<tr>
<td>No. of SLE criteria met, mean ± SD</td>
<td>4.9 ± 1.2</td>
<td>5.2 ± 1.2</td>
</tr>
<tr>
<td>SDI score, mean ± SD</td>
<td>0.8 ± 1.2</td>
<td>0.9 ± 1.2</td>
</tr>
<tr>
<td>SLEDAI score, mean ± SD (range)</td>
<td>3.5 ± 3.3 (0–10)</td>
<td>3.1 ± 3.4 (0–15)</td>
</tr>
<tr>
<td>SLEDAI score distribution</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>20 (38)</td>
<td>22 (41)</td>
</tr>
<tr>
<td>1–5</td>
<td>16 (31)</td>
<td>18 (33)</td>
</tr>
<tr>
<td>6–10</td>
<td>16 (31)</td>
<td>13 (24)</td>
</tr>
<tr>
<td>≥11</td>
<td>0</td>
<td>1 (2)</td>
</tr>
<tr>
<td>Positive antiphospholipid antibody test result¶</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anticardiolipin antibodies</td>
<td>13 (25)</td>
<td>18 (33)</td>
</tr>
<tr>
<td>Anti-β2-GPI</td>
<td>8 (15)</td>
<td>8 (15)</td>
</tr>
<tr>
<td>Lupus anticoagulant</td>
<td>6 (12)</td>
<td>5 (9)</td>
</tr>
<tr>
<td>Any antiphospholipid antibody</td>
<td>20 (38)</td>
<td>23 (43)</td>
</tr>
<tr>
<td>Treatment at baseline</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prednisone</td>
<td>23 (44)</td>
<td>27 (50)</td>
</tr>
<tr>
<td>Azathioprine</td>
<td>11 (21)</td>
<td>14 (26)</td>
</tr>
<tr>
<td>Antimalarial drugs</td>
<td>22 (42)</td>
<td>23 (43)</td>
</tr>
<tr>
<td>NSAIDs</td>
<td>18 (35)</td>
<td>17 (31)</td>
</tr>
</tbody>
</table>

* Except where indicated otherwise, values are the number (%) of patients. BMI = body mass index; SDI = Systemic Lupus International Collaborating Clinics/American College of Rheumatology Damage Index; anti-β2-GPI = anti–β-2-glycoprotein I; NSAIDs = nonsteroidal antiinflammatory drugs. Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) scores range from 0 to 105, with higher scores indicating more severe disease.
† Estimated only for patients who were in early (n = 33) or late (n = 50) postmenopause.
‡ Women who presented with endometrial bleeding in the 12 months prior to study initiation were considered to be in the menopausal transition. Women in early postmenopause included those with ≤5 years of amenorrhea, and women in late postmenopause were those with >5 years of amenorrhea.
§ Follicle-stimulating hormone (FSH) levels were measured in 45 women in the menopause hormonal therapy group and in 40 women in the placebo group.
¶ Low-titer antiphospholipid antibodies were considered positive.

Tations of SLE were similar between the 2 treatment groups (Table 1). There were 2,053 patient-months of followup (81% of the possible 2,544 total patient-months of followup). The mean ± SD length of followup in the menopause hormonal therapy and placebo groups was 19.7 ± 7.9 and 19.1 ± 8.2 months, respectively (P = 0.70). Forty-four patients (85%) in the menopause hormonal therapy group finished the first year of the trial, while 45 patients (83%) in the placebo group finished the first year (P = 0.86). Thirty-seven patients (71%) in the menopause hormonal therapy group finished the second year of the trial, as did 38 patients (70%) in the placebo group (P = 0.91) (Figure 1).

Mean ± SD serum estradiol levels (pg/ml) at baseline were 31.6 ± 35.3 and 36.6 ± 73.4 (P = 0.62), in the menopause hormonal therapy and placebo groups, respectively. During the followup, mean serum estradiol levels ranged between 55.7 and 80.9 in the menopause
hormonal therapy group and 28.0 and 45.1 in the placebo group. Compared with baseline, estradiol levels at each followup assessment were higher in the menopause hormonal therapy group (\(P < 0.001\)) but not in the placebo group; between-group comparison also showed higher estradiol levels in the menopause hormonal therapy group than in the placebo group (\(P < 0.001\)) (data not shown).

**Disease activity.** At baseline, the mean ± SD SLEDAI score was 3.5 ± 3.3 in the group receiving menopause hormonal therapy and 3.1 ± 3.4 in the group receiving placebo (\(P = 0.54\)) (Table 1). There were no significant differences in the SLEDAI AUC or in the maximum SLEDAI score between the groups throughout the study period (Table 2). No patient was withdrawn from the study because of severe disease activity. SLEDAI scores remained mild and stable during the trial, and there were no significant differences between the groups (Figure 2).

Changes in SLEDAI scores from baseline at each followup visit were assessed in both groups. The mean change in SLEDAI score ranged between \(-0.31 (P = 0.42)\) and \(-1.02 (P = 0.07)\) in the group receiving menopause hormonal therapy and between \(-0.38 (P = 0.47)\) and 0.47 (\(P = 0.25\)) in the group receiving placebo.

When the data on patients with active disease (SLEDAI score \(\geq 1\)) at baseline were analyzed separately, the baseline SLEDAI scores and the SLEDAI AUC and maximum SLEDAI scores during followup did not differ between treatment groups (Table 2). Disease activity did not differ either, when analyzed separately by SLEDAI strata (0, 1–5, 6–11) at baseline, and the results obtained using the SLEDAI-2K and the

<table>
<thead>
<tr>
<th></th>
<th>Menopause hormonal therapy</th>
<th>Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>All treated patients</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No.</td>
<td>52</td>
<td>54</td>
</tr>
<tr>
<td>SLEDAI AUC, mean ± SD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 month</td>
<td>3.0 ± 2.4</td>
<td>2.9 ± 2.7</td>
</tr>
<tr>
<td>2 months</td>
<td>5.6 ± 4.5</td>
<td>5.6 ± 4.7</td>
</tr>
<tr>
<td>3 months</td>
<td>8.1 ± 6.8</td>
<td>8.2 ± 6.6</td>
</tr>
<tr>
<td>6 months</td>
<td>13.8 ± 10.8</td>
<td>15.8 ± 12.3</td>
</tr>
<tr>
<td>9 months</td>
<td>20.9 ± 15.5</td>
<td>23.9 ± 18.7</td>
</tr>
<tr>
<td>12 months</td>
<td>28.5 ± 20.4</td>
<td>31.9 ± 26.0</td>
</tr>
<tr>
<td>15 months</td>
<td>35.2 ± 24.5</td>
<td>38.5 ± 31.7</td>
</tr>
<tr>
<td>18 months</td>
<td>41.4 ± 29.3</td>
<td>44.4 ± 36.4</td>
</tr>
<tr>
<td>21 months</td>
<td>47.0 ± 34.5</td>
<td>50.3 ± 41.4</td>
</tr>
<tr>
<td>24 months</td>
<td>52.8 ± 38.9</td>
<td>56.7 ± 47.1</td>
</tr>
<tr>
<td>Maximum SLEDAI score</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>6.7 ± 3.0</td>
<td>6.7 ± 3.1</td>
</tr>
<tr>
<td>Range</td>
<td>0–16</td>
<td>2–18</td>
</tr>
<tr>
<td><strong>Treated patients with active disease at baseline</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No.</td>
<td>32</td>
<td>32</td>
</tr>
<tr>
<td>Baseline SLEDAI score</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>5.6 ± 2.2</td>
<td>5.2 ± 3.0</td>
</tr>
<tr>
<td>Range</td>
<td>2–10</td>
<td>1–15</td>
</tr>
<tr>
<td>SLEDAI AUC, mean ± SD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 month</td>
<td>4.2 ± 2.2</td>
<td>4.1 ± 2.8</td>
</tr>
<tr>
<td>2 months</td>
<td>7.3 ± 4.8</td>
<td>7.3 ± 5.0</td>
</tr>
<tr>
<td>3 months</td>
<td>10.4 ± 7.5</td>
<td>10.7 ± 7.0</td>
</tr>
<tr>
<td>6 months</td>
<td>17.1 ± 11.8</td>
<td>19.9 ± 12.9</td>
</tr>
<tr>
<td>9 months</td>
<td>25.3 ± 16.2</td>
<td>30.2 ± 19.9</td>
</tr>
<tr>
<td>12 months</td>
<td>34.7 ± 19.5</td>
<td>41.3 ± 28.2</td>
</tr>
<tr>
<td>15 months</td>
<td>42.8 ± 22.8</td>
<td>49.3 ± 34.1</td>
</tr>
<tr>
<td>18 months</td>
<td>49.8 ± 27.6</td>
<td>56.2 ± 38.9</td>
</tr>
<tr>
<td>21 months</td>
<td>55.8 ± 33.7</td>
<td>63.7 ± 43.7</td>
</tr>
<tr>
<td>24 months</td>
<td>63.2 ± 38.4</td>
<td>72.8 ± 49.5</td>
</tr>
<tr>
<td>Maximum SLEDAI score</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>10.2 ± 2.4</td>
<td>12.0 ± 3.0</td>
</tr>
<tr>
<td>Range</td>
<td>6–16</td>
<td>8–18</td>
</tr>
</tbody>
</table>

* Active disease at baseline was defined as a Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) score of ≥1. AUC = area under the curve.
M-SLEDAI were also similar to those of the primary analysis (data not shown).

**Flares.** The number of patients who had flares and who had multiple flares during the trial was similar in both groups. Only 1 severe flare occurred during the trial, in a patient who received placebo; thus, the flares were analyzed altogether. The number of flares and the incidence-density rate of flares per patient-year were similar in both treatment groups. There were 79 flares, with an incidence-density rate of 0.93, in the group that received menopause hormonal therapy, and 83 flares, with an incidence-density rate of 0.97, in the group that received placebo. Compared with the placebo group, the group that received menopause hormonal therapy had a RR of flares of 0.96 (95% CI 0.70–1.32) (P = 0.80). The median time to the first flare was 3 months in both groups. The probability of flare at 1 and 2 years in the group receiving menopause hormonal therapy and the group receiving placebo was 0.91 and 0.75 (P = 0.29) and 0.94 and 0.79 (P = 0.25), respectively (Table 3).

Among the patients with active disease at baseline, there were no differences according to treatment group in the probability of flares. The number and incidence rate of flare were also similar between the 2 groups (Table 3).

**Other secondary outcome measures.** The rate of use and the dosage of prednisone, immunosuppressants, chloroquine, and nonsteroidal antiinflammatory drugs were similar between the groups during the study. Three patients in the menopause hormonal therapy group developed thromboses, 1 venous (lower limb) and 2 arterial (left middle cerebral and mesenteric arteries, respectively), for an incidence-density rate of 35.2 per 1,000 patient-years. Thromboses developed after 2, 15, and 18 months of treatment. One patient in the placebo group developed bilateral lacunar infarctions after 12 months of treatment (incidence-density rate of 11.6 per 1,000 patient-years; RR 3.0 [95% CI 0.24–158.9], P = 0.37). Only 1 patient in the menopause hormonal therapy group was both a smoker and had a history of thrombosis (transient ischemic attack 2 years before

### Table 3. Flare characteristics and cumulative net probabilities of flares at 3, 6, 12, and 24 months in the 106 treated women and in the subgroup of 64 women who had active disease at baseline*

<table>
<thead>
<tr>
<th></th>
<th>Menopause hormonal therapy</th>
<th>Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td>All treated patients</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No.</td>
<td>52</td>
<td>54</td>
</tr>
<tr>
<td>Patients with flares, no. (%)</td>
<td>44 (85)</td>
<td>39 (72)</td>
</tr>
<tr>
<td>Patients with multiple flares, no. (%)</td>
<td>24 (46)</td>
<td>26 (48)</td>
</tr>
<tr>
<td>Severe flares</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of flares</td>
<td>79</td>
<td>83</td>
</tr>
<tr>
<td>Incidence-density rate per patient-year</td>
<td>0.93</td>
<td>0.97</td>
</tr>
<tr>
<td>RR (95% CI)</td>
<td>0.96 (0.70–1.32)</td>
<td></td>
</tr>
<tr>
<td>Probability of flare</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 months</td>
<td>0.47</td>
<td>0.54</td>
</tr>
<tr>
<td>6 months</td>
<td>0.64</td>
<td>0.60</td>
</tr>
<tr>
<td>12 months</td>
<td>0.91</td>
<td>0.75</td>
</tr>
<tr>
<td>24 months</td>
<td>0.94</td>
<td>0.79</td>
</tr>
<tr>
<td>Treated patients with active disease at baseline</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No.</td>
<td>32</td>
<td>32</td>
</tr>
<tr>
<td>Patients with flares, no. (%)</td>
<td>29 (91)</td>
<td>24 (75)</td>
</tr>
<tr>
<td>Patients with multiple flares, no. (%)</td>
<td>18 (56)</td>
<td>16 (50)</td>
</tr>
<tr>
<td>No. of flares</td>
<td>54</td>
<td>54</td>
</tr>
<tr>
<td>Incidence-density rate per patient-year</td>
<td>1.05</td>
<td>0.99</td>
</tr>
<tr>
<td>RR (95% CI)</td>
<td>1.06 (0.71–1.58)</td>
<td></td>
</tr>
</tbody>
</table>

* Active disease was defined as a Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) score of ≥1. A flare was defined as an increase in the SLEDAI score of ≥3 points from the previous visit. A severe flare was defined as an increase in the SLEDAI score of ≥12 points from the previous visit. RR = relative risk; 95% CI = 95% confidence interval.
participating in the trial); only the patient receiving placebo had low titers of antiphospholipid antibodies (aPL). Nine patients in the menopause hormonal therapy group were hospitalized (ducto thrombosis in 3, elective surgery in 2, acute abdomen in 1, pneumonia in 1, pyelonephritis in 1, and vaginal carcinoma in 1), as were 6 patients in the placebo group (elective surgery in 2, dehydration in 1, thrombocytopenia in 1, acute abdomen in 1, and lacunar stroke in 1). Two patients died during the trial due to sepsis; the deaths occurred after 1 and 3 months of receiving placebo and menopause hormonal therapy, respectively.

**DISCUSSION**

We evaluated the effects of menopause hormonal therapy as compared with placebo on disease activity in women with SLE who were in the menopausal transition or in early or late postmenopause. Over 24 months of followup, disease activity remained mild and stable, and no clinically significant difference was seen between treatment groups.

Disease activity was assessed by a validated measure that was sensitive to change over time (26), and the incidence of flares was estimated using a validated definition (31). We also analyzed disease activity according to the SLEDAI-2K (32) and M-SLEDAI, and in the subgroup of patients with active disease at entry. The results of these analyses were similar to those of the primary analyses. We believe that the assessments of disease activity were adequate and that menopause hormonal therapy does not increase lupus activity to a clinically significant degree. Our results are consistent with those of observational studies of menopause hormonal therapy (21–23), our clinical trial of contraceptive methods in women with SLE (19), and the contraception trial conducted by the SELENA group (20), in which no increase in disease activity was observed.

Recently, the SELENA group, using the same hormone regimen in postmenopausal women, found that the rate of severe flares and the mean change in the activity index score were not different between women who received menopause hormonal therapy and those who received placebo; nevertheless, women receiving menopause hormonal therapy experienced an increased rate of mild or moderate flares (24). In our study, although the number of patients with flares and the probability of flares tended to be higher among women receiving menopause hormonal therapy, especially when the lupus was active at baseline, the incidence rate of flares was similar in both groups. The difference in these results might be explained by the more sensitive definition of flare, the larger number of patients, or the higher rates of stopping the study medication and loss to followup (35% for the menopause hormonal therapy group and 27% for the placebo group) in the SELENA trial in comparison with our study. Nevertheless, this statistical difference is not clinically relevant and overall, these studies show that the use of menopause hormonal therapy by women with SLE does not elicit disease exacerbation.

Disease activity in the study population was mild/moderate at entry; since we did not restrict study participation unless the SLEDAI score was >30, and no patient was rejected for this reason, this result reflects the mildness of lupus activity at various postreproductive stages (33,34). So, we consider that the population studied represents most women with SLE in the menopausal transition or in early or late postmenopause, in terms of disease activity.

Although the risk–benefit profile of menopause hormonal therapy has been established by the results of the HERS and the WHI trials (3,4), the population included in those studies does not exactly correspond with that included in this study. Our population was composed of younger women, half of whom were in the menopausal transition or in early postmenopause (28), when menopause symptoms are most severe and bone loss is accelerated.

No differences in adverse events were observed between treatment groups in this study. However, the study was not adequately powered to detect differences in the incidence rates of most adverse events between the groups.

A result from our study and the SELENA trial (24) that is worth discussion is that thrombotic events occurred more often and at an identical ratio (3:1) in women receiving menopause hormonal therapy than in those receiving placebo. This result is consistent with results of observational studies (35–37) and clinical trials (4) of the use of menopause hormonal therapy among healthy women. Also, in our trial of contraceptive methods in women with SLE, thrombosis occurred only among women assigned to hormonal methods (2 patients in the combined oral contraceptives group and 2 in the progestin-only pill group); none occurred in the copper intrauterine device group (19).

Thrombosis is unusual in the general population. The incidence rate among women ≤30 years of age is 0.05 per 1,000 person-years (38), and in postmenopausal women, it is 0.08–0.11 per 1,000 person-years (35–37). In stark contrast, thrombosis has been reported in 10–20%
of lupus patients (39–41), and the incidence rate among patients in whom the disease is prevalent is 5.1 per 1,000 patient-years (39) and up to 51.9 per 1,000 patient-years in an inception cohort of lupus patients (40). Considering that the use of exogenous estrogens is associated with a thrombosis RR of 2.1–3.6 (35–37), the absolute risk imposed by menopause hormonal therapy in women with SLE seems to be unacceptable. In our study, 1 episode of thrombosis was observed among every 28 women who received menopause hormonal therapy.

Avoiding menopause hormonal therapy in women who are positive for aPL would not be enough, since other factors, such as smoking, older age, disease activity over time, and glucocorticoid dose, are also associated with the occurrence of venous thrombosis in lupus patients (42). Therefore, we consider the real threat of menopause hormonal therapy in women with SLE to be the risk of developing thrombosis, not the effect of menopause hormonal therapy on disease activity. Whether this hazard can be diminished by alternative routes of estrogen administration or preparations of lower dosages needs to be explored.

Our study has several limitations. Since women who had previously received menopause hormonal therapy were allowed to participate, a selection bias toward women in whom this therapy was safe and well tolerated may exist. Nevertheless, the percentage of women who received menopause hormonal therapy in the past was similar between those who declined to participate and those who enrolled in the study; thus, we do not consider that this situation influenced our results.

We used a continuous-sequential estrogen-progestogen regimen, in which medroxyprogesterone acetate may attenuate estrogen’s proinflammatory effects (43); therefore, our results should not be extrapolable to regimens containing only estrogens. The present trial evaluated the effect of a continuous-sequential estrogen-progestogen regimen on lupus activity during most of the recommended period of treatment for menopause symptoms; however, the risks and benefits of long-term use in SLE patients still need to be addressed. Treatment of SLE was administered at the discretion of the rheumatologist, and no differences in the treatment administered between the patient groups were evident at entry or throughout the followup period. Because this study was conducted at a single center with limited ethnic variation among the patients, one must be circumspect about extrapolating the results to all women with SLE. However, our results are consistent with those of studies conducted in populations of other ethnicities (21–24).

Our study was a nonequivalence trial; however, considering the actual level of disease activity in the study population at baseline, the sample size we achieved provided a 90% chance of detecting a difference in the SLEDAI score of ≥2.10, instead of the originally planned 3.0. Therefore, although we did not detect any clinically significant differences in disease activity (25) between treatment groups, the study had limited power to detect a smaller difference. The study was underpowered to assess the risk for cardiovascular events, cancer, and other disorders associated with menopause hormonal therapy. Also, in this report we do not describe the effects of this therapy on menopause symptoms, bone mineral density, lipid profile, and mammographic breast density in women with lupus. Such analysis is under way.

Some strengths of our study also need to be considered. The participation rate throughout the trial was high, and the adherence to treatment was assessed by estradiol measurements. We offered participation in the study to all women with SLE at different postproductive stages, including the menopausal transition and early or late postmenopause (28), who attended our center, including those with variable disease activity and those who tested positive for aPL, provided they had no history of recent thrombosis. Twenty-seven percent of the patients had undergone premature menopause, and the mean BMI was <27 kg/m². Our patients were all Mexican women, and we think the results can be generalized to most Hispanic women with SLE at different menopause-related stages.

In conclusion, since women with SLE are at an increased risk of developing premature menopause (2), osteoporotic fractures (44), cognitive dysfunction (45), premature atherosclerosis (46), thrombosis (40), and cardiovascular events (47), and their quality of life often is poorer than that of the general population (48), the effects of menopause in addition to these conditions, and the risk–benefit profile of menopause hormonal therapy in these women and those with other chronic diseases, need to be explored in depth. Menopause hormonal therapy did not affect the course of disease activity, at a clinically significant level, in women with SLE who were in the menopausal transition or in early or late postmenopause. Nonetheless, the potential increase in the risk of developing thrombosis may outweigh any benefits.

ACKNOWLEDGMENTS

The menopause hormonal therapy and calcium were kindly provided by Wyeth Mexico and Whitehall-Robbins, respectively.
AUTHOR CONTRIBUTIONS

Dr. Sánchez-Guerrero had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Sánchez-Guerrero, González-Pérez, Cravioto.

Acquisition of data. Sánchez-Guerrero, González-Pérez, Durand-Carbajal, Lara-Reyes, Jiménez-Santana, Cravioto.


REFERENCES


Association of a CD24 Gene Polymorphism With Susceptibility to Systemic Lupus Erythematosus

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Objective. To determine the potential role of the CD24 A57V gene polymorphism in systemic lupus erythematosus (SLE).

Methods. We studied 3 cohorts of Caucasian patients and controls. The Spanish cohort included 696 SLE patients and 539 controls, the German cohort included 257 SLE patients and 317 controls, and the Swedish cohort included 310 SLE patients and 247 controls. The CD24 A57V polymorphism was genotyped by polymerase chain reaction, using a predeveloped TaqMan allele discrimination assay. Odds ratios (ORs) and 95% confidence intervals (95% CIs) were calculated.

Results. In the Spanish cohort there was a statistically significant difference in the distribution of the CD24 V allele between SLE patients and controls (OR 3.6 [95% CI 2.13–6.16], P < 0.0001). In addition, frequency of the CD24 V/V genotype was increased in SLE patients compared with controls (OR 3.7 [95% CI 2.16–6.34], P < 0.00001). We sought to replicate this association with SLE in a German population and a Swedish population. A similar trend was found in the German group. The CD24 V/V genotype and the CD24 V allele were more frequent in SLE patients than in controls, although this difference was not statistically significant. No differences were observed in the Swedish group. A meta-analysis of the Spanish and German cohorts demonstrated that the CD24 V allele has a risk effect in SLE patients (pooled OR 1.25 [95% CI 1.08–1.46], P = 0.003). In addition, homozygosity for the CD24 V allele significantly increased the effect (pooled OR 2.19 [95% CI 1.50–3.22], P = 0.00007).

Conclusion. These findings suggest that the CD24 A57V polymorphism plays a role in susceptibility to SLE in a Spanish population.

Systemic lupus erythematosus (SLE) is a prototypic autoimmune disease with a complex pathogenesis involving multiple genetic and environmental factors. The disease is characterized by enhanced autoantibody production, abnormalities of immune/inflammatory system function, and inflammation in several organs. Al-
though the pathogenesis of SLE is unknown, there is
evident familial clustering and a high concordance of
SLE in monozygotic twins, providing evidence of the
role of genetic factors in this disorder (1). The genetic
background of SLE is complex and involves multiple
genes encoding different molecules with significant func-
tions in the regulatory pathway of the immune system
(1–4). During the past few years, genome-wide linkage
studies have identified several loci as being associated
with SLE (3). In this respect, findings of several studies
support the notion that regulatory T and B cell genes
play an important role in the pathogenesis of SLE.

One of the chromosome regions suggested to be
associated with SLE and other autoimmune diseases is
6q21–25 (5,6), where the CD24 gene maps. CD24 (heat-
stable antigen) is a glycosyl phosphatidylinositol (GPI–
linked protein that is anchored to the cell surface and is
expressed in a wide variety of cell types, including
activated T cells, B-lineage cells, mature granulocytes,
macrophages, and dendritic cells, among others (7–10).
The biologic function of CD24 is unclear, but it has been
shown that CD24 is a ligand for P-selectin on tumor cells
(11). This binding could be important in the dissemina-
tion of tumor cells and could be a key factor in recruiting
leukocytes into inflamed tissue. CD24 has also been
implicated in the activation and differentiation of B
lymphocytes (12) and has been identified as an import-
ant mediator in a CD28-independent costimulatory
pathway in the activation of both CD4 and CD8 T cells
(9). In addition, CD24 plays an important role in binding
the adhesion molecules very late activation antigen 4
and vascular cell adhesion molecule 1 (13). These adhe-
sion molecules are important in lymphocyte costimula-
tion in specific tissue and sites of inflammation in SLE
patients (14,15).

A recent study has identified a coding polymor-
phism of the CD24 gene (226C→T, single-nucleotide polymorphism [SNP] no. rs8734), which results in the
replacement of an alanine (CD24 A) amino acid by valine
(CD24 V) (A57V) (16). This nonconservative amino acid
change at a position that precedes the putative cleavage
site for the GPI anchor has been found to be associated
with susceptibility to and progression of multiple sclerosis
(MS) (16). Taking into account these findings, in this study
we aimed to investigate the potential implication of the
functional CD24 polymorphism for susceptibility to SLE.

PATIENTS AND METHODS

Patients. Peripheral blood samples were obtained from
3 unrelated cohorts of European Caucasian patients with SLE
and from healthy controls. The Spanish cohort consisted of 696
SLE patients and 539 healthy controls, the German cohort
consisted of 257 SLE patients and 317 healthy controls, and
the Swedish cohort consisted of 310 SLE patients and 247 healthy
controls. All 1,263 SLE patients met the American College of
Rheumatology criteria for SLE (17). Patients in the Spanish
cohort were recruited from the following 5 Spanish hospitals:
Hospital Virgen de las Nieves, Hospital Clínico San Cecilio,
Hospital Virgen del Rocio, Hospital Carlos-Haya, and Hospital
Xeral-Calde. Patients in the German cohort were recruited from
a North German multicenter study on the genetics of SLE. Members of the German Systemic Lupus Erythematosus
Study Group are shown in Appendix A. Patients in the Swedish
cohort were recruited from 3 centers in central and southern
Sweden (Stockholm, Eskilstuna, and Lund). Control samples
were obtained from a total of 1,103 healthy blood bank or bone
marrow donors in the corresponding cities. Patients and con-
trols were all Caucasian and were matched for age (by mean
age) and for sex (by frequency matching). Written informed
consent was obtained from all subjects, and the study was
approved by the local ethics committee of each center. Demo-
graphic characteristics of the patients and controls in each
population have been described previously (5,18,19).

Genotyping. DNA was obtained from peripheral
blood, using standard methods. Samples were genotyped for
the CD24 A57V variant using a TaqMan 5′-allele discrimina-
tion method (Sigma-Aldrich, St. Louis, MO). The primer
sequences were 5′-CCC-AAA-TCC-TAA-TGC-C-3′
and 5′-TAA-GAG-TAG-AGA-TGC-AGA-AGA-G-3′. The
TaqMan minor groove binder probe sequences were 5′-ACC-
AAG-GCG-GCT-GGT-GGT-G-3′ and 5′-ACC-AAG-GTG-
GCT-GGT-GGT-G-3′; the probes were labeled with the fluo-
rescent dyes FAM and JOE, respectively.

The polymerase chain reaction (PCR) was carried out
in a total reaction volume of 10 μl using the following
amplification protocol: denaturation at 95°C for 10 minutes,
followed by 40 cycles of denaturation at 92°C for 15 seconds,
and annealing and extension at 60°C for 90 seconds. Following
PCR, the genotype of each sample was automatically deter-
mined by measuring the allele-specific fluorescence in the ABI
Prism 7000 Sequence Detection System (Applied Biosystems,
Foster City, CA), using SDS, version 1.1 software (Applied
Biosystems) for allele discrimination. To confirm the genotype
obtained by the TaqMan 5′-allele discrimination assay, direct
sequencing using the ABI Prism 3100 Genetic Analyzer (Ap-
plied Biosystems) was performed. The German samples were
typed at Uppsala University using the Custom TaqMan SNP
genotyping assay (Applied Biosystems). To verify the genotype-
ing consistency, 96 of the Swedish control samples were typed
in both centers (Uppsala University and Instituto de Parasito-
logía y Biomedicina López-Neyra, CSIC), and for 95 of the 96
samples (99%) the 2 genotypes were identical.

Statistical analysis. Allele and genotype frequencies
were obtained by direct counting. Hardy-Weinberg equili-
brum was assessed using the chi-square test, and statistical
analysis was performed to compare allele and genotype distrib-
utions. Odds ratios (ORs), with 95% confidence intervals
(95% CIs), were calculated by Woolf’s method, using StatCalc
and logistic regression software programs (Epi Info 2002;
Centers for Disease Control and Prevention, Atlanta, GA).
The pooled OR was calculated according to a fixed-effects
model (Mantel-Haenszel meta-analysis) as well as a random-effects model (DerSimonian-Laird), using StatsDirect software (StatsDirect, Cheshire, UK). P values less than 0.05 were considered significant. Homogeneity of ORs among cohorts was calculated by the Breslow-Day test and Woolf Q method, using StatsDirect software. The power of each analysis was estimated using Quanto, version 0.5 (Department of Preventive Medicine, University of Southern California, Los Angeles, CA).

RESULTS

CD24 genotypes were in Hardy-Weinberg equilibrium in patients and in controls in the 3 populations studied. We first analyzed the cohorts individually and then combined the samples in a meta-analysis. Table 1 shows the CD24 A57V allele and genotype distributions in SLE patients and healthy controls in all 3 cohorts.

An association was found when we compared genotype frequencies in Spanish patients with SLE with those in Spanish controls (P = 0.0004 by chi-square test using a 2 × 3 contingency table), with a higher frequency of the CD24 V/V genotype in the SLE group (10.2% versus 4.3%) (OR 3.6 [95% CI 2.1–6.34]). Analyses of allele frequencies produced similar results, showing an increased frequency of the CD24 V allele in SLE patients compared with healthy controls (29.5% versus 23.8%) (OR 3.6 [95% CI 2.13–6.16], P < 0.0001).

Additionally, we sought to replicate the association of the CD24 A57V polymorphism with SLE in 2 different populations from Germany and Sweden. The same trend was found in the German cohort, in which the frequencies of the CD24 V/V genotype and CD24 V allele were slightly increased in SLE patients compared with healthy controls (8.9% versus 5.7% for the CD24 V/V genotype and 29.4% versus 27.4% for the CD24 V allele). Although this difference did not reach statistical significance (OR 1.54 [95% CI 0.70–3.40], P = 0.2 for the CD24 V/V genotype and OR 1.45 [95% CI 0.67–3.13], P = 0.3 for the CD24 V allele), meta-analysis of the ORs in the Spanish and German cohorts demonstrated that the CD24 V allele has a risk effect in SLE patients (pooled OR 1.25 [95% CI 1.08–1.46], P = 0.003) and the CD24 V/V genotype has an increased risk effect (pooled OR 2.19 [95% CI 1.50–3.22], P = 0.00007) (Table 2). However, we did not find any significant difference in genotype and allele frequencies between SLE patients and healthy controls in the Swedish cohort.

Estimation of OR homogeneity between the cohorts showed the combinability of all 3 cohorts at the allele level (CD24 V), but only of the Spanish and German cohorts at the genotype level (CD24 V/V). Consequently, meta-analysis of the CD24 V/V genotype under a fixed-effects model should not include the Swedish cohort, but analysis of the CD24 V allele can include all 3 cohorts. Meta-analysis of all 3 cohorts using the Mantel-Haenszel test revealed a significant association of SLE with the CD24 V allele (pooled OR 1.20 [95% CI 1.05–1.36], P = 0.007), although a separate analysis of the Spanish and German cohorts showed the strongest association (Table 2).

Table 1. Genotype and allele frequencies of the CD24 A57V polymorphism in SLE patients and healthy controls*

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Genotype</th>
<th>Spanish cohort</th>
<th>German cohort</th>
<th>Swedish cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SLE patients</td>
<td>Healthy</td>
<td>SLE patients</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(n = 696)</td>
<td>controls (n = 539)</td>
<td>(n = 257)</td>
</tr>
<tr>
<td>CD24 A/A</td>
<td></td>
<td>356</td>
<td>305</td>
<td>129</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(51.1)</td>
<td>(56.6)</td>
<td>(50.2)</td>
</tr>
<tr>
<td>CD24 A/V</td>
<td></td>
<td>269</td>
<td>211</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(38.7)</td>
<td>(39.1)</td>
<td>(40.9)</td>
</tr>
<tr>
<td>CD24 V/V</td>
<td></td>
<td>71</td>
<td>23</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(10.2)</td>
<td>(4.3)</td>
<td>(8.9)</td>
</tr>
<tr>
<td>Allele</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD24 A</td>
<td></td>
<td>981</td>
<td>821</td>
<td>363</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(70.5)</td>
<td>(76.2)</td>
<td>(70.6)</td>
</tr>
<tr>
<td>CD24 V</td>
<td></td>
<td>411</td>
<td>257</td>
<td>151</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(29.5)</td>
<td>(23.8)</td>
<td>(29.4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Values are the number (%) of genotypes or alleles. OR = odds ratio; 95% CI = 95% confidence interval.
† P values indicate the difference between systemic lupus erythematosus (SLE) patients and healthy controls.
Clinical features of Spanish patients with SLE were analyzed for possible association with the different alleles or genotypes of the CD24 A57V polymorphism. When we stratified Spanish SLE patients according to the presence or absence of renal involvement, no statistically significant differences were observed in the distribution of CD24 polymorphism between SLE patients with and without lupus nephritis (Table 3). Similar results were found after comparisons of other demographic and clinical features (data not shown).

**DISCUSSION**

This is the first study to attempt to determine the potential role of the CD24 A57V polymorphism in SLE. Our results showed that in the Spanish population the CD24 V allele was associated with an increased risk of SLE (OR 3.6). These results were further supported by analysis of a separate SLE cohort from Germany. The trend toward an increase in CD24 V frequency was repeated in the German cohort, although this trend did not reach statistical significance. This could be explained by the sample size, which was smaller in the German cohort (n = 257) than in the Spanish cohort (n = 696), resulting in a lack of power to detect a susceptibility association. In addition, we demonstrated that the frequency of the CD24 V/V genotype in Spanish SLE patients is more than twice that in the control population. This implies that the effect of the CD24 gene on SLE predisposition is dose dependent, since homozygosity for the CD24 V allele confers a 2-fold increased risk of SLE (OR 3.7).

Similar results have been found for MS in other Caucasian populations (16), and the CD24 A57V polymorphism has also recently been shown to be associated with MS in a Spanish population (20). However, a recent replication study of CD24 in 2 large, unrelated cohorts of Caucasian patients with MS living in Belgium and the UK failed to demonstrate the reported association (21). The lack of replication in that study was probably not due to insufficient power to detect an association, since the sample sizes (n = 334 patients living in Belgium and n = 846 patients living in the UK) were large enough to reach >90% statistical power to detect a relative risk similar to that found in the original study.

Population heterogeneity may explain the conflicting results of these studies, since Swedish, Belgian, and British populations showed different allele frequencies compared with Spanish and German populations. Northern European populations had a higher frequency of the T allele (CD24 V) than did the other groups, exhibiting a clear north–south gradient. For example, the frequency of the T allele in the original study (20) (26.8%) was very similar to that found in controls from northern Spain (25.3%) and our controls from southern Spain (23.8%), but was slightly different from that found in controls from Germany (27.4%), and differed to a

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**Table 2.** Meta-analysis using fixed-effects and random-effects models

<table>
<thead>
<tr>
<th>CD24 A57V meta-analysis</th>
<th>Pooled OR (95% CI)</th>
<th>P</th>
<th>Pooled OR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD24 V versus CD24 A</td>
<td>1.25 (1.08–1.46)</td>
<td>0.003</td>
<td>1.20 (1.05–1.36)</td>
<td>0.007</td>
</tr>
<tr>
<td>CD24 V/V versus CD24 A/V</td>
<td>2.19 (1.50–3.22)</td>
<td>0.00007</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

**Table 3.** Relationship between the CD24 A57V polymorphism and the presence of nephritis in Spanish patients with SLE

<table>
<thead>
<tr>
<th>Genotype</th>
<th>SLE with nephritis (n = 271)</th>
<th>SLE without nephritis (n = 425)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD24 A/A</td>
<td>142 (52.4)</td>
<td>211 (49.6)</td>
</tr>
<tr>
<td>CD24 A/N</td>
<td>96 (35.4)</td>
<td>169 (39.8)</td>
</tr>
<tr>
<td>CD24 V/V</td>
<td>33 (12.2)</td>
<td>45 (10.6)</td>
</tr>
<tr>
<td>Allele</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD24 A</td>
<td>380 (70.1)</td>
<td>591 (69.5)</td>
</tr>
<tr>
<td>CD24 V</td>
<td>162 (29.9)</td>
<td>259 (30.5)</td>
</tr>
</tbody>
</table>

* Values are the number (%) of genotypes or alleles. SLE = systemic lupus erythematosus.
greater extent from those found in controls from Belgium (30%), Sweden (30.8%), and the UK (34%).

However, in the present study the frequencies of the CD24 V allele among SLE patients in all 3 cohorts were similar (29.5% in the Spanish cohort, 29.4% in the German cohort, and 31.6% in the Swedish cohort), suggesting the existence of clines among controls, with an association of the CD24 V allele with SLE. In fact, the same trend has been observed in MS, where a difference has been found among controls but not among MS patients in 4 independent cohorts. The frequency of the CD24 V allele was 33.2% in American Caucasian patients with MS, 32% in Belgian patients with MS, 30% in British patients with MS, and 30.7% in Spanish patients with MS (16,20,21). Regarding SLE, a study of the PDCD1 gene (22) showed a north–south gradient in the distribution of the PD1.3A allele in healthy individuals. This gradient of frequencies in European Caucasians in relation to their geographic origin has not been observed in SLE patients, causing divergent results when different populations are analyzed. This pattern of variation has been characterized in a large panel of SNPs, defining primarily 2 groups of European subpopulations, from southern Europe and from northern Europe (23).

We believe that there could be 3 possible explanations for the observed phenomenon. First, CD24 V might be associated with a gene for SLE not in linkage disequilibrium with CD24 and with which CD24 interacts. Second, CD24 V could be associated with an environmental factor which confers selection of this allele in patients with SLE. Finally, there may be a founder effect of CD24 V in SLE.

Alternatively, lack of replication in different populations may be considered evidence of a Type I error, but this requires the implicit assumption that the variant that causes disease is shared and has an equal effect in all populations, i.e., that it is ancestral before divergence of the populations. However, regional founder effects may underlie differences even within European populations, as has been suggested for NOD2 and DLG5 (24,25). Furthermore, allele frequencies of NOD2 risk alleles have been reported to vary significantly between European populations (26). Therefore, the inconsistent results of CD24 A57V association studies may indicate that allelic heterogeneity, regional founder effects, or both, underlie the conflicting results. Analysis of more European subjects from other southern and eastern regions is needed to determine the gradient effect of this allele.

There is also the possibility that a second functional variant of CD24 plays a role in disease risk in populations other than the Spanish population because of allelic heterogeneity. Complete analysis of the CD24 gene would be required to clarify the existence of 1 or several risk haplotypes.

The observation that an inherited variation in CD24 predisposes to both MS and SLE is intriguing, and supports current hypotheses that common cellular and molecular pathogenetic mechanisms might be involved in multiple inflammatory diseases. The general observation that other inflammatory and autoimmune diseases are associated with the same genes (e.g., CTLA4 [27], PTPN22 [28–30], and PDCD1 [31–34]) suggests it may be useful to evaluate the role of CD24 in other diseases.

The role of CD24 in autoimmunity has not been clearly elucidated. Bai et al (35) previously reported the critical role of CD24 in the development of a mouse model of MS (experimental autoimmune encephalomyelitis). CD24 has been demonstrated to function as an important regulator during the early stages of B and T cell lymphopoiesis (36). Murine CD24 is expressed during the initial stages of T and B cell development and its expression is reduced in CD4+CD8+ thymocytes. Current evidence suggests that CD24 could affect both adhesion and signaling (37). To date, P-selectin is the only ligand that has been identified that exhibits increased levels of expression in response to acute mediators of inflammation in SLE (15). It is, however, unlikely that this CD24–P-selectin interaction accounts for all of the biologic activities attributed to CD24. Since CD24 is localized in lipid rafts, the absence or presence of CD24 may influence membrane raft composition and thereby affect important signaling pathways within the cell (37). It has also been reported that CD24 is involved in intracellular signaling by direct binding to tyrosine kinases (38).

In conclusion, it appears that the CD24 A57V polymorphism plays a role in susceptibility to SLE in a Spanish population. The evidence of association of the CD24 genetic variation with SLE was strongest in the Spanish cohort, although broadly similar allele frequencies were observed in the German cohort. Nevertheless, conflicting results have been found in different populations, suggesting that allele frequency clines are common in other polymorphisms and that their impact should be carefully considered in genetic epidemiology studies. Therefore, it might be of interest to further investigate the role of the CD24 gene variant in additional ethnic groups to confirm this hypothesis.
ACKNOWLEDGMENTS

We thank all of the DNA donors for making this study possible. We also thank Mari Paz Ruiz for excellent technical assistance.

AUTHOR CONTRIBUTIONS

Dr. Martín had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Sánchez, González-Escribano, Alarcón-Riquelme, Martín.

Acquisition of data. Sánchez, Abelson, Gunnarsson, Svenungsson, Sturfelt, Truedsson, Jönsson, Witte.


Statistical analysis. Sánchez, Abelson.


REFERENCES


APPENDIX A: THE GERMAN SYSTEMIC LUPUS ERYTHEMATOSUS STUDY GROUP

Members of the German Systemic Lupus Erythematos Study Group are as follows: Wolfgang L. Gross, MD (University Hospital of Schleswig-Holstein, Campus Luebeck, Rheumaklinik Bad Bramstedt, Luebeck), Erika Gromnica-Ihle, MD (Rheumaklinik Berlin-Buch, Berlin), Sebastian Schnarr, MD, Henning Zeidler, MD (Abteilung Rheumatologie, Medizinische Hochschule Hannover, Hannover), Reinhold E. Schmidt, MD (Abteilung Klinische Immunologie, Medizinische Hochschule Hannover, Hannover).
Adapted Versions of the Sharp/van der Heijde Score Are Reliable and Valid for Assessment of Radiographic Progression in Juvenile Idiopathic Arthritis

Angelo Ravelli,1 Maka Ioseliani,2 Ximena Norambuena,2 Juliana Sato,2 Angela Pistorio,2 Federica Rossi,2 Nicolino Ruperto,2 Silvia Magni-Manzoni,3 Nicola Ullmann,2 and Alberto Martini1

Objective. To develop adapted versions of the Sharp/van der Heijde radiographic scoring system for use in juvenile idiopathic arthritis (JIA), and to investigate their validity in JIA patients with polyarticular disease.

Methods. The study group comprised 177 patients with polyarticular JIA. Radiographs of the wrist/hand of each patient were obtained at baseline (first observation) and then at 1, 3, 5, 7/8, and 10 years and were assessed independently by 2 pediatric rheumatologists according to different adaptations of the Sharp/van der Heijde method. To facilitate score assignment, the radiograph for each patient was compared with a bone age–related standard. Validation procedures included analysis of reliability, construct validity, and score progression over time.

Results. Interobserver and intraobserver agreement on longitudinal score values and score changes was good for all of the adapted scoring versions (intraclass correlation coefficient >0.85). Score changes over time were moderately to strongly correlated with the clinical indicators of long-term joint damage and with the amount of long-term radiographic damage as measured with the carpo:metacarpal ratio, thereby demonstrating good construct validity. A steady increase in scores over time was observed, with joint space narrowing being the most common form of damage throughout the disease course. The inclusion of 5 new areas appeared to increase the overall construct validity of erosion scores.

Conclusion. Our results show that the adapted versions of the Sharp/van der Heijde score are reliable and valid for the assessment of radiographic progression in patients with JIA.

Juvenile idiopathic arthritis (JIA) is a chronic and heterogeneous disease characterized by prolonged synovial inflammation that may lead to destructive lesions of joint structures (1). Because the prevention or retardation of joint changes is a major objective of treatment of chronic arthritis, evaluation of radiographic joint damage has become an important tool for assessing disease severity and progression in patients with JIA. The assessment of structural joint damage is considered the gold standard of treatment efficacy studies in patients with chronic arthritis (2) and is now required by the US Food and Drug Administration to be used as a measure of disease progression in clinical trials of potential disease-modifying drugs (3). The evaluation of radiographic progression has never been included in controlled trials in JIA, reflecting primarily the paucity of established radiographic scoring systems for use in the pediatric age group. However, because new potent therapeutic agents are now available for children with JIA (4), there is a growing need for a reliable radiographic assessment standard to investigate thoroughly the effectiveness of these new agents.
In recent years, there has been a great deal of effort to devise new radiographic scoring systems or validate existing methods for use in JIA (5–13). However, only a few of these measures have undergone a detailed validation process or have been tested in sufficient numbers of patients. It is commonly believed that the traditional scoring methods used for adult patients with rheumatoid arthritis, which are based on the assessment of joint space narrowing (JSN) and erosions, may not be suitable for the evaluation of pediatric patients with joint diseases. In contrast to the situation in adults, it is difficult to reliably determine cartilage loss and erosions in children by simple examination of radiographs, because growing joints change anatomically over time (14,15). In a recent pilot study, however, we observed that the Sharp and Larsen scoring methods are potentially reliable and valid for the assessment of radiographic progression in children with JIA (16).

In the present study, we describe the development of adapted versions of the Sharp/van der Heijde radiographic scoring method (17) for use in JIA and provide preliminary evidence of their validity in a large sample of children with polyarticular disease.

PATIENTS AND METHODS

Study design and patient selection. Because we were principally interested in examining the value of the scoring system for assessing radiographic progression, we chose a longitudinal design and aimed at analyzing continuous data. We reviewed the radiology records of patients seen at the study units from January 1986 to December 2004, to identify those who had a diagnosis of JIA according to the International League of Associations for Rheumatology revised criteria (18) and polyarthritis with wrist and/or hand joint involvement, and who had ≥2 standard radiographs of both wrists and hands in the posteroanterior view, one that was obtained at baseline (first observation) and one or more that were obtained at years 1, 3, 5, 7 or 8.

Radiograph scoring. All films were scored using the wrist/hand component of the Sharp/van der Heijde method (i.e., feet were excluded). This method applies to 15 areas for JSN and 16 areas for erosion in each hand and wrist (17,19). Scores for JSN and erosion in each area range from 0 to 4 and from 0 to 5, respectively. The total Sharp/van der Heijde score is calculated as the sum of the scores for JSN (range 0–120) and erosion (range 0–160) and ranges from 0 to 280. These scores will be referred to as “original” Sharp/van der Heijde scores.

We previously observed that JSN was more common than erosive changes in patients with JIA (16), and that erosions were frequent in some wrist areas not included in the adult scores (Ravelli A, et al: unpublished observations). Furthermore, erosive damage has been shown to be a better predictor than JSN of the long-term outcome of chronic arthritis (20,21). For these reasons, we devised a modified version of the Sharp/van der Heijde erosion score, which included 5 additional areas in each wrist: the second, third, and fourth metacarpal bases, the capitate bone, and the hamate bone. Values for this modified erosion score range from 0 to 210. The sum of the JSN and modified erosion scores yields the total modified Sharp/van der Heijde score, which ranges from 0 to 330. The areas included in the original and modified radiographic scores are shown in Figure 1.

In younger children (generally boys with a bone age <5 years and girls with a bone age <6 years), some of the wrist areas were not assessable due to incomplete ossification of the carpal bones. In such cases, each area that was not assessable was assigned the average score of assessable areas, rounded to the closest integer.

As noted previously (16), in younger children the changes in carpal bones and, to a lesser extent, in distal metacarpal epiphyses, were frequently seen as deformity in shape, from squaring to squeezing to gross deformity, rather than as discrete erosions. Although bone deformity and erosion are presumably caused by different pathogenetic mechanisms, for practical reasons they were considered as equivalent, and the severity of bone deformity was graded in the Sharp/van der Heijde erosion score, on the same 0–5-point severity scale.
Because in childhood the degree of ossification and the width of joint spaces vary with age, the evaluation of time progression of JSN and bony erosion in an individual patient is difficult; the same applies to comparison of films from patients of different ages. To facilitate assignment of Sharp/van der Heijde scores, we compared each study patient’s radiograph with a wrist/hand radiograph from a healthy child with the same bone age. Radiographs obtained from healthy boys and girls of all bone ages, ranging from 1.5 to 16 years according to the atlas of Greulich and Pyle (22), were identified by reviewing a large sample of radiographs in the study units. All children had undergone a bone age evaluation for short stature and were found to have a constitutional growth delay without endocrinologic abnormalities or had a radiograph (disclosing no abnormalities) that was obtained after wrist/hand trauma.

Reading strategy. Two observers (MI and XN) independently assigned the original and modified Sharp/van der Heijde scores to all study radiographs. Radiographs from each patient were read in sequential order, and previous radiographs and scores were available to observers when examining and scoring followup radiographs. Both observers are pediatric rheumatologists with >5 years of clinical experience in the field, but they were not familiar with radiographic scoring. Before the beginning of the study, the observers had a training session with the principal investigator (AR), a pediatric rheumatologist with ~20 years of clinical experience and familiarity with radiographic scoring, in order to gain experience with the Sharp/van der Heijde method.

Interobserver reliability of each scoring method was assessed for all of the films read by the 2 observers. Intraobserver reliability was based on the scores of radiographs obtained from a subset of 39 randomly selected patients, whose films were read a second time in a blinded manner by the 2 observers (81 films from 20 patients for observer MI and 71 films from 19 patients for observer XN), 3 months after the previous review.

Clinical assessment. Patient information included age at disease onset, sex, disease duration at baseline and at last followup visit, JIA subtype, and therapy with second-line medications and systemic corticosteroids throughout the study period. The following clinical assessments made at the last followup visits were recorded: physician’s global assessment of overall disease activity, as measured on a 10-cm visual analog scale (0 = no activity and 10 = maximum activity); count of joints with swelling, pain on motion/tenderness, restricted motion, and active disease (23); assessment of functional ability, using the Italian version of the Childhood Health Assessment Questionnaire (C-HAQ; 0 = best and 3 = worst) (24,25); Steinbrocker functional class (26); erythrocyte sedimentation rate (ESR) (Westergren method); C-reactive protein (CRP) level (as determined by nephelometry); the Juvenile Arthritis Damage Index, Articular (JADI-A) score (27); and the Poznanski score for radiographic damage (28). Briefly, the JADI-A assessed 36 joints or joint groups for the presence of damage, and the damage observed in each joint was scored on a 3-point scale (0 = no damage, 1 = partial damage, and 2 = severe damage, ankylosis, or prosthesis). The maximum total score is 72. The Poznanski score is a measure of the carpo:metacarpal ratio (29) and reflects the amount of radiographic damage in the wrist. Poznanski scores that are more negative represent more severe radiographic damage.

Statistical analysis. Validation procedures were primarily based on the analysis of reliability, construct validity, and score progression over time. Interobserver agreement and intraobserver agreement for the Sharp/van der Heijde scores were analyzed by computing the intraclass correlation coefficient (ICC) (30) for both longitudinal score values and score changes between study time points. For interpretation of the ICC values, the following classification was used: <0.4 = poor agreement, 0.4–<0.75 = moderate agreement, and ≥0.75 = good agreement (31). To visualize observer agreement, we plotted the scoring values (both absolute and changes) using the method described by Bland and Altman (32). The independent scores determined by the 2 observers for each radiograph were then averaged, and this average was used for the analyses.

Construct validity is a form of validation that examines whether the construct in question, in this case the Sharp/van der Heijde score, is related to other measures in a manner consistent with a priori prediction. Given that the Sharp/van der Heijde score is a measure of structural joint damage, it was predicted that the correlation between baseline Sharp/van der Heijde scores and score changes over time with the values at the last followup visit for the count of joints with restricted motion, Steinbrocker functional class, the JADI-A, and the Poznanski score, which measure closely related constructs, would be in the moderate-to-high range. Correlations with disease activity parameters at the final visit, such as the physician’s global assessment of disease activity, the count of swollen and tender joints, the ESR, and the CRP level, were predicted to be poor. No predictions were made for the correlation with the C-HAQ, because this measure was found to reflect both disease activity and damage in all stages of JIA (33). The level of radiographic damage at baseline and the rate of radiographic progression over time were expected to be associated with the amount of long-term radiographic damage. All correlations were assessed using Spearman’s rank correlation coefficient. For the purpose of this analysis, correlations >0.7 were considered high, correlations ranging from 0.4 to 0.7 were considered moderate, and correlations <0.4 were considered low (34). Agreement between predicted and observed correlations was taken as evidence of construct validity.

The time course of radiographic scores was assessed by calculating the average score value at each study time point and the change between time points, and was described by plotting the median score values and changes over time, using the cumulative probability plots method (35). Because the range of scores differs according to various versions of the Sharp/van der Heijde score, we compared the grading of joint damage for each score by normalizing each score by its possible range, according to the following formula: (observed value – minimum value)/possible range × 100. Likewise, the comparison of score changes between study time points was made after normalization of each observed change by the maximum possible change. Because the study sample comprised JIA patients with polyarthritis, who represent the subset of patients with the most severe form of JIA, an average steady increase in scores over time was expected. Statistical analysis was performed with Statistica (StatSoft, Tulsa, OK).
RESULTS

A total of 180 patients who were eligible for the study were identified. Three patients were excluded because they had only a baseline radiograph available for review. Of the 177 patients included (57 boys and 120 girls), 55 had systemic arthritis, 55 had polyarthritis (8 were rheumatoid factor positive), 52 had extended oligoarthritis, 9 had psoriatic arthritis, and 6 had undifferentiated arthritis. The mean age at disease onset was 3.7 years (range 0.3–15.7 years), and the mean disease duration at baseline was 1.4 years (range 0.6–6.5 years). During the study period, 170 patients (96%) had received ≥1 second-line drugs, and 96 patients (54%) had received systemic corticosteroids. At 1 year, 3 years, 5 years, 7/8 years, and 10 years, 147 patients (83%), 103 patients (58%), 75 patients (42%), 44 patients (25%), and 22 patients (12%) had radiographs available; a total of 568 radiographs were available for study.

Interobserver and intraobserver reliability. The interobserver agreement and intraobserver agreement (as assessed by the ICC) for the radiographic scores, either absolute values or score changes, were good, with 90% of the ICCs >0.9 and the remaining ICCs >0.85 (data not shown). The ICCs for radiographs examined separately at single time points and for all radiographs combined (and for the original and modified scores) were comparable. Table 1 shows the results obtained for the total original and modified scores, using the Bland and Altman method. 95% limits of agreement for absolute score values and score changes were comparable. Assessment of intraobserver reliability revealed better results, in terms of both the average difference and 95% limits of agreement, for observer 1 than for observer 2.

Construct validity. Table 2 presents Spearman’s correlations between the baseline radiographic score values, the change in radiographic scores between baseline and 1 year, the change in radiographic scores...
between baseline and 5 years, and the clinical measures of JIA severity at the last followup visit, ≥5 years (range 5–21 years) after the baseline visit. This analysis involved 96 patients who were followed up for ≥5 years and for whom followup clinical data were available. The radiography scores for these patients were comparable with those for the 81 patients who could not be included because of a followup period <5 years or a lack of clinical information (data not shown). Radiography score changes in the first year and in the first 5 years, but not the baseline score values, were moderately correlated, as predicted, with the clinical indicators of disease damage, such as the number of joints with restricted motion, the JADI-A, the Steinbrocker functional class, and the Poznanski score. Also as predicted, radiographic damage was poorly correlated with disease activity measures such as the physician’s global assessment of disease activity, the swollen and tender joint counts, and the laboratory indicators of inflammation. Unexpectedly, all correlations for the functional ability tool (the C-HAQ) were in the poor range. Looking at the correlations concerning change in radiography scores between baseline and 1 year, which are the most meaningful clinically, the modified erosion score demonstrated overall better results than the original erosion score.

Spearman’s correlations between the baseline radiography score values, the changes from baseline to 1 year and the changes from baseline to 5 years, and the absolute radiography score values at 5 years are shown in Table 3. As expected, the level of correlation increased progressively, revealing that the amount of radiographic damage at 5 years was predicted poorly by the level of radiographic damage at baseline, moderately by the level of radiographic progression during the first year, and strongly by the level of radiographic progression during the first 5 years.

### Table 2. Spearman’s correlations of baseline score values and changes over time with measures of JIA severity at last followup visit in 96 patients with JIA

<table>
<thead>
<tr>
<th>No.</th>
<th>No.</th>
<th>No.</th>
<th>C-HAQ</th>
<th>JADI-A</th>
<th>Steinbrocker score</th>
<th>Poznanski score</th>
<th>ESR</th>
<th>CRP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Physician’s global assessment</td>
<td>Total original score</td>
<td>Total modified score</td>
<td>Change 0–1 year</td>
<td>Total original score</td>
<td>Total modified score</td>
<td>Change 0–1 year</td>
<td>Total original score</td>
</tr>
<tr>
<td></td>
<td></td>
<td>baseline to 5 years†</td>
<td>baseline to 5 years‡</td>
<td></td>
<td>baseline to 5 years†</td>
<td>baseline to 5 years‡</td>
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<td>baseline to 5 years†</td>
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</tbody>
</table>

* JIA = juvenile idiopathic arthritis; C-HAQ = Childhood Health Assessment Questionnaire; JADI-A: Juvenile Arthritis Damage Index, Articular; ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; total original score = sum of joint space narrowing (JSN) score in 30 areas plus erosion score in 42 areas; total modified score = sum of JSN score in 30 areas plus erosion score in 42 areas.
† Moderate correlation (r ≥ 0.4 and ≤0.7).
‡ All correlations were high (r ≥ 0.7).

### Table 3. Spearman’s correlations between scores at baseline, changes from baseline to 1 year, changes from baseline to 5 years, and score values at 5 years in 75 patients with JIA

<table>
<thead>
<tr>
<th>Score</th>
<th>Total original score</th>
<th>Total modified score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline value</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JSN score</td>
<td>0.43†</td>
<td>0.42†</td>
</tr>
<tr>
<td>Original erosion score</td>
<td>0.27</td>
<td>0.27</td>
</tr>
<tr>
<td>Modified erosion score</td>
<td>0.35</td>
<td>0.37</td>
</tr>
<tr>
<td>Total original score</td>
<td>0.38</td>
<td>0.39</td>
</tr>
<tr>
<td>Total modified score</td>
<td>0.42†</td>
<td>0.40†</td>
</tr>
<tr>
<td>Change, baseline to 1 year</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JSN score</td>
<td>0.49†</td>
<td>0.51†</td>
</tr>
<tr>
<td>Original erosion score</td>
<td>0.49†</td>
<td>0.50†</td>
</tr>
<tr>
<td>Modified erosion score</td>
<td>0.60†</td>
<td>0.62†</td>
</tr>
<tr>
<td>Total original score</td>
<td>0.61†</td>
<td>0.61†</td>
</tr>
<tr>
<td>Total modified score</td>
<td>0.66†</td>
<td>0.66†</td>
</tr>
<tr>
<td>Change, baseline to 5 years‡</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JSN score</td>
<td>0.83</td>
<td>0.82</td>
</tr>
<tr>
<td>Original erosion score</td>
<td>0.79</td>
<td>0.81</td>
</tr>
<tr>
<td>Modified erosion score</td>
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<td>0.84</td>
</tr>
<tr>
<td>Total original score</td>
<td>0.89</td>
<td>0.88</td>
</tr>
<tr>
<td>Total modified score</td>
<td>0.88</td>
<td>0.89</td>
</tr>
</tbody>
</table>

* JIA = juvenile idiopathic arthritis; JSN = joint space narrowing; original erosion score = erosion score in 32 areas; modified erosion score = erosion score in 42 areas; total original score = sum of JSN score in 30 areas plus erosion score in 32 areas; total modified score = sum of JSN score in 30 areas plus erosion score in 42 areas.
† Moderate correlation (r ≥ 0.4 and ≤0.7).
‡ All correlations were high (r > 0.7).
tion during the first 5 years. Looking again at the correlations concerning the changes in radiography scores between baseline and 1 year, there was a tendency for the modified scores to yield better correlations than did the original scores. Furthermore, the modified erosion scores, but not the original erosion scores, yielded overall better correlations compared with the JSN scores.

**Absolute radiography score values and score changes over time.** The amount of radiographic damage at baseline was comparable across the different JIA subtypes (data not shown). Plotting of the median normalized values of absolute score values at different study time points showed a steady increase in scores during the study period (Figure 2). The JSN scores increased more rapidly and remained consistently higher over time than both the original and modified erosion scores, suggesting that cartilage loss remained the most common and severe form of radiographic damage throughout the entire disease course. Modified erosion scores offered a slight advantage over original erosion scores in capturing erosive changes over time. Analysis of the median normalized change in scores over time confirmed that the rate of JSN change was constantly greater than that of erosive change (data not shown). Figure 3 depicts the cumulative probability plot of individual baseline-to-1 year changes in total original and modified scores. Five percent to 15% of the patients, depending on the study interval, showed improvement (positive change) in radiography scores over time.

**DISCUSSION**

The course and prognosis of JIA are highly variable: some patients experience a benign course and recover fully, whereas others have unremitting illness and carry a significant risk of joint destruction and permanent disability (36,37). Although the outcome of JIA is generally unpredictable, especially early in the disease course (38), it is well known that patients with polyarticular arthritis are those in whom progressive destructive disease is more likely to develop (36,37,39). Furthermore, a higher than expected percentage of these patients have been found to have JSN and erosions early in their illness (40,41). The presence of polyarthritis is a prerequisite for a patient's inclusion in controlled trials of second-line or biologic agents (42–44).

JIA patients with polyarthritis and wrist disease are at high risk of experiencing radiographic progression (10,45). As many as 85% of the 633 patients enrolled in a controlled trial aimed at comparing intermediate and high doses of methotrexate in polyarticular JIA (44) had active disease in the wrist and/or hand joints (Ruperto N, et al: unpublished observations). Thus, the wrist and hand joints represent optimal sites at which to investigate radiographic progression in patients with polyarticular JIA.

We observed that the Sharp/van der Heijde score is a reliable and valid method for assessing radiographic progression in children with chronic polyarthritis. The study sample represents 30–40% of the entire population of JIA patients seen by the authors during the study period. Furthermore, the sample includes the majority of the most severe cases, as shown by the fact that as many as 96% of the patients had received second-line...
medications, and roughly half had received systemic corticosteroids.

We chose to investigate the Sharp/van der Heijde scoring method, because we believed that its manner of grading bony erosion, which not only is based on the count of the number of erosions but also takes into account their size in relation to bone surface, is particularly suited for application in pediatric patients. The bone size in children changes with skeletal maturation. Furthermore, in younger children with JIA, the changes in carpal bones and, to a lesser extent, in distal metacarpal epiphyses, are seen most frequently as deformity in shape rather than as discrete erosions (16). This phenomenon is unique to JIA and is likely attributable to a combination of growth abnormalities, ossification of previous cartilage injury, and true bony erosions (46–48). We excluded the foot component of the Sharp/van der Heijde score, because foot joints are rarely involved in JIA.

The assessment of JSN and erosions in growing children is challenging, owing to the changes in the morphology of bones and joints during skeletal maturation. To overcome this problem, we compared each patient’s radiograph with a wrist/hand radiograph obtained in a healthy child of the same sex and bone age. We chose bone age–related instead of age-related or size-related standards, because patients with JIA frequently have advanced skeletal maturation (45,46) and are small for their age (with their bones being correspondingly small), making these standards unreliable.

In the investigational setting chosen, the radiography scores under study proved to be reliable. Interobserver and intraobserver agreement, as assessed by the ICC, were good for both absolute values and score changes. The overall good concordance among observers was confirmed using the Bland and Altman method and the cumulative probability plots method.

The results of construct validity analysis were consistent with our expectations. Radiography score changes over time were strongly correlated with the clinical indicators of long-term joint damage and were poorly correlated with clinical measures of disease activity at the last followup visit. Similarly, radiographic progression over time was highly correlated with the amount of long-term radiographic damage. Evaluation of the rate of radiographic progression over time confirmed our previous observation (16) that JSN, which captures cartilage resorption, is the most common form of damage throughout the disease course in children with JIA. A sizable proportion of patients experienced improvement in radiographic progression over time, which may reflect both the effectiveness of antirheumatic therapies and the distinctive regenerative capacity of articular cartilage in growing children (39,46).

Of the different radiography scoring methods tested, the modified versions (which included more areas at which erosion can be scored) appeared to be overall advantageous compared with the original versions. The modified scores revealed slightly wider 95% limits of agreement on the Bland and Altman analysis of interobserver reliability and were only modestly superior to the original scores in capturing radiographic progression over time. However, the modified scores revealed better construct validity in the clinically most meaningful subset of correlations between radiographic change from baseline to 1 year and long-term clinical and radiographic damage. Furthermore, 4 of the 5 new erosion areas included in the modified score were shown to be the most frequent sites of erosive changes within the entire hand and wrist areas (data not shown).

Our study should be viewed in the context of certain limitations. We chose a longitudinal design, because our aim was to examine the reliability of scores in the assessment of radiographic progression. The reading of serial radiographs may have facilitated concordance among readers, whereas agreement on scoring of cross-sectional films might have been more difficult to achieve. Readers examined the radiographs in chronological sequence and were allowed to see the previous scores. There is no definite consensus regarding whether readers should be aware of the time order of radiographs (49). However, blinding of films to chronological order in children is impossible due to readily apparent growth and maturation of the skeleton. We recognize that our choice of considering bone deformity as equivalent to bony erosion represents a potential limitation of our work, because these forms of damage are presumably unrelated pathogenetically. We also acknowledge that because the study scores assess radiographic damage only in the wrist/hand joints, our findings are of value only for patients with wrist and/or hand disease.

We conclude that in our cohort of JIA patients with polyarthritis, the adapted versions of the Sharp/van der Heijde scoring method proved reliable and valid and performed well in terms of capturing radiographic damage and its progression. These findings confirm and extend our previous demonstration that scoring methods for adults can be used to assess radiographic progression in JIA. Furthermore, they support the use of quantitative measures of radiographic damage in pediatric rheumatology care and their inclusion in future observational studies and therapeutic trials in JIA.
AUTHOR CONTRIBUTIONS

Dr. Ravelli had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Ravelli, Pistorio, Ruperto, Magni-Manzoni, Martini.

Acquisition of data. Ioseliani, Norambuena, Sato, Rossi, Ullmann.

Analysis and interpretation of data. Ravelli, Sato, Pistorio, Magni-Manzoni.

Manuscript preparation. Ravelli, Martini.

Statistical analysis. Pistorio, Ruperto.

REFERENCES

A Randomized, Placebo-Controlled Trial of Infliximab Plus Methotrexate for the Treatment of Polyarticular-Course Juvenile Rheumatoid Arthritis


Objective. To evaluate the safety and efficacy of infliximab in the treatment of juvenile rheumatoid arthritis (JRA).

Methods. This was an international, multicenter, randomized, placebo-controlled, double-blind study. One hundred twenty-two children with persistent polyarticular JRA despite prior methotrexate (MTX) therapy were randomized to receive infliximab or placebo. ClinicalTrials.gov identifier: NCT00036374. Supported by Centocor, Inc.

Drs. Ruperto, Lovell, Woo, Prieur, Petty, Martini, and Giannini have received honoraria and/or consulting fees (less than $10,000) from Centocor for serving as members of the study steering committee. Dr. Lovell has received consulting fees, speaking fees, and/or honoraria (less than $10,000 each) from Hoffmann-LaRoche, Amgen, Novartis, Bristol-Myers Squibb, Abbott, and Xoma and (more than $10,000) from Regeneron. Dr. Saurenmann has received honoraria (less than $10,000) from Essex for serving as a member of the Remicade “Switzerland” advisory board. Drs. Travers, Beutler, Keenan, Clark, Visvanathan, Fasanmade, Raychaudhuri, and Mendelsohn own stock or stock options in Johnson & Johnson, of which Centocor, Inc., is a subsidiary.

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for 14 weeks, after which all children received infliximab through week 44. Patients received MTX plus infliximab 3 mg/kg through week 44, or MTX plus placebo for 14 weeks followed by MTX plus infliximab 6 mg/kg through week 44.

Results. Although a higher proportion of patients in the 3 mg/kg infliximab group than in the placebo group had achieved responses according to the American College of Rheumatology (ACR) Pediatric 30 (Pedi 30) criteria for improvement at week 14 (63.8% and 49.2%, respectively), the between-group difference in this primary efficacy end point was not statistically significant ($P = 0.12$). By week 16, after the crossover from placebo to infliximab 6 mg/kg when all patients were receiving infliximab, an ACR Pedi 30 response was achieved in 73.2% of all patients. By week 52, ACR Pedi 50 and ACR Pedi 70 responses had been reached in 69.6% and 51.8%, respectively, of patients. Infliximab was generally well tolerated, but the safety profile of infliximab 3 mg/kg appeared less favorable than that of infliximab 6 mg/kg, with more frequent occurrences of serious adverse events, infusion reactions, antibodies to infliximab, and newly induced antinuclear antibodies and antibodies to double-stranded DNA observed with the 3 mg/kg dose.

Conclusion. While infliximab at 3 mg/kg and 6 mg/kg showed durable efficacy at 1 year, achievement of the primary efficacy end point at 3 months did not differ significantly between infliximab-treated and placebo-treated patients. Safety data indicated that the 6-mg/kg dose may provide a more favorable risk/benefit profile. These results warrant further investigation in children with JRA.

Juvenile rheumatoid arthritis (JRA) is the most common chronic rheumatic disorder in children (1) and is also a major cause of acquired disability in childhood (2–4). Weekly methotrexate (MTX), at parenteral dosages of up to 15 mg/m²/week, has been established as an effective and safe therapy for polyarticular-course JRA (5–7). For children whose disease does not respond to MTX, anti–tumor necrosis factor (anti-TNF) therapy is a treatment option (8–11). One such anti-TNF agent, infliximab, is a chimeric monoclonal antibody that specifically and potently binds and neutralizes soluble TNFα and its membrane-bound precursor. Infliximab has been shown to be effective in combination with MTX for the treatment of adult RA (12,13), and open-label studies of infliximab in JRA have been conducted (14,15).

Infliximab has some potentially attractive features for treatment of JRA, including infrequent administration under the direct supervision of a health care professional and, in contrast to etanercept, lack of binding to lymphotoxin α, which is important in immune defense. The current trial was undertaken to investigate the efficacy and safety of infliximab in children with polyarticular-course JRA.

PATIENTS AND METHODS

Patients. A total of 122 children with JRA from 34 sites (9 in North America, 3 in South America, and 22 in Europe) were enrolled between October 2001 and April 2004. An independent ethics committee at each center approved the protocol. Patients provided assent (if age appropriate and per local regulations), and parents/legal guardians provided written informed consent.

Criteria for study eligibility were as follows: age ≥4 years but <18 years, a diagnosis of JRA (16), suboptimal response to MTX after ≥3 months of treatment, ≥5 active joints, and no active systemic symptoms. Exclusion criteria included active uveitis, serious infection including tuberculosis, malignancy, or prior treatment with any TNF inhibitor. Disease-modifying drugs other than MTX and intraarticular corticosteroid injections were not permitted within 4 weeks prior to study entry and during the trial. Any patient with a positive purified protein derivative (PPD) skin test result had to receive adequate therapy for tuberculosis prior to receipt of study agent, according to the Centers for Disease Control and Prevention 2000 guidelines (17) or from the local tuberculosis control agency as appropriate. Concomitant treatment with low-dose corticosteroids (up to 0.2 mg/kg/day or 10 mg/day, whichever was less), 1 nonsteroidal antiinflammatory drug (NSAID), 1 analgesic that was not an NSAID, folic acid prophylaxis (required for all patients taking MTX), and narcotic or opioid analgesics was permitted during the study. If a mild or moderate reaction (e.g., headache, nausea, itching, urticaria) had been observed during a previous infusion, pretreatment with acetaminophen and an antihistamine was recommended for the patient prior to the start of all subsequent infusions.

Study design. This was a phase III, international, multicenter, randomized, double-blind, placebo-controlled study of infliximab therapy administered for 14 weeks, followed by a double-blind, all–active treatment extension through 44 weeks, in JRA patients receiving concomitant oral or parenteral MTX therapy (10–15 mg/m²/week). Patients were randomly assigned to 1 of 2 groups (Figure 1). Group 1 received placebo infusions plus MTX at weeks 0, 2, and 6, followed by an induction regimen of infliximab 6 mg/kg plus MTX at weeks 14, 16, and 20 and then every 8 weeks (termed “placebo/infliximab 6 mg/kg” or simply “infliximab 6 mg/kg”). Group 2 received infliximab 3 mg/kg plus MTX at weeks 0, 2, 6, and 14, placebo at week 16, and infliximab 3 mg/kg at week 20 and then every 8 weeks (termed “infliximab 3 mg/kg”). Patients completing treatment through week 44 who, in the judgment of the investigator, could benefit from continued...
treatment were eligible for an open-label extension beginning at week 52; this report contains data collected through week 52 only.

Infliximab was supplied as a lyophilized solid formulation containing 100 mg. Placebo was supplied as a lyophilized solid. Study medication was administered over a period of 40–120 minutes.

Serum samples were tested for antibodies to infliximab, using a bridging immunoassay (18), at weeks 0, 52, and 64. Serum infliximab concentrations were determined with an enzyme-linked immunoassay (18) at weeks 0, 2, 6, 14, 16, 20, 28, 36, 44, and 52. Clinical response evaluations, results of laboratory analyses (including testing for antinuclear antibodies [ANAs] and anti–double-stranded DNA [anti-dsDNA]), and adverse events were recorded throughout the study.

**Definitions of response to therapy.** Response to therapy was defined based on the combination of the following 6 JRA core set parameters (19,20): 1) physician global assessment of disease activity, on a 10-cm visual analog scale (VAS); 2) parent/patient global assessment of overall well-being, on a 10-cm VAS; 3) a measure of physical function, which for this trial was the cross-culturally adapted and validated version of a measure of functional ability in children with JRA, i.e., the Childhood Health Assessment Questionnaire (21,22); 4) the number of joints with active arthritis, defined by the presence of swelling or, if no swelling was present, limitation of motion accompanied by pain, tenderness, or both (16); 5) the number of joints with limited range of motion; and 6) a laboratory measure of inflammation, which for this trial was the erythrocyte sedimentation rate. Whenever possible, each patient’s joints were evaluated by the same assessor at each visit.

The primary end point of the trial was the proportion of patients meeting the American College of Rheumatology (ACR) Pediatric 30 (Pedi 30) criteria for improvement at week 14, defined as improvement of ≥30% in at least 3 of 6 core variables, with no more than 1 of the remaining variables worsened by >30% (19,20). Patients were also evaluated using more stringent definitions of improvement, i.e., ≥50% or ≥70% improvement in at least 3 of 6 core variables, with no more than 1 of the remaining variables worsened by >30% (ACR Pedi 50 and ACR Pedi 70, respectively). At week 52, the number of patients who had 0 joints with active arthritis was determined.

**Statistical analysis.** This report complies with the recommendations of the CONSORT (Consolidated Standards of Reporting Trials group) statement (23). Statistical analyses were carried out according to the intent-to-treat principle, by 2 of the authors at Centocor, Inc. (Drs. Clark and Raychaudhuri). Members of the study steering committee (Drs. Ruperto, Lovell, Woo, Prieur, Petty, Martini, and Gianinni) had full access to all statistical analysis reports.

For the primary efficacy end point, efficacy data were excluded due to withdrawal of consent for 1 patient in the placebo group (no efficacy data were available) and due to potential patient unblinding issues at a single site for 4 patients (2 in the placebo group and 2 in the infliximab 3 mg/kg group) (Figure 1). Thus, 117 patients were included in the primary efficacy analysis. In the analysis of the primary end point (the

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**Figure 1.** Disposition of the 122 juvenile rheumatoid arthritis patients enrolled in the study. MTX = methotrexate.
proportion of patients meeting the ACR Pedi 30 at week 14), patients who did not return for evaluation or patients with missing data were considered nonresponders. For efficacy analyses other than analysis of the primary end point, patients with available data at the given time point were included.

Statistical comparisons were made using the Cochran-Mantel-Haenszel chi-square test for categorical data and the van der Waerden test for continuous data. Ninety-five percent confidence intervals were calculated.

Patients were randomly assigned, at a 1:1 ratio, to 2 treatment groups. Patient allocation was performed using adaptive stratified randomization, with investigational site and age group (≥4 years but <8 years, ≥8 years but <12 years, and ≥12 years but <18 years) as the strata. Since age was considered as an independent variable that would affect probability of response, stratification according to age was implemented to assure proper balance among the treatment groups. It was determined that each treatment group would have to include at least 60 patients, to provide 79–97% power to detect a difference in the proportions of patients achieving the ACR Pedi 30 (α = 0.05, 2-sided) if the infliximab group had a frequency of response of at least 55–60% and the placebo group had a frequency of response of 25–30%.

RESULTS

Baseline characteristics of the study patients.
One hundred twenty-two patients were randomized to 1 of 2 treatment groups: 62 to the placebo/infliximab 6 mg/kg group and 60 to the infliximab 3 mg/kg group. Prior to week 52, 13 (11%) of the 122 patients withdrew from the study (Figure 1). Baseline demographic and clinical characteristics were comparable between the 2 treatment groups (Table 1).

Efficacy. A rapid response was observed in the infliximab 3 mg/kg group, with 58.6% of the patients (34 of 58) exhibiting an ACR Pedi 30 response by week 6 (Figure 2). Although an ACR Pedi 30 response had been achieved in a higher proportion of patients in the infliximab 3 mg/kg group (37 of 58 [63.8%]) when compared with patients in the placebo group (29 of 59 [49.2%]) at week 14, the between-group difference in the proportion of patients achieving the primary efficacy

<table>
<thead>
<tr>
<th>Table 1. Baseline demographic and clinical characteristics of the JRA patients*</th>
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<tbody>
<tr>
<td>Treatment randomization</td>
</tr>
<tr>
<td>Placebo/infliximab 6 mg/kg + MTX (n = 62)</td>
</tr>
<tr>
<td>Infliximab 3 mg/kg + MTX (n = 60)</td>
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<tr>
<td>Age at study entry, years</td>
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<tr>
<td>Disease duration, years</td>
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<tr>
<td>Age subgroup, no. (%)</td>
</tr>
<tr>
<td>≥4 years, &lt;8 years</td>
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<tr>
<td>≥8 years, &lt;12 years</td>
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<tr>
<td>≥12 years, &lt;18 years</td>
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<tr>
<td>Female, no. (%)</td>
</tr>
<tr>
<td>White, no. (%)</td>
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<tr>
<td>JRA onset subtype, no. (%)</td>
</tr>
<tr>
<td>Systemic onset</td>
</tr>
<tr>
<td>Pauciarticular onset with polyarticular course</td>
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<tr>
<td>Polyarticular onset</td>
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<tr>
<td>Physician's global assessment of disease activity, 10-cm VAS</td>
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<tr>
<td>Parent's assessment of overall well-being, 10-cm VAS</td>
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<tr>
<td>C-HAQ score, 0–3 scale</td>
</tr>
<tr>
<td>No. of joints with active arthritis</td>
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<tr>
<td>No. of joints with limited range of motion</td>
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<tr>
<td>Erythrocyte sedimentation rate, mm/hour</td>
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<tr>
<td>Rheumatoid factor positive, no. (%)</td>
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<tr>
<td>Oral corticosteroid treatment, no. (%)</td>
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<tr>
<td>Prior DMARD treatment other than MTX, no. (%)</td>
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<tr>
<td>Weekly MTX dosage, mg/m² (range 10–15)</td>
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<tr>
<td>Duration of MTX therapy, no. (%)</td>
</tr>
<tr>
<td>&gt;6 months</td>
</tr>
<tr>
<td>≤6 months</td>
</tr>
<tr>
<td>Unknown†</td>
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</tbody>
</table>

* All patients had involvement of multiple joints. Except where indicated otherwise, values are the mean ± SD. JRA = juvenile rheumatoid arthritis; MTX = methotrexate; VAS = visual analog scale; C-HAQ = childhood Health Assessment Questionnaire; DMARD = disease-modifying antirheumatic drug.
† Exact duration of therapy unknown, but known to be ≥3 months per study inclusion criteria.
end point was not statistically significant ($P = 0.12$). Also at week 14, higher proportions of patients in the infliximab 3 mg/kg group than in the placebo group met the ACR Pedi 50 improvement criteria (29 of 58 [50%] versus 20 of 59 [33.9%]; $P = 0.078$) and the ACR Pedi 70 improvement criteria (13 of 58 [22.4%] versus 7 of 59 [11.9%]; $P = 0.130$).

By week 16, following crossover from placebo to infliximab 6 mg/kg and at which time all patients were receiving infliximab, 73.2% of all patients (82 of 112) exhibited an ACR Pedi 30 response. By week 52, clinical responses meeting the ACR Pedi 50 and ACR Pedi 70 criteria were reached by 69.6% (78 of 112) and 51.8% (58 of 112) of the patients, respectively. There were no statistically significant differences between the infliximab dose groups when the proportions of patients with an ACR Pedi 30, ACR Pedi 50, or ACR Pedi 70 response at week 52 were compared. In addition, similar proportions of patients in the infliximab 3 mg/kg and infliximab 6 mg/kg groups had 0 joints with active arthritis at week 52 (26 of 59 [44.1%] and 25 of 58 [43.1%], respectively).

Findings for the individual components of the JRA core set are shown in Figure 3. By week 14, improvements in 4 of the 6 JRA core set components were greater in the infliximab 3 mg/kg group than in the placebo group. At week 14, the number of joints with active arthritis differed significantly between patients in the infliximab 3 mg/kg group and those in the placebo group ($P = 0.016$), whereas there were no significant differences for the other core set variables. By the end of the study, following crossover of placebo-treated patients to infliximab 6 mg/kg, improvement in the JRA core set components was comparable between the treatment groups.

Results of pharmacokinetic analyses demonstrated that, as expected, the infliximab 3 mg/kg group had lower median serum concentrations of infliximab early in the study compared with the infliximab 6 mg/kg group (at relative time frames). The majority of patients receiving infliximab 3 mg/kg had detectable serum infliximab concentrations up to week 36, after which more patients in this group had lower-than-quantifiable trough levels of infliximab in their sera. At these later sampling time points, more than half of the patients receiving infliximab 3 mg/kg (i.e., 51.1% at week 44 and 52.4% at week 52) did not maintain quantifiable infliximab concentrations by the end of the 8-week interval separating infusions (Figure 4). Overall, the median half-life of infliximab was 9.5 days with the 6-mg/kg

Figure 2. Proportion of juvenile rheumatoid arthritis patients meeting the American College of Rheumatology (ACR) Pediatric 30 (Pedi 30) criteria for improvement, the ACR Pedi 50 criteria for improvement, and the ACR Pedi 70 criteria for improvement over time, by treatment group. Bars show the 95% confidence intervals. MTX = methotrexate.
dose, compared with 6.9 days with the 3-mg/kg dose. When the proportion of patients in whom an ACR Pedi 30 response was achieved was plotted against different categories of infliximab trough serum levels, a progressive increase in the proportion of patients achieving improvement was found to correspond to
Antibodies to infliximab and infusion reactions. Overall, of the patients with evaluable samples, 25% (26 of 102) had antibodies to infliximab, with a higher incidence in the infliximab 3 mg/kg group (20 of 53 [38%]) compared with the infliximab 6 mg/kg group (6 of 49 [12%]). In addition, the antibody titers were notably higher among patients in the infliximab 3 mg/kg group, with 8 of 20 patients (40%) having a titer of 1:320 or higher (3 patients had titers of 1:20,480), compared with only 2 of 6 patients (33%) in the infliximab 6 mg/kg group.

Patients who did not test positive for antibodies to infliximab but following their last infusion had detectable serum concentrations of infliximab (data not shown).

Antibodies to infliximab and infusion reactions. Among patients who had positive test results for antibodies to infliximab, the incidence of infusion reactions was 60% (12 of 20) in the infliximab 3 mg/kg group, compared with 50% (3 of 6) in the infliximab 6 mg/kg group. Across all treatment groups, there was a 3-fold higher incidence of infusion reactions among patients who tested positive for antibodies to infliximab (15 of 26 [58%]) than among patients who tested negative for antibodies to infliximab (5 of 26 [19%]) or who had inconclusive test results (6 of 50 [12%]). Similarly, serious infusion reactions occurred in 20% of patients (4 of 20) who were antibody positive, compared with none of the patients who had either negative or inconclusive test results.

Additional safety assessments. The time frames during which adverse events were monitored, as shown in Table 2, differed among the treatment groups. The overall occurrence of adverse events was similar among the 3 treatment groups. More patients in the infliximab 6 mg/kg group (5 patients) discontinued study agent due to adverse events, compared with patients in the placebo and infliximab 3 mg/kg groups (1 patient and 2 patients, respectively) (Table 2). In addition, 2 patients receiving infliximab 3 mg/kg discontinued study agent as a result of serious infusion reactions related to treatment. Among these patients, 1 patient developed a delayed-type hypersensitivity reaction, and 1 patient had a possible anaphylactic reaction. In general, infusion-related reactions resolved upon slowing or stopping of the infusion.
of abnormal laboratory test values, i.e., markedly abnormal alanine aminotransferase/aspartate aminotransferase levels that normalized after the MTX dosage was lowered (it should be noted that these laboratory abnormalities were not considered adverse events). After adjustment for the length of followup (i.e., incidence divided by length of followup), serious adverse events were approximately 2 times more frequent in the infliximab 3 mg/kg group (62.4%) than in the placebo group (32.9%) or the infliximab 6 mg/kg group (24.0%).

One patient died during the study, ∼10 days after the placebo infusion at week 2. After hospitalization for septic shock, cardiac function deteriorated, leading to death. A second patient, with systemic-onset JRA, died following study participation (infliximab 3 mg/kg group). This child experienced a severe JRA flare 3 months after the final infusion of infliximab and was hospitalized and treated. The patient withdrew from the study during participation in the open-label extension and 3 months later (6 months after the last infliximab infusion) died in the hospital, of cardiac arrest. The child had been placed on the stem cell transplant list because of JRA severity.

Infections occurred in 66.6% of patients treated with infliximab, compared with 46.7% of those receiving placebo (Table 2). The most commonly reported infection was upper respiratory tract infection. Three patients in the infliximab 3 mg/kg group had potential opportunistic infections: 1 with moniliasis presenting as vaginal thrush, 1 with moniliasis presenting as oral thrush, and 1 with herpes zoster. Serious infections occurred in 6 patients treated with infliximab and 2 patients receiving placebo. Among the serious infections reported, there were 4 cases of pneumonia in the infliximab 3 mg/kg group; 1 case followed a varicella zoster infection.

There was 1 report of asymptomatic pulmonary tuberculosis with negative mycobacterial cultures; this patient had a negative PPD skin test result at screening but a positive result during the routine screening that was performed prior to the start of the second year of the open-label extension. At that time, the patient was noted to have a PPD skin test result of 13 mm, as well as chest radiography and magnetic resonance imaging evidence of interstitial infiltration and nodularity. Results of polymerase chain reaction testing of a sputum sample from the patient were positive for *Mycobacterium tuberculosis* complex (*Mycobacterium tuberculosis*, *Mycobacterium africanum*, *Mycobacterium bovis*, and *Mycobacterium microti*), but all sputum cultures were read as negative. A subsequent lung scan revealed improvement of lung infiltration, and findings on repeat chest radiog-

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**Table 2. Summary of safety findings***

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Placebo + MTX (0–14 weeks) (n = 60)</th>
<th>Infliximab 3 mg/kg + MTX (0–52 weeks) (n = 60)</th>
<th>Infliximab 6 mg/kg + MTX (14–52 weeks) (n = 57)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± SD weeks of followup</td>
<td>15.2 ± 7.2</td>
<td>50.8 ± 4.6</td>
<td>36.7 ± 4.5</td>
</tr>
<tr>
<td>Adverse events</td>
<td>49 (81.7)</td>
<td>58 (96.7)</td>
<td>54 (94.7)</td>
</tr>
<tr>
<td>Adverse events leading to discontinuation of study agent</td>
<td>1 (1.7)</td>
<td>2 (3.3)</td>
<td>5 (8.8)</td>
</tr>
<tr>
<td>Infusion reactions†</td>
<td>0 (0.0)</td>
<td>2 (3.3)</td>
<td>4 (7.0)</td>
</tr>
<tr>
<td>Circulatory failure</td>
<td>1 (1.7)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Depression</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>1 (1.8)</td>
</tr>
<tr>
<td>Serious adverse events‡</td>
<td>3 (5.0)</td>
<td>19 (31.7)</td>
<td>5 (8.8)</td>
</tr>
<tr>
<td>Infections</td>
<td>28 (46.7)</td>
<td>41 (68.3)</td>
<td>37 (64.9)</td>
</tr>
<tr>
<td>Serious infections</td>
<td>2 (3.3)</td>
<td>5 (8.3)</td>
<td>1 (1.8)</td>
</tr>
<tr>
<td>No. (%) of infusions with infusion reactions§</td>
<td>6/177 (3.4)</td>
<td>46/503 (9.1)</td>
<td>13/313 (4.2)</td>
</tr>
<tr>
<td>Patients with infusion reactions</td>
<td>5 (8.3)</td>
<td>21 (35.0)</td>
<td>10 (17.5)</td>
</tr>
<tr>
<td>Antinuclear antibodies (newly positive)</td>
<td>0/30 (0)</td>
<td>8/54 (14.8)</td>
<td>1/46 (2.2)</td>
</tr>
<tr>
<td>Anti–double-stranded DNA (newly positive)</td>
<td>0/30 (0)</td>
<td>7/54 (13.0)</td>
<td>0/46 (0)</td>
</tr>
</tbody>
</table>

*Except where indicated otherwise, values are the number (%) of patients. MTX = methotrexate.
†The following infusion reactions were reported, with some patients reporting >1 symptom: allergic reaction, hypotension, abdominal pain, anaphylactoid reaction, coughing, face edema, fever, rash, urticaria, and vomiting.
‡Any adverse event that resulted in death, any life-threatening event, inpatient hospitalization or prolongation of existing hospitalization, persistent or significant disability/incapacity, or congenital anomaly/birth defect.
§Any adverse event that occurred during an infusion or within 1 hour after completion of an infusion.
raphy were reported as being normal after 9 months of antituberculosis therapy. There were no reports of congestive heart failure or malignancy in this study.

Newly positive ANAs (titer ≥1:320) occurred in 8 of 54 patients (14.8%) and 1 of 46 patients (2.2%) during treatment with infliximab 3 mg/kg and infliximab 6 mg/kg, respectively, compared with none of the patients receiving placebo. Similarly, newly positive anti-dsDNA occurred in 7 of 54 patients (13.0%) and 0 of 46 patients (0%) during treatment with infliximab 3 mg/kg and infliximab 6 mg/kg, respectively, and in no patients during treatment with placebo. Overall, markedly abnormal changes in hematologic and clinical chemistry parameters were infrequent and were generally evenly distributed across treatment groups. A higher proportion of patients in the infliximab groups (1.7% and 8.8% in the 3 mg/kg and 6 mg/kg groups, respectively) as compared with placebo (0%) had at least 1 decrease in the absolute neutrophil count (<1.5 × 10^3 cells/µl and decrease ≥33%). Elevations in alanine aminotransferase levels to ≥90 IU/liter and elevations of ≥100% from baseline were observed in 3.3% of the patients treated with infliximab 3 mg/kg, 8.8% of those treated with infliximab 6 mg/kg, and no patients during placebo treatment.

**DISCUSSION**

This was a randomized placebo-controlled trial of a chimeric monoclonal anti-TNFα agent for the treatment of children with severe JRA that has been resistant to previous therapies. Patients treated with infliximab 3 mg/kg exhibited rapid improvement as early as week 2, based on the ACR Pedi 30 response. At week 14, an ACR Pedi 30 response had been achieved in a higher proportion of patients in the infliximab 3 mg/kg group compared with patients in the placebo group, but this difference in the primary efficacy end point was not statistically significant. At week 16, following crossover from placebo to the first dose of infliximab 6 mg/kg, 73% of all patients exhibited an ACR Pedi 30 response; this level of response was maintained for the remainder of the study. Similar trends were observed when higher levels of ACR Pedi response were assessed. Indeed, by week 52, an ACR Pedi 50 response had been achieved in just under 70% of the patients, and an ACR Pedi 70 response in just under 52%. Similar proportions of patients in the infliximab 3 mg/kg and infliximab 6 mg/kg groups had 0 joints with active disease at week 52.

These findings, despite the difference in the study design, are similar to those observed during the open-label phase of a trial assessing the use of etanercept in JRA (8), and demonstrate the value of infliximab in maintaining efficacy for up to 1 year. Of note, 78 of the 122 children enrolled in the study (63.9%) subsequently entered a 3-year open-label extension.

As noted above, the difference between the infliximab 3 mg/kg and placebo groups with regard to the percentage of patients meeting the ACR Pedi 30 criteria at week 14, the primary outcome measure for this trial, was not statistically significant. The ACR Pedi 30 criteria have been validated, and their reliability in discriminating between active agent and placebo has been established in studies of NSAIDs (24,25), MTX (6), etanercept (8), and more recently, other biologic agents (26,27). We believe the lack of statistical significance between treatment groups in the present study resulted from the combination of a small sample size (60 patients per treatment group) and a higher-than-anticipated rate of response (49.2%) in the placebo group (28), which was likely due to a greater placebo effect associated with agents administered by infusion and a placebo treatment phase that was too brief. Given that the study population, by design, consisted of patients with MTX-resistant disease, a placebo response of ~20% was anticipated.

Overall, infliximab proved to be a beneficial treatment option, consistent with the risk/benefit profile in adult patients with RA (29). However, the safety profile for infliximab 3 mg/kg appeared to be less favorable in comparison with the 6-mg/kg dose, with an 2-fold higher incidence of infusion reactions (often severe), a higher incidence of antibodies to infliximab, and more frequent new formation of antinuclear and anti-dsDNA antibodies.

As implied by our pharmacokinetic findings and infusion reaction data, and as recently demonstrated in adults with RA (30), there is a correlation between maintenance of drug levels and avoidance of the development of antibodies to infliximab. In our study, children who received infliximab 6 mg/kg achieved better maintenance of drug levels and had a lower incidence of antibodies to infliximab. Indeed, if infliximab acts as an immunomodulator, the higher 6-mg/kg dose will be more likely than the 3-mg/kg dose to influence the incidence and titer of antibodies to infliximab. In addition, once development of infliximab antibodies is initiated, the neutralizing activity of the antibodies may lead to even more rapid elimination of infliximab at the lower dose. The suppressive effect of a higher infliximab dose on infliximab antibody formation may thus lead to a reduction in the incidence of adverse events associated with the presence of the antibodies.

In our study, 25% of the children developed
antibodies to infliximab, with a higher incidence in the infliximab 3 mg/kg group (38%) than in the 6 mg/kg group (12%). These findings are consistent with trends previously observed in adult patients with Crohn’s disease (18). However, since the increased incidence of antibody formation in JRA patients receiving the lower dose of infliximab in the present study led to comparatively lower median trough serum infliximab levels, we cannot rule out the possibility that if lower-dose treatment is given, a shorter interval between administrations might be necessary to maintain a more appropriate trough serum infliximab concentration, as has been recommended for the adult RA population (29), or that a higher dose of infliximab might be required at the beginning of the treatment regimen. Further evaluations in a larger group of patients will be needed to confirm or refute this unanticipated finding of a more favorable safety profile with infliximab 6 mg/kg than with infliximab 3 mg/kg.

In conclusion, while both infliximab 3 mg/kg and infliximab 6 mg/kg, in combination with MTX, produced an important, rapid, and durable clinical effect in children with JRA at 1 year, the primary efficacy end point of this study was not significantly different between infliximab treatment and placebo treatment at week 14. The 3-mg/kg dose of infliximab was associated with a substantially higher risk of serious adverse events, infusion reactions, and development of antibodies to infliximab, ANAs, and anti-dsDNA compared with the 6-mg/kg dose. Thus, the use of infliximab in children warrants further investigation.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS


REFERENCES

13. Lipsky PE, van der Heijde DM, St.Clair EW, Durst DE, Breedveld


Rituximab for the Treatment of Juvenile Dermatomyositis

A Report of Four Pediatric Patients

Megan A. Cooper, Donna L. Willingham, Diane E. Brown, Anthony R. French, Fei F. Shih, and Andrew J. White

Objective. Juvenile dermatomyositis (DM) is a chronic inflammatory myopathy of childhood primarily affecting the muscles and skin. Treatment for juvenile DM is often difficult, and conventional therapies include corticosteroids and other immune suppressants. We reviewed the records of 4 patients with juvenile DM who received the B cell–depleting anti-CD20 monoclonal antibody rituximab to determine whether this therapy resulted in improved control of their juvenile DM.

Methods. This is a retrospective review of 4 pediatric patients ages 10–17 years with juvenile DM who were treated with rituximab. All patients were tested for myositis autoantibodies and received weekly rituximab infusions for a total of 4 doses. Two patients received repeat courses of rituximab 1 year after their first dose. Patients were followed up between 12 and 24 months after their first course of rituximab, and their strength, muscle enzymes, and rash were reviewed.

Results. One patient was positive for a myositis-specific antibody, anti–Mi-2, and demonstrated striking reductions in her muscle enzyme levels for 1 year after rituximab therapy. Following a second course of rituximab, this patient remained disease free for 14 months before requiring a third course of rituximab. Two myositis antibody–negative patients showed clinical improvement and tolerated lower doses of corticosteroids following treatment with rituximab. Finally, 1 patient had worsening of her disease following rituximab.

Conclusion. These cases highlight the potential for anti-B cell therapies in the treatment of juvenile DM in both myositis-specific autoantibody–positive and –negative patients.

Juvenile dermatomyositis (DM) is a chronic inflammatory disease affecting the muscles and skin (1). While juvenile DM is relatively rare, with an incidence of 2.5–4.1 cases per million children in the US (2), it is the most common inflammatory myopathy of childhood. This disease is characterized by proximal muscle weakness and pathognomonic rashes, including a scaly rash over the dorsal aspect of the finger joints (Gottron’s papules) and heliotrope discoloration of the eyelids. The course of juvenile DM is variable, and patients can have multiorgan system involvement with clinical manifestations including fatigue, calcinosis, visceral vasculitis, and lipodystrophy.

The use of corticosteroids over the last 40 years has dramatically reduced the morbidity and mortality of juvenile DM (for review, see ref. 3); however, long-term use of corticosteroids is associated with multiple complications. Therefore, steroid-sparing agents are needed for the long-term management of juvenile DM. While there have been no randomized controlled studies of other immunosuppressive agents for the treatment of juvenile DM, retrospective reports have indicated that methotrexate (MTX) and intravenous immunoglobulin (IVIG) may be beneficial for decreasing the dosage of corticosteroids needed to control disease, and a variety of other therapies, such as cyclosporine and cyclophosphamide, have been used with mixed results in children with refractory disease (for review, see ref. 4).
The pathogenesis of juvenile DM remains unclear, although an increasing role of humoral immunity has been suggested by the association of myositis-specific and myositis-associated antibodies with myopathies, including juvenile DM and adult DM (5). While the majority of adults with DM have circulating myositis autoantibodies, <10% of children with juvenile DM have these autoantibodies, and their significance with regard to clinical diagnosis and disease course is unclear (1,6). A recent open trial by Levine (7) and 3 case reports (8–10) of adults with DM have shown a potential benefit with the B cell–depleting anti-CD20 monoclonal antibody rituximab, with improvement of muscle strength and/or skin manifestations following therapy. The only pediatric patient described was a 16-year-old girl with juvenile DM treated with rituximab for persistent cutaneous disease (10) who experienced remission of her skin disease following therapy.

Here we review our experience treating 4 pediatric juvenile DM patients ages 10–17 years with rituximab. One patient was positive for the myositis-specific autoantibody anti–Mi-2 and demonstrated dramatic normalization of her muscle enzyme levels and clinical improvement of her strength and rash following each of 2 courses of rituximab 1 year apart. Two myositis antibody–negative patients demonstrated improvement in strength and rash. The final patient had progression of her disease after rituximab.

TABLE 1. Demographic characteristics, prior and concurrent therapies, myositis antibody panel results, CD19 B lymphocyte count, and daily prednisone doses in 4 patients with juvenile dermatomyositis treated with rituximab*

<table>
<thead>
<tr>
<th>Patient/sex, age at rituximab treatment†</th>
<th>Disease duration</th>
<th>Prior therapies‡</th>
<th>Concurrent therapies</th>
<th>Myositis panel</th>
<th>CD19 cells/mm³§</th>
<th>Prednisone, mg/day¶</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/F</td>
<td>10 years 10 months</td>
<td>27 months</td>
<td>Pred., MTX, IVIG, MP</td>
<td>MTX, IVIG</td>
<td>Anti–Mi-2 positive</td>
<td>634</td>
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<tr>
<td>11 years 10 months</td>
<td>39 months</td>
<td>Pred., MTX, IVIG, MP, CYC</td>
<td>MTX</td>
<td>ND</td>
<td>475</td>
<td>0</td>
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<tr>
<td>2/M</td>
<td>14 years 11 months</td>
<td>5 weeks</td>
<td>Pred., MTX, HCQ</td>
<td>Pred., MTX, MP</td>
<td>Negative</td>
<td>1,289</td>
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<td>3/F</td>
<td>14 years 1 month</td>
<td>4 months</td>
<td>Pred., MTX, IVIG, MP</td>
<td>Pred., MTX, IVIG, MP</td>
<td>Negative</td>
<td>NA</td>
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<tr>
<td>15 years 2 months</td>
<td>17 months</td>
<td>Pred., MTX, IVIG, MP</td>
<td>Pred., MTX, IVIG, MP</td>
<td>ND</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>4/F</td>
<td>17 years 9 months</td>
<td>1.5 months</td>
<td>Pred., MTX, IVIG, MP, HCQ</td>
<td>Pred., MTX, MP</td>
<td>Negative</td>
<td>88</td>
</tr>
</tbody>
</table>

* Pred. = prednisone; MTX = methotrexate; IVIG = intravenous immunoglobulin; MP = intravenous methylprednisolone; CYC = cyclophosphamide; ND = not determined; HCQ = hydroxychloroquine; RA = not available.
† Patients 1 and 3 had two courses of treatment.
‡ Any medications given from diagnosis until treatment with rituximab.
§ Pre = CD19 count measured at the start of rituximab infusions; post = CD19 count measured at the fourth dose of rituximab, with the exception of the first course in patient 3 (measured 3 months after rituximab) and patient 4 (measured 5 weeks after rituximab).
¶ At the start of rituximab infusions and 6 months later.

PATIENTS AND METHODS

We retrospectively reviewed the records of patients at our institution with a diagnosis of juvenile DM alone, without an overlap syndrome, who received rituximab. A summary of the patient demographics, therapies, and immunologic parameters is shown in Table 1. All patients were diagnosed as having juvenile DM based on the presence of characteristic rash (heliotrope discoloration around the eyes and/or Gottron’s papules), proximal muscle weakness, and elevated muscle enzyme levels. Myositis antibody profiles (Oklahoma Medical Research Facility, Oklahoma City, OK) were performed on all patients to assay for myositis-specific and myositis-associated autoantibodies specific for the following antigens: Jo-1, PL-7, PL-12, EJ, OJ, Mi-2, signal recognition particle, PM-Scl, Ku, U1 RNP, U2 RNP, and Ro. All patients received 375 mg/m² of rituximab weekly by IV infusion for a total of 4 weeks (cumulative dose 1,500 mg/m²). Patients were pretreated with hydrocortisone (0.7–2 mg/kg) or dexamethasone (0.25 mg/kg in patient 1, first course) with each infusion. This study was conducted in accordance with the guidelines for case studies from the Human Resources Research Protection Office at Washington University, and informed consent was obtained from the patients for inclusion in this study.

RESULTS

All patients tolerated rituximab well, with no hospitalizations for serious infections in the 6 months after treatment. Only patient 1, who was positive for the myositis-specific antibody anti–Mi-2, had positive results...
of the myositis panel. Patients 1, 2, and 4 had full depletion of their B cells, and patient 3 had depletion of B cells after her second course of rituximab (Table 1). All patients who were receiving daily prednisone at the start of rituximab treatment tolerated a lower dose after 6 months (Table 1). Three patients (patients 1–3) had clinical improvement of their juvenile DM and/or laboratory parameters after treatment with rituximab, while patient 4 had progression of her disease, as described below.

A summary of the treatment course and muscle enzyme levels in patient 1 is shown in Figure 1. The patient presented at age 8.5 years with globally elevated muscle enzyme levels, profound proximal muscle weakness, and Gottron’s papules on her hands. Prior to receiving rituximab, her disease was controlled with IV methylprednisolone, oral prednisone, monthly high-dose (2 gm/kg) IVIG, and weekly MTX (Table 1). Two years after diagnosis, she had a relapse of her juvenile DM, with increasing muscle enzyme levels (time 0 in Figure 1), proximal muscle weakness with strength of 3/5 in her upper extremities and 4/5 in her lower extremities, and increased rash. She could not tolerate daily corticosteroid therapy due to osteopenia and vertebral compression fractures. Rituximab was started, and she also received IV methylprednisolone and 3 months of cyclophosphamide (Figure 1) due to profound muscle weakness and muscle enzyme elevation. However, she did not receive any further corticosteroids 2 months after starting rituximab (Figure 1).

The patient’s muscle enzyme levels began to decrease 1 month after starting rituximab (Figure 1), and her strength and rash started to improve 2 months after completing rituximab. Her muscle enzyme levels normalized after 6 months (Figure 1), and she regained 4+ to 5/5 strength throughout, with resolution of her rash. However, after 9 months while maintained on treatment with MTX alone, her muscle enzyme levels began to rise (Figure 1) while she maintained normal strength. By 12 months, she had significant elevation of all of her muscle enzyme levels (Figure 1), 4/5 proximal muscle strength in her upper and lower extremities, increasing erythema of her Gottron’s papules, and recovery of her B cells (Table 1). She subsequently received a second 4-week course of rituximab. She again began to have improvement of her muscle enzyme levels 1 month after restarting rituximab, and her muscle strength improved after 2.5 months. Ten months after her second course of rituximab, she had normalization of all of her muscle enzyme levels (Figure 1) and normal strength. At her last followup visit, 14 months after her second course of rituximab, she had normal strength on examination but worsening rash and significantly elevated muscle enzyme levels (Figure 1). Her CD19 count was 564 cells/mm³, and she is starting a third course of rituximab. She has continued receiving weekly MTX and
has not received any other therapies for her juvenile DM since starting her second course of rituximab.

Patients 2 and 3 had normal muscle enzyme levels at the time of treatment with rituximab but demonstrated clinical responses. Patient 2 started rituximab 5 weeks after diagnosis due to worsening weakness and rash (heliotrope rash, Gottron’s papules, and shawl rash) despite therapy with 1 mg/kg/day of oral corticosteroids and weekly MTX. He received IV methylprednisolone (250 mg for 7 doses) with his first infusion. After starting rituximab, he continued taking prednisone 20 mg twice daily and MTX, but he received no further methylprednisolone. Two months after starting rituximab, he had improved proximal muscle strength of 4/5 in his upper and lower extremities and decreased rash. Six months after starting rituximab, he had normal proximal muscle strength and his prednisone was decreased to 10 mg daily (Table 1). Twelve months after starting rituximab, he had full strength with fading Gottron’s papules and faint heliotrope discoloration around his eyes, and all medications were stopped.

Patient 3 received 2 courses of rituximab 1 year apart (Table 1). She was treated with her first course of rituximab 4 months after diagnosis due to persistent fatigue and proximal muscle weakness (4/5 upper extremity strength) with normal muscle enzyme levels after therapy with weekly MTX, prednisone, IV methylprednisolone, and 3 months of high-dose IVIG (2 gm/kg/month). IV methylprednisolone was given with her first and fourth doses of rituximab (500 mg for 3 doses and 500 mg for 1 dose, respectively). One month after completing rituximab, she continued to receive MTX, prednisone, and monthly IVIG with subjective improvement of her fatigue, normal proximal muscle strength, and improving Gottron’s papules and heliotrope rash. Six months after completing rituximab, she was maintained on a lower dose of prednisone (Table 1), weekly MTX, monthly IVIG, and hydroxychloroquine, with normal strength and trace rash.

Approximately 1 year after completing rituximab, the patient had increased fatigue and rash and subjective decreased strength with normal muscle enzyme levels. She received a second course of rituximab and IV methylprednisolone with her first, second, and fourth doses of rituximab (2 500-mg doses with each infusion). One month after her second course of rituximab, her weakness and fatigue had subjectively improved. By 6 months, she had resumed competitive sports and was maintained on monthly IVIG, MTX, and prednisone (1 mg daily). At her last followup visit, 1 year after her second course of rituximab, she is no longer taking prednisone.

Patient 4 had progression of her disease after receiving rituximab. She was started on rituximab 1.5 months after diagnosis and received 1 course of IV methylprednisolone (250 mg for 3 doses) with her first infusion. Two months after starting rituximab, her strength improved to 4/5–5/5, but she had persistent rash, weakness, and fatigue and an elevated lactate dehydrogenase level, and monthly IVIG (2 gm/kg) was started. One year after diagnosis (10.5 months after the start of rituximab), cyclosporine was started due to increased rash and fatigue. Now, 14 months following her diagnosis, she has developed vasculitic skin lesions and interstitial lung disease and is receiving cyclophosphamide.

**DISCUSSION**

Juvenile DM is a multisystem inflammatory disorder that is often difficult to treat, and traditional therapies including corticosteroids and immunosuppressive agents often have unacceptable side effects (1,3,4). To the best of our knowledge, this case series represents the first report of the use of the B cell–depleting anti-CD20 monoclonal antibody rituximab for the treatment of the muscle and skin manifestations of juvenile DM in children. All of the patients described in this report tolerated rituximab infusions well, with good depletion of their peripheral blood B cells after a 4-week course of rituximab (Table 1), and we observed no treatment-related infections. Three of 4 patients described in this report had clinical improvement following rituximab, while the fourth patient had progression of her disease requiring escalation of her immunosuppressive therapy.

Patients had perhaps the most significant clinical symptoms and treatment-refractory juvenile DM and also had the most notable response following rituximab. While she did receive additional immunosuppressive therapies concurrent with her first course of rituximab (Figure 1), she had no additional medications aside from her baseline MTX during or after her second course of rituximab, when she had a clear clinical and laboratory response for 14 months (Figure 1), implicating rituximab as playing a role in remission of her disease. One possible explanation for her dramatic response may be the presence of the myositis-specific autoantibody anti-Mi-2, suggesting a strong B cell–driven component to her disease. This hypothesis is supported by the recurrence of disease coincident with recovery of her
peripheral blood B cells 12 months after her first course of rituximab and 14 months after her second course.

While the significance of myositis autoantibodies in pediatric patients remains unclear, these antibodies can be useful for classifying disease and predicting outcome in adults with myositis (5,6). Two of the 3 myositis autoantibody–negative patients also had clinical improvement. We hypothesize that these patients may have had unidentified autoantibodies that were depleted and/or that eliminating B cells may have altered the activity of other immune cells, as has been suggested by a recent report of altered macrophage function in adult rheumatoid arthritis patients receiving rituximab (11). However, B cell dysfunction alone is unlikely to fully explain the pathogenesis of juvenile DM, as demonstrated by patient 3, who had relatively low numbers of circulating B cells at the time of relapse of her disease, and as demonstrated by patient 4, who had progression of her disease despite B cell depletion. Larger studies will be required to see whether the presence of myositis autoantibodies predicts a better response to anti–B cell therapies and to determine the mechanism of rituximab-induced remission in juvenile DM. A recently opened phase II trial of rituximab for the treatment of refractory juvenile DM, adult DM, and polymyositis (Clinical Trials.gov identifier NCT00106184) may help to address some of these questions.

All of the patients presented here who had clinical responses showed improved strength and decreased rash within 2 months after completing rituximab. Furthermore, their remissions persisted for 12 months or longer. Two patients (patients 1 and 3) received a second course of rituximab after 1 year due to disease relapse, while the third patient (patient 2) continues to do well 12 months after rituximab and has discontinued all medications for his juvenile DM. These observations of remission for up to 1 year are consistent with Levine’s open-label pilot study of rituximab in adults with DM, in which 2 of the 6 evaluable patients had clinical responses for at least 1 year after rituximab (7). Since rituximab therapy may not induce a durable cure for juvenile DM, it remains to be determined whether there is an optimal treatment schedule for administering repeated courses based on recurrence of clinical symptoms, periodic monitoring of B cell numbers, or predetermined intervals, or a combination thereof.

The case series presented here supports the further investigation of anti–B cell therapies such as rituximab for the treatment of pediatric patients with juvenile DM through clinical trials. Such therapies may be particularly beneficial for pediatric patients with myositis-specific autoantibodies, although the favorable clinical responses of 2 antibody-negative patients suggest that rituximab may ultimately be considered for all patients with refractory juvenile DM.

AUTHOR CONTRIBUTIONS

Dr. White had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Cooper, Brown, French, Shih, White.

Acquisition of data. Cooper, Willingham, Brown, French, Shih, White.

Analysis and interpretation of data. Cooper, White.


REFERENCES

A Possible Mechanism for Endogenous Activation of the Type I Interferon System in Myositis Patients With Anti–Jo-1 or Anti–Ro 52/Anti–Ro 60 Autoantibodies

Maija-Leena Eloranta,1 Sevim Barbasso Helmers,2 Ann-Kristin Ulfgren,2 Lars Rönnblom,1 Gunnar V. Alm,3 and Ingrid E. Lundberg2

Objective. To investigate type I interferon (IFN) system activation and its correlation with autoantibodies and organ manifestations in polymyositis (PM), dermatomyositis (DM), and inclusion body myositis.

Methods. Sera from 30 patients and 16 healthy controls, or purified IgG, were combined with material released from necrotized cells to stimulate IFNα production by peripheral blood mononuclear cells (PBMCs) from healthy blood donors. Muscle biopsy specimens from 25 patients and 7 healthy controls were investigated for blood dendritic cell antigen 2 (BDCA-2)–positive plasmacytoid dendritic cells (PDCs) and IFNα/β-inducible myxovirus resistance 1 (MX-1) protein.

Results. Sera from 13 patients who were positive for anti–Jo-1 or anti–Ro 52/anti–Ro 60 autoantibodies induced IFNα production in PBMCs when combined with necrotic cell material. In addition, IgG prepared from anti–Jo-1–positive PM sera induced IFNα with necrotic material, but not when the latter was treated with RNase. BDCA-2 expression in PDCs in muscle tissue was increased in PM patients with anti–Jo-1 autoantibodies, while MX-1 staining in capillaries was increased in DM patients, compared with healthy individuals. IFNα-inducing capacity correlated with interstitial lung disease, while MX-1 expression in the capillaries correlated with DM.

Conclusion. Immune complexes containing anti–Jo-1 or anti–Ro 52/anti–Ro 60 autoantibodies and RNA may act as endogenous IFNα inducers that activate IFNα production in PDCs. These PDCs could be of importance for inducing myositis, whereas in DM patients without autoantibodies the presence of MX-1 protein in capillaries suggests another cellular IFNα source and induction mechanism. Consequently, the type I IFN system may be of importance in both PM and DM, but via different pathways.

The idiopathic inflammatory myopathies polymyositis (PM), dermatomyositis (DM), and inclusion body myositis (IBM) are characterized by symmetric, proximal muscle weakness and decreased muscle endurance. Other organs are frequently involved, such as skin in DM and lungs in PM and DM. Shared classic histopathologic features are the presence of inflammatory cell infiltrates in skeletal muscle tissue, dominated by T cells and macrophages, and regenerating and degenerating muscle fibers (1). Another characteristic feature is the presence of autoantibodies, of which anti–histidyl–transfer RNA synthetase (anti–HisRS or anti–Jo-1) is one of the most frequent (2). This autoantibody is considered myositis specific and is associated with a distinctive clinical phenotype, the so-called antisynthetase syndrome, which is characterized by myositis, Raynaud’s phenomenon, interstitial lung disease (ILD), and...
arthritis, and skin changes of the hands (mechanic’s hands) (3). Other autoantibodies often present in myositis, but less specific, are anti-SSA (anti–Ro 52, anti–Ro 60) and anti-SSB (anti-La) autoantibodies. The role of autoantibodies in disease mechanisms in myositis is not clear.

Muscle fibers of most myositis patients, in contrast to those of healthy individuals, express class I major histocompatibility complex (MHC) antigen (4,5), which could have a pathogenic role. This is supported by the association between muscle weakness and up-regulation of class I MHC on muscle fibers in a transgene mouse model (6). The explanation for the class I MHC expression on muscle fibers in human myositis is unknown. One cause could be type I interferon (IFN), one of the strongest inducers of class I MHC expression (7,8). In fact, type I IFN appears to be involved in both adult and juvenile DM, because muscle biopsy specimens display up-regulation of type I IFN–induced genes, induction of IFNα/β-inducible human myxovirus resistance 1 (MX-1) protein, and the presence of plasmacytoid dendritic cells (PDCs), the major type I IFN producers (9,10). An additional role for IFNα in PM is suggested by the induction of this disease in patients treated with IFNα (11–13).

Although the cause of type I IFN production in myositis is unknown, one interesting mechanism in the context of autoimmune diseases is the well-documented IFNα-inducing capacity of immune complexes containing antibodies directed against nucleic acids, DNA or RNA, and associated proteins (14,15). This suggests an endogenous IFNα production in patients who have anti-RNA binding autoantibodies, as reported in patients with systemic lupus erythematosus (SLE) or primary Sjögren’s syndrome (SS) (14,15).

Because patients with myositis often have autoantibodies directed against RNA binding proteins, such as Jo-1 or Ro 52/ Ro 60 antigens, we postulated that sera from myositis patients with these autoantibodies may have an IFNα-inducing capacity. Furthermore, we aimed to investigate whether these patients have IFNα production in muscle tissue, and, if so, if this was related to class I MHC expression in muscle fibers. Finally, another objective was to investigate whether the IFNα-inducing capacity correlated with clinical manifestations.

PATIENTS AND METHODS

Patients and controls. Patients were followed up regularly at the Rheumatology Unit, Karolinska University Hospital in Solna, Stockholm, and fulfilled the diagnostic criteria for definite or probable PM/DM (16,17) or sporadic IBM (18). We used sera from 30 patients (19 women and 11 men) with inflammatory myopathies (14 with PM, 9 with DM, and 7 with IBM) and from 16 healthy controls (8 women, 4 men, and 4 healthy blood donors). Patients were recruited to this study based on the presence or absence of anti–Jo-1, anti–Ro 52, and anti–Ro 60 autoantibodies according to their records. The most recent available sera were chosen for this investigation. At the time of serum sampling, the median age of the patients was 61 years (range 26–79 years) and that of the healthy controls was 61 years (range 44–80 years). The median duration from diagnosis until serum sampling was 5.3 years (range 0–23 years). Demographic data, clinical information, and treatment of the patients at the time of serum sampling are shown in Table 1.

Muscle biopsy specimens were available from 25 of these patients (17 women and 8 men; 11 with PM, 7 with DM, and 7 with IBM), and, in most cases, were obtained close to or at the same time point at which sera were collected. Three patients were untreated prior to biopsy sampling; all others received immunosuppressive treatment. Bio specimens from 7 healthy individuals (4 women and 3 men) were included as controls. All patients gave their informed consent to participate, and the local ethics committee at the Karolinska Hospital Nord, Stockholm, approved the study.

Clinical and laboratory data. The patients’ overall disease activity at the time of serum sampling was determined by the physician’s global assessment based on a retrospective evaluation of patient records, including muscle function, serum levels of creatine phosphokinase (CPK) and C-reactive protein (CRP), and the erythrocyte sedimentation rate (ESR), as well as treatment decisions. Global disease activity was scored as low, moderate, or high. The levels of CPK and CRP and the ESR were analyzed as routine tests at the Department of Clinical Chemistry, Karolinska University Hospital in Solna, Stockholm (see Table 1 for reference values).

ILD was defined as lung pathology as seen by radiography or high-resolution computed tomography and/or an abnormal lung function test result. Radiographic signs of ILD were defined as the presence of one of the following abnormalities: increased lung attenuation, honeycombing, traction bronchiectasis, and architectural distortion. An abnormal lung function test result was defined as diminished lung volume (<80% of predicted values) with reduced diffusing capacity for carbon monoxide (<80% of predicted values).

Autoantibody determination. Patient and control sera were analyzed for antibodies to SmB, SmD, U1 RNP 70K, U1-A RNP, U1-C RNP, Ro 52/SSA, Ro 60/SSA, La/SSB, centromere B, topoisomerase I/Scl-70, Jo-1/HisRS, ribosomal P antigen, and histones using the Inno-Lia ANA Update line blot assay (Innogenetics, Ghent, Belgium). In addition, patient sera were routinely tested for anti–Jo-1 antibody activity, at the Department of Clinical Immunology at Karolinska University Hospital in Solna, Stockholm, where enzyme-linked immunosorbet assay (ELISA) and immunodiffusion are employed, with similar results. Antibodies to Ro 52 and Ro 60 were measured by both the Inno-Lia assay and the ELISA method (19). The results using both methods were incongruent in 3 sera. One was positive for anti–Ro 52 by Inno-Lia assay but negative by ELISA. A second was negative for anti–Ro 52 by Inno-Lia assay but positive by ELISA. A third was negative for...
Table 1. Demographic characteristics and clinical and laboratory data of patients at the time of serum sampling

<table>
<thead>
<tr>
<th>Patient/age/sex</th>
<th>Disease duration, years</th>
<th>Diagnosis</th>
<th>Disease activity†</th>
<th>CPK, (\mu)cat/liter</th>
<th>ESR, mm/hour</th>
<th>CRP, mg/liter</th>
<th>Medication‡</th>
<th>Cumulative clinical manifestations§</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/44/F</td>
<td>1.6</td>
<td>Definitive PM</td>
<td>High</td>
<td>3.8</td>
<td>45</td>
<td>10</td>
<td>Pred. 6.25, CYC</td>
<td>ILD, ED, T</td>
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<td>Definitive PM</td>
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<td>Pred. 5, MTX</td>
<td>ILD, A, RP, MH, SS</td>
</tr>
<tr>
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<td>18.0</td>
<td>Definitive PM</td>
<td>Low</td>
<td>1.2</td>
<td>5</td>
<td>&lt;7</td>
<td>Pred. 10</td>
<td>ILD</td>
</tr>
<tr>
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<td>Definitive PM</td>
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<td>6.7</td>
<td>10</td>
<td>54</td>
<td>Pred. 20, CYC</td>
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<td>A</td>
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<td>28</td>
<td>12</td>
<td>Pred. 7.5, CSA</td>
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<td>Low</td>
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<td>18</td>
<td>Pred. 6.25, AZA</td>
<td>ILD, RP</td>
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<tr>
<td>11/52/F</td>
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<td>Probable PM</td>
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<td>27</td>
<td>7</td>
<td>Pred. 20, MTX</td>
<td>A, primary SS</td>
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<td>&lt;6</td>
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<td>ILD</td>
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<tr>
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<td>Low</td>
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<td>&lt;7</td>
<td>None</td>
<td>SR, A</td>
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<td>Definitive DM</td>
<td>Moderate</td>
<td>2.4</td>
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<td>&lt;4</td>
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<td>Pred. 5, IVIG</td>
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<td>IVIG</td>
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* CPK = creatine phosphokinase (normal levels: men <2.5 \(\mu\)cat/liter, women <2.0 \(\mu\)cat/liter; factor by 60 to units/liter); ESR = erythrocyte sedimentation rate (normal <30 mm/hour); CRP = C-reactive protein (normal level <10 mg/liter); PM = polymyositis; NA = not assessed; DM = dermatomyositis; IBM = inclusion body myositis.† By physician’s global assessment.‡ Pred. = prednisolone (dosage given in mg/day); CYC = cyclophosphamide; MTX = methotrexate; AZA = azathioprine; CSA = cyclosporin A; IVIG = intravenous immunoglobulin.§ ILD = interstitial lung disease; ED = esophageal dysmotility; T = thrombosis; A = arthritis; RP = Raynaud’s phenomenon; MH = mechanic’s hands; SS = Sjogren’s syndrome; SR = skin rash.

anti–Ro 52/anti–Ro 60 by Inno-Lia assay but positive by ELISA. A sample that tested positive against an autoantigen by either method was considered positive.

**IFNa inducers and culture of peripheral blood mononuclear cells (PBMCs).** PBMCs from 4 normal blood donor buffy coats were prepared and cultured as previously described (15). Monocytic U937 cells (no. CRL-1593.2; American Type Culture Collection, Manassas, VA) were used as a source for necrotic cell material. Supernatants containing necrotic material were prepared by freeze-thawing U937 cells (3–4 cycles) at 50 \(\times\) 10^6/ml, and the necrosis was verified by flow cytometry using fluorescein isothiocyanate–labeled annexin V and propidium iodide stainings. The necrotic cell culture supernatant was centrifuged at 400\(\times\)g for 5 minutes and used at a final concentration of 10% in the cell cultures.

In the PBMC cultures, patient or control sera were used in combination with or without necrotic material for IFNa induction. Initially, 6 sera were tested in 4 different concentrations (0.01%, 0.1%, 1%, or 10%), with or without necrotic U937 cell material, to stimulate PBMCs from 2 healthy blood donors. The highest IFNa induction was obtained with 1% serum, followed by 0.1% serum, in combination with 10% necrotic cell material. All sera from patients and healthy controls were used in these 2 concentrations, while only results with 1% serum will be presented. For a control IFNa inducer, ultraviolet-inactivated herpes simplex virus type 1 (HSV-1) was prepared and used as described previously (15). The cell cultures were incubated for 20 hours at 37°C in 7% CO₂.

**Preparation of IgG.** Sera from 3 anti–Jo-1–positive but otherwise antinuclear antibody– and autoantibody-negative PM patients or healthy controls were used for IgG preparation. The IgG was purified using protein G columns (Amersham Biosciences, Uppsala, Sweden) as described earlier (20). The final concentration of IgG used in the cell cultures was 1 mg/ml.

**RNase treatment of IFNa inducers.** The necrotic cell material as well as the control IFNa inducers poly(I-C)
(Sigma-Aldrich, Steinheim, Germany), pcDNA3 plasmid, and HSV-1 were treated with DNase-free RNase A (8 μg/ml; AB-Gene, Surrey, UK) as previously described (20). The inducers were incubated with or without the RNase for 3 hours at 37°C before addition of IgG or the transfection agent lipofectin (Invitrogen, San Diego, CA), as indicated.

**Immunofluorescence and Immunohistochemistry.** The section was stored at −80°C. For each biopsy specimen, 7-μm-thick cryostat sections were mounted on gelatin-coated glass slides (Cel-line Associates, Newfield, NJ) and air-dried for 30 minutes. The sections were stored at −80°C until stained. Sections intended for analysis of MX-1 and class I MHC antigen were fixed in 2% formaldehyde prior to storage at −80°C.

**Immunohistochemistry.** PDCs were identified using antibodies to blood dendritic cell antigen 2 (BDCA-2)/CD303 (4 antibodies to blood dendritic cell antigen 2 (BDCA-2)/CD303, alternatively, many positive cells in at least 2 infiltrates; 4 = a large number of evenly distributed stained capillaries, other vessels, and some scattered cells, or, alternatively, some or many scattered positive cells plus positive cells in at least 3 infiltrates.

The proportion of muscle fibers expressing class I MHC was graded as follows: 0 = no positive fibers; 1 = 1–20% positive fibers; 2 = 21–40% positive fibers; 3 = 41–60% positive fibers; 4 = 61–80% positive fibers; 5 = 81–100% positive fibers. The presence of infiltrates was estimated by hematoxylin and eosin staining. In addition, quantitative evaluation giving the percentage of the total tissue area that stained positively for MX-1 or BDCA-2 expression was performed by computerized image analysis, using the Leica QWin software and DMRBE microscope (Leitz, Cambridge, UK). All sections were analyzed in a coded manner. There was a high degree of correlation between the results of conventional microscopic evaluation and those of computerized image analysis of BDCA-2–positive cells (r = 0.855, P < 0.0001) and of total MX-1 expression (r = 0.940, P < 0.0001).

**Statistical analysis.** Data were analyzed using GraphPad Prism 4.0 statistical software (GraphPad Software, San Diego, CA). A Kruskal-Wallis test (multiple analysis of variance) with Dunn’s post hoc test, the Mann-Whitney U test, and Fisher’s exact test were used to compare the patient groups. Spearman’s rank correlation coefficient was used to test for correlations. P values less than or equal to 0.05 were considered significant.

**RESULTS**

**Clinical, laboratory, and serological findings.** Fourteen patients were positive and 16 patients were negative for the autoantibodies tested (Table 2). Seven patients (all with PM) were positive for anti-Jo-1 autoantibodies only. One patient (with DM) was anti-Jo-1 and anti-Ro 52 positive; 1 patient (with DM) was anti-Jo-1, anti-Ro 52, anti-Ro 60, and anti-La positive; 2 patients (1 with PM and 1 with IBM) were anti-Ro 52 positive only; and 3 patients (1 with PM, 1 with DM, and 1 with IBM) were anti-Ro 52 and anti-Ro 60 positive. All patients were negative for the other tested autoantibodies. Ten patients had ILD, and all were positive for anti-Jo-1 or anti-Ro 52 (Tables 1 and 2). Skin rash was recorded in 9 patients, all of whom were classified as having DM. Twenty-two patients had low disease activity, 5 had moderate disease activity, and 3 had high disease activity at the time of serum sampling. The median serum level of CPK was 2.9 μcat/liter (range...
Table 2. IFNα production, serum antibodies, and BDCA-2, MX-1, and class I MHC expression in muscle tissue of the study patients*

<table>
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<th>Patient</th>
<th>Diagnosis</th>
<th>IFNα production in vitro, units/ml</th>
<th>IFNα in sera, units/ml</th>
<th>Anti–Jo-1</th>
<th>Anti–Ro 52</th>
<th>Anti–Ro 60</th>
<th>BDCA-2†</th>
<th>MX-1†</th>
<th>Class I MHC on muscle fibers</th>
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<td></td>
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<td>+</td>
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* IFNα = interferon-α; BDCA-2 = blood dendritic cell antigen 2; MX-1 = myxovirus resistance 1; MHC = major histocompatibility complex; NA = not available (see Table 1 for other definitions).
† See Patients and Methods for a description of scoring systems for BDCA-2, MX-1, and class I MHC.
‡ Cellular infiltrates present.
§ Perifascicular distribution.
0.5–61.2), and the median serum level of CRP was 7 mg/liter (range 4–54). The median ESR was 14 mm/hour (range 2–90) (Table 1).

**IFN-α-inducing ability of sera.** Forty-three percent (13 of 30) of the patient sera induced IFN-α production in the PBMC cultures when combined with necrotic cell material with or without autoantibodies against Jo-1, anti-Ro 52/anti-Ro 60) (A and B). C, Comparison of IFN-α induction by sera from patients with polymyositis (PM), dermatomyositis (DM), or inclusion body myositis (IBM). D, IFN-α production in PBMCs stimulated with IgG purified from 3 anti-Jo-1–positive myositis patients (M1, M2, and M13) or with normal IgG (N IgG) combined with necrotic cell material with or without RNase treatment. Each data point in A–C represents the mean IFN-α production by PBMCs from 4 healthy blood donors in response to a single serum. Horizontal bars represent the median IFN-α production for each group of sera. Values in D are the mean and SD of triplicate cultures from 1 representative experiment of 3 experiments performed. Ultraviolet irradiation–inactivated herpes simplex virus type 1 (HSV) (B and D) and lipofected poly(I:C) (poly I:C+lipo) and lipofected plasmid DNA (pcDNA3+lipo) (D) were used as control IFN-α inducers. * = P < 0.05; ** = P < 0.01; *** = P < 0.001.

**Figure 1.** A–D, Interferon-α (IFN-α) production in normal peripheral blood mononuclear cells (PBMCs) in vitro, stimulated by necrotic cell material combined with sera (A–C) or IgG (D) from healthy controls or myositis patients, with or without autoantibodies (anti-Jo-1, anti-Ro 52/anti-Ro 60) (A and B). C, Comparison of IFN-α induction by sera from patients with polymyositis (PM), dermatomyositis (DM), or inclusion body myositis (IBM). D, IFN-α production in PBMCs stimulated with IgG purified from 3 anti-Jo-1–positive myositis patients (M1, M2, and M13) or with normal IgG (N IgG) combined with necrotic cell material with or without RNase treatment. Each data point in A–C represents the mean IFN-α production by PBMCs from 4 healthy blood donors in response to a single serum. Horizontal bars represent the median IFN-α production for each group of sera. Values in D are the mean and SD of triplicate cultures from 1 representative experiment of 3 experiments performed. Ultraviolet irradiation–inactivated herpes simplex virus type 1 (HSV) (B and D) and lipofected poly(I:C) (poly I:C+lipo) and lipofected plasmid DNA (pcDNA3+lipo) (D) were used as control IFN-α inducers. * = P < 0.05; ** = P < 0.01; *** = P < 0.001.
any significant IFNα production when incubated with PBMCs from healthy donors at any of the tested concentrations.

IgG prepared from anti-Jo-1–positive PM sera (n = 3) induced IFNα production in PBMC cultures when combined with necrotic cell material (Figure 1D). No significant IFNα induction could be detected with IgG or necrotic material alone. Furthermore, healthy control IgG alone (not shown) or in combination with necrotic material failed to induce any IFNα production. Finally, the IFNα-inducing capacity of myositis IgG and necrotic material was abolished when the necrotic material was treated with RNase (Figure 1D). The specificity of RNase treatment was verified by showing that it destroyed the IFNα-inducing capacity of lipofected poly(I-C) RNA, but not that of HSV-1 or lipofected pcDNA3 DNA.

**IFNα in sera.** Three patients, 1 PM patient and 1 DM patient with anti–Ro 52 and anti–Ro 60 antibodies and 1 DM patient with anti–Jo-1, anti–Ro 52, anti–Ro 60, and anti-La antibodies, had detectable but low levels of IFNα in the serum (range 1.1–2.8 units/ml) (Table 2). None of the healthy controls had any detectable IFNα in serum.

**Presence of PDCs in muscle tissue.** With conventional microscopic evaluation, BDCA-2–positive PDCs were observed in 16 of 25 myositis patients. These cells were round or had an elongated shape (Figure 2A) and were scattered with a predominantly endomysial and/or perivascular localization (Table 2). In 5 of 8 patients (2 with PM and 3 with IBM) with large cellular infiltrates, BDCA-2–positive cells were also observed within such infiltrates. In 4 of 7 healthy individuals, few scattered BDCA-2–positive PDCs were detected.
The total expression of BDCA-2 estimated by computerized image analysis was significantly increased in patients with detected autoantibodies compared with that in healthy controls ($P < 0.03$) (Figure 3A). Also, the total BDCA-2 expression in the autoantibody-positive PM patients was significantly increased compared with that in healthy controls ($P < 0.05$) (Figure 3B). This was also true for PM patients with anti–Jo-1 autoantibodies only compared with that in healthy controls ($P = 0.02$).

**IFNα/β-inducible MX-1 protein in muscle tissue.**

As an indicator of IFNα production in vivo, type I IFN–induced MX-1 protein expression was investigated. By conventional microscopic evaluation, positive MX-1 staining in inflammatory mononuclear cells was observed in 17 of 24 myositis patients (1 patient was not included due to lack of a biopsy specimen). This staining was observed in scattered cells with endomysial and/or perivascular localization (Figure 2B and Table 2). Positive cells were also present within infiltrates in 5 of 8 patients (2 with PM and 3 with IBM) with large cellular infiltrates. Positive MX-1 staining was also seen in endothelial cells of capillaries (Table 2) in 14 patients, and in other vessels in 6 patients. In 5 of 7 healthy individuals, MX-1 was expressed in few inflammatory

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**Figure 3.** Total expression of blood dendritic cell antigen 2 (BDCA-2) (A and B) or myxovirus resistance 1 (MX-1) (C and D) in muscle tissue, as evaluated by immunohistochemical staining and computerized image analysis. BDCA-2 or MX-1 (MxA) expression is shown for healthy controls and either all myositis patients (A and C) or patients with PM, DM, or IBM (B and D) with (+) or without (−) autoantibodies. Horizontal bars represent the median IFNα production for each group of sera. Dotted lines indicate the cutoff level (the mean value of positively stained area of all healthy controls + 2.5 SD). * = $P < 0.05$. See Figure 1 for other definitions.
cells, and in few capillaries in 1 individual (Figure 4C). The MX-1 capillary expression in patients with detected autoantibodies as well as in DM patients irrespective of autoantibodies was significantly increased compared with the MX-1 capillary expression in healthy individuals (both \( P = 0.03 \)) (Figure 4A). No MX-1 staining was detected in muscle fibers.

Total MX-1 expression in the muscle tissue assessed by computerized image analysis was significantly higher in autoantibody-positive patients compared with healthy individuals (\( P = 0.008 \)) (Figure 3C). Patients with DM without the presence of autoantibodies also had significantly increased total MX-1 expression (\( P = 0.02 \)) (Figure 3D).

**Class I MHC antigen expression in muscle tissue.** Muscle biopsy specimens from 20 patients were available for class I MHC staining, and 15 of these had class I MHC–positive muscle fibers as assessed by conventional microscopic evaluation (Figure 2 and Table 2). Two of the healthy controls had 1 or 2 fibers positive for class I MHC antigen expression (median score 0, range 0–0). In patients positive for autoantibodies, the class I MHC expression on muscle fibers (median score 2, range 0–4) was significantly increased compared with that in healthy individuals (\( P < 0.05 \)).

**Correlations between IFNα-inducing ability of sera and BDCA-2, MX-1, and class I MHC expression in muscle tissue.** Patients with IFNα-inducing capacity (>10 units IFNα/ml) had significantly higher expression of total MX-1 as well as class I MHC on fibers in muscle tissue compared with patients without IFNα-inducing capacity (Table 3). MX-1– or BDCA-2–positive cells were in many cases localized adjacent to class I MHC–positive fibers (Figures 2A–C). Expression of BDCA-2–
positive PDCs and MX-1 expression correlated with class I MHC expression on muscle fibers ($r = 0.631 [P < 0.001]$ and $r = 0.624 [P = 0.001]$), respectively. The MX-1 and BDCA-2 scores by conventional microscopic evaluation correlated well ($r = 0.582, P < 0.001$).

**Correlation between clinical manifestations and IFNα-inducing ability of sera.** Sera from the patients with ILD had a significantly higher IFNα-inducing capacity compared with that in sera from those without ILD (median 872 IFNα units/ml [range 10–1,628] versus median 5.5 IFNα units/ml [range 2–1,769]; $P = 0.004$) (Tables 1 and 2). There was a correlation between IFNα levels induced in vitro and the presence of ILD ($r = 0.537, P < 0.002$). There were no correlations between IFNα-inducing ability and other clinical manifestations, physician’s global assessment, or immunosuppressive treatment (Tables 1 and 2).

**Correlations between clinical manifestations and BDCA-2, MX-1, and class I MHC expression in muscle tissue.** DM patients had significantly higher MX-1 scores in capillaries, by conventional microscopic evaluation, compared with patients with PM or IBM considered together (median score 2 [range 0–4] versus median score 0 [range 0–4]; $P = 0.033$). No correlations were detected between BDCA-2 or class I MHC expression and clinical manifestations, disease duration, or treatment (Tables 1 and 2).

**DISCUSSION**

We present data that strongly suggest a role of the type I IFN system in disease mechanisms in myositis patients. This was supported in 3 ways. First, sera containing anti-Jo-1 or anti-Ro 52/anti-Ro 60 autoantibodies had an IFNα-inducing capacity in vitro when combined with necrotic cell material. Second, a major cellular source of IFNα production, PDCs, was present in muscle tissue. Third, there was an increased expression of the IFN-inducible protein MX-1 in muscle tissue. The IFNα-inducing capacity of sera was associated with high total expression of the MX-1 protein and with class I MHC expression on muscle fibers. Two different patterns of MX-1 and PDC expression were observed. The MX-1 expression in DM patients without detected autoantibodies was predominantly localized to endothelial cells of capillaries without a simultaneously increased expression of BDCA-2–positive PDCs in muscle tissue, whereas in patients with anti-Jo-1 or anti–Ro 52/anti–Ro 60 autoantibodies, the MX-1 expression was localized to both mononuclear inflammatory cells and capillaries, and the BDCA-2–positive cells were increased. These observations, together with IFNα-inducing capacity by autoantibody-positive sera, indicate different mechanisms for IFNα induction in different subsets of myositis.

We have previously reported a similar IFNα-inducing capacity of sera from SLE or primary SS patients with autoantibodies to RNA binding proteins (e.g., anti-SSA [anti–Ro 52/anti–Ro 60], anti-SSB, anti-RNP, or anti-Sm autoantibodies) (14,15). In those studies, we demonstrated that the interferogenic activity of patient sera correlated with immune complexes composed of autoantibodies to RNA binding proteins and required the presence of RNA and IgG (14,15). Furthermore, we have shown that IFNα induction by these immune complexes involves Fcγ receptor type IIa and
also found a correlation between the IFN-γ expression and the presence of anti–Jo-1 antibodies (29). We previously showed that sera and IgG from anti–Jo-1 antibody–positive patients did not have any IFN-γ-inducing capacity. It is still being debated whether anti–Jo-1 antibodies have a pathogenic role in disease mechanisms in myositis, or whether they are merely an epiphenomenon. Increased expression of the Jo-1 antigen in muscle from PM or DM patients compared with that in healthy muscle was recently reported (24). This was mainly dependent on an increased expression in regenerating muscle fibers in myositis patients. Further support for a role of anti–Jo-1 antibodies in disease mechanisms comes from earlier observations that anti–Jo-1 antibodies are usually present at the time of myositis diagnosis and could even precede the myositis symptoms (25). Furthermore, it was shown that these autoantibodies underwent affinity maturation to the target autoantigen, histidyl–transfer RNA synthetase, and that the anti–Jo-1 response was mainly restricted to the IgG1 heavy-chain isotype. Moreover, the levels of IgG1 anti–Jo-1 varied in relation to myositis disease activity (25,26). In addition, Jo-1 antigen can serve as a chemokine for immature dendritic cells, T cells, and activated monocytes, all of which have been shown to be present in the muscle tissue of myositis patients (27,28).

To these observations we can now add our finding that sera and IgG from anti–Jo-1 antibody–positive patients can induce IFN-γ production from PBMCs, when cultured with necrotic cell material, possibly by forming immune complexes containing RNA and anti–Jo-1 antibodies. These immune complexes may act as endogenous IFN-γ inducers by activating BDCA-2–positive PDCs and perpetuating an immune response directed against a ubiquitous antigen. The same mechanism could also be of relevance for anti–Ro 52/anti–Ro 60 autoantibodies in myositis, and anti–Ro 52 antibodies frequently co-occur with anti–Jo-1 antibodies (29). We also found a correlation between the IFN-γ-inducing capacity of sera and the presence of ILD, one of the major extramuscular features of anti–Jo-1 antibody–positive patients (3,30), suggesting that IFN-γ production could have a role in the development of ILD in these patients. Another explanation could be that IFN-γ induction and ILD independently correlate with the presence of anti–Jo-1 antibodies. Notably, in a previous study, it was demonstrated that pulmonary manifestations in patients with primary SS with anti-SSA autoantibodies were associated with the IFN-γ-inducing capacity of these patients’ sera in combination with apoptotic cell material (14).

A local production of IFN-γ in muscle, the target organ of myositis, was supported by the presence of BDCA-2–positive PDCs and the expression of MX-1 protein in muscle tissue. This was found in patients with or without autoantibodies, but with slightly different expression patterns, and was present even during immunosuppressive treatment. Thus, we confirmed previously reported increased expression of MX-1 in capillaries in muscle tissue of DM patients (10). The IFN-γ in the muscle tissue of these patients did not seem to be produced locally by BDCA-2–positive cells, since these were only seen as scattered cells in a few DM patients. The IFN-γ in DM patients may therefore be produced not only in muscles, but also in other organs, as previously observed (e.g., in the skin of SLE patients and in salivary glands of primary SS patients) (14,31–33). A similar pattern of MX-1 expression in capillaries was also seen in the skin of psoriasis patients without autoantibodies, indicating another type I IFN–inducing mechanism (34). One such proposed mechanism is viral or bacterial infection. The low number of BDCA-2–positive cells in muscle tissue of DM patients is in contrast to a previous finding (10). One possible explanation for this discrepancy is a difference in the selection of patients. Consistent with this, the DM patients in our study lacked anti-RNP or anti-DNA autoantibodies, in contrast to the DM patients studied by Greenberg et al (10). Anti-RNP and anti-DNA autoantibodies can have an IFN–inducing capacity (14,15). Furthermore, our PM patients may have received more extensive immunosuppressive therapy. This possibility is perhaps less likely, because the PM patients in the present study received similar therapy but still had marked infiltration of BDCA-2–positive PDCs. Interestingly, the class I MHC expression on muscle fibers observed for many years in myositis patients could be a consequence of the local IFN-γ production in muscle tissue, supported by the strong class I MHC expression in muscle fibers in the
vicinity of BDCA-2– and MX-1–positive cells (Figures 2A–C).

We also included sera from IBM patients. IBM is suggested to be a myopathy with pathogenic mechanisms different from those of PM and DM, due to distinct clinical and histopathologic features. However, IBM patients occasionally have autoantibodies and even features characteristic of SS, suggesting that at least in some IBM patients immune mechanisms may be shared with PM or DM (35). Sera from 2 of the IBM patients had IFNα-inducing capacity. One of these patients had ant–Ro 52 antibodies and the other had ant–Ro 52 and ant–Ro 60 antibodies. They also had increased expression of MX-1 protein in muscle tissue (Figure 4B), but only one had BDCA-2–positive cells. Since the number of IBM patients with or without autoantibodies was low, we cannot draw any conclusions regarding the source of IFNα production in these patients.

In conclusion, we hypothesize that immune complexes containing RNA and autoantibodies may act as endogenous IFNα inducers in ant–Jo-1– or ant–Ro 52/ant–Ro 60–positive patients with myositis. Together with an increased number of the BDCA-2–positive PDCs (the major type I IFN–producing cells) and MX-1 expression in muscle biopsy specimens, this suggests that the type I IFN system may play an important role in pathogenesis not only in DM, as previously suggested, but also in PM patients with these autoantibodies.

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AUTHOR CONTRIBUTIONS

Mrs. Barbasso Helmers had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Eloranta, Barbasso Helmers, Ulfgren, Rönblom, Alm, Lundberg.

Acquisition of data. Eloranta, Barbasso Helmers, Lundberg.

Analysis and interpretation of data. Eloranta, Barbasso Helmers, Rönblom, Alm, Lundberg.


Statistical analysis. Eloranta, Barbasso Helmers.

REFERENCES

Anti–Jo-1 Antibody Levels Correlate With Disease Activity in Idiopathic Inflammatory Myopathy

Kerry B. Stone, Chester V. Oddis, Noreen Fertig, Yasuhiro Katsumata, Mary Lucas, Molly Vogt, Robyn Domsic, and Dana P. Ascherman

Objective. Previous case series have examined the relationship between anti–Jo-1 antibody levels and myositis disease activity, demonstrating equivocal results. Using enzyme-linked immunosorbent assays (ELISAs) and novel measures of myositis disease activity, the current study was undertaken to systematically reexamine the association between anti–Jo-1 antibody levels and various disease manifestations of myositis.

Methods. Serum anti–Jo-1 antibody levels were quantified using 2 independent ELISA methods, while disease activity was retrospectively graded using the Myositis Disease Activity Assessment Tool, which measures disease activity in 7 different organ systems via the Myositis Disease Activity Assessment Visual Analog Scale (VAS) and the Myositis Intention-to-Treat Index (MITAX) components. Spearman's rank correlation coefficients and mixed linear regression analysis were used to identify associations between anti–Jo-1 antibody levels and organ-specific disease activity in cross-sectional and longitudinal analyses, respectively.

Results. Cross-sectional assessment of 81 patients with anti–Jo-1 antibody revealed a modest correlation between the anti–Jo-1 antibody level and the serum creatine kinase (CK) level, as well as muscle and joint disease activity. Correlation coefficients were similar for CK levels ($r_s = 0.38$, $P = 0.002$), myositis VAS ($r_s = 0.36$, $P = 0.002$), and arthritis VAS ($r_s = 0.40$, $P = 0.001$). In multiple regression analyses of 11 patients with serial samples, anti–Jo-1 antibody levels correlated significantly with CK levels ($R^2 = 0.65$, $P = 0.0002$), myositis VAS ($R^2 = 0.53$, $P = 0.0008$), arthritis VAS ($R^2 = 0.53$, $P = 0.006$), pulmonary VAS ($R^2 = 0.69$, $P = 0.005$), global VAS ($R^2 = 0.63$, $P = 0.002$), and global MITAX ($R^2 = 0.64$, $P = 0.0003$).

Conclusion. In this large series of patients with idiopathic inflammatory myopathy, anti–Jo-1 antibody levels correlated modestly with muscle and joint disease, an association confirmed by a custom ELISA using recombinant human Jo-1. More striking associations emerged in a smaller longitudinal subset of patients that link anti–Jo-1 antibody levels to muscle, joint, lung, and global disease activity.

Idiopathic inflammatory myopathy (IIM) represents a group of disorders characterized by immune-mediated destruction and/or dysfunction of muscle (1). The autoimmune process extends beyond muscle disease, with additional clinical features involving the skin, joints, lungs, and circulation (2). These protean manifestations are often difficult to manage, particularly because different features may not be simultaneously present or active in all patients. Further complicating disease management is the imprecise nature of current markers of disease activity. For example, manual muscle testing has poor reproducibility and is not specific for active inflammatory muscle disease versus muscle damage (3). Moreover, while creatine kinase (CK) levels may be helpful in following muscle disease activity, they do not correlate with systemic manifestations such as lung disease. The latter complication can be especially difficult to assess because pulmonary function tests and high-resolution computed tomography cannot easily distinguish infection, active inflammatory disease, or scarring. Overall, these diagnostic and management issues...
underscore the lack of a clinically useful biomarker for component and global disease activity.

Anti–Jo-1 antibody is a myositis-specific antibody that was first described in 1980 (4). Initially thought to be a marker of inflammatory myopathy alone, anti–Jo-1 antibody is now associated with a distinct clinical entity known as the antisynthetase syndrome, which includes fever, myositis, interstitial lung disease (ILD), nonerosive arthropathy, mechanic’s hands, and Raynaud’s phenomenon (5). ILD is especially prevalent in the antisynthetase syndrome, occurring in ~75% of patients with anti–Jo-1 antibody compared to ~30% of patients with IIM in the absence of antisynthetase antibodies (6–8).

Anti–Jo-1 antibody is one of several antisynthetase antibodies that have been described, each of which has been associated with overlapping features of the antisynthetase syndrome (5,9). Anti–Jo-1 antibody recognizes different epitopes of histidyl-tRNA synthetase (Jo-1), including the subunit that catalyzes binding between the amino acid histidine and its cognate tRNA in the process of protein synthesis (5,10,11). Based on a range of immunologic and immunogenetic data, Jo-1 likely plays a direct role in the induction and maintenance of autoimmunity in the antisynthetase syndrome. For example, the antibody response to histidyl-tRNA synthetase undergoes class switching, spectrototype broadening, and affinity maturation, all of which are indicators of a T cell–dependent, antigen-driven process (10–13). Therefore, although data are lacking that directly implicate anti–Jo-1 antibody in the pathogenesis of the antisynthetase syndrome, this patterned B cell reactivity reflects an underlying T cell response directed against Jo-1 that may drive autoantibody formation and direct tissue damage.

In turn, the likely role of Jo-1 in the immunopathogenesis of the antisynthetase syndrome suggests that anti–Jo-1 antibody levels may correlate with disease activity. A limited number of previous studies that address this question have been hampered by factors such as small sample size, imprecise indices of disease activity, and variable and/or unreliable methods of quantifying anti–Jo-1 antibody levels (12,14,15). Furthermore, none of the earlier studies was designed to specifically examine the correlation between antibody levels and disease activity. In contrast, the current analysis directly addresses the association between enzyme-linked immunosorbent assay (ELISA)–determined anti–Jo-1 antibody levels and disease activity as measured by the Myositis Disease Activity Assessment Tool (MDAAT) (16), a partially validated index for measuring disease activity in IIM.

**PATIENTS AND METHODS**

**Patients.** Serum was collected from patients with suspected autoimmune disease during inpatient and outpatient encounters at the University of Pittsburgh Medical Center from 1975 through 2006. Serum obtained at the time of clinical evaluation was tested for the presence of anti–Jo-1 antibody using immunodiffusion. Patients were included in the current study if they provided written consent and tested positive for anti–Jo-1 antibody by this method, but were excluded if adequate clinical or laboratory data were unavailable.

**Clinical indices of disease activity.** Disease activity was measured through chart review and retrospective application of the 2005 MDAAT. The MDAAT consists of the Myositis Disease Activity Assessment Visual Analog Scale (MYOACT) and the Myositis Intention-to-Treat Index (MITAX) components, each of which contains specific guidelines for physician scoring of disease activity. While the MYOACT measures myositis-associated disease activity during the previous 4 weeks in 7 organ systems (constitutional, cutaneous, joint, gastrointestinal, pulmonary, cardiac, and muscle) using a continuous 10-cm visual analog scale (VAS), the MITAX incorporates the physician’s intention to treat active disease present during the preceding month in the same 7 organ systems using a 4-point ordinal scale (0 = disease not present, 1 = disease improving, 2 = disease unchanged, 3 = disease worsening, 4 = new disease manifestations).

**ELISAs.** Anti–Jo-1 antibody levels were determined in stored serum samples with a commercially available ELISA kit (Inova Diagnostics, San Diego, CA) that uses calf thymus extract. Samples were prepared according to the manufacturer’s instructions. To confirm the results from this system, a custom ELISA was independently devised using full-length recombinant human Jo-1 (rHuJo-1)/maltose binding protein fusion protein as the substrate. Briefly, 96-well microtiter plates (Nunc, Rochester, NY) were coated with rHuJo-1 (0.1 mg/ml) in carbonate buffer (50 mM NaHCO3/Na2CO3, pH 9.6) and incubated overnight at 4°C. Plates were washed 4 times with phosphate buffered saline (PBS) containing 0.05% Tween 20. After blocking wells with PBS containing 1% bovine serum albumin (Sigma-Aldrich, St. Louis, MO) at room temperature for 2 hours, appropriately diluted serum samples (1:10,000) were added to triplicate wells for 2 hours.

Following repeated washes with PBS–Tween 20 and a 60-minute incubation with a 1:10,000 dilution of horseradish peroxidase–conjugated goat anti-human IgG (stock = 0.4 mg/ml; Santa Cruz Biotechnology, Santa Cruz, CA), the enzymatic reaction was visualized using tetramethylbenzidine (Sigma-Aldrich) and subsequently terminated with 1N H2SO4. Color development was measured at 450 nm using a Wallac 1420 multilabel counter (PerkinElmer, Wellesley, MA). Quantification of the rHuJo-1–based ELISA and assignment of relative unit values were based on comparisons of readings at an optical density of 450 nm with those obtained from serial dilutions of a control anti–Jo-1 antibody serum sample obtained from the Centers for Disease Control and Prevention (Atlanta, GA). The specificity of this ELISA for human Jo-1 was confirmed through pilot experiments that showed no serum reactivity to plates coated with the maltose binding protein fusion partner alone.
Statistical analysis. The mean ± SD anti–Jo-1 antibody level was determined using both the commercial Jo-1 and the rHuJo-1 ELISAs. Spearman’s rank correlation coefficients were calculated to quantify relationships between disease activity and anti–Jo-1 antibody levels at a single point in time. In patients for whom serial samples were available, mixed linear regression analysis for repeated measures was performed to determine correlations between anti–Jo-1 antibody levels and disease activity. All statistical analyses were performed using SAS software (SAS Institute, Cary, NC). P values less than 0.05 were considered significant.

RESULTS

Patient selection and demographics. Ninety-four patients with anti–Jo-1 antibody (detected by immuno-diffusion) from the University of Pittsburgh database were evaluated for this study, but 13 individuals were excluded from this analysis due to incomplete clinical information or lack of informed consent. Of the 81 patients who satisfied the inclusion requirements, ≥3 serum samples were available from 11 patients. Based on the criteria established by Bohan and Peter (17), 55 patients (68%) had definite or probable polymyositis, 16 (20%) had definite or probable dermatomyositis, and 10 (12%) had undifferentiated connective tissue disease or overlap syndromes. Among the patients with overlap diagnoses, 6 had myositis and systemic sclerosis (SSc), 1 had myositis and systemic lupus erythematosus (SLE), and 1 had combined features of myositis, SSc, and SLE. Additional serologic abnormalities in the overlap group included the presence of anti–double-stranded DNA (anti-dsDNA) antibody (n = 1), anti–PM-Scl antibody (n = 1), and anticentromere antibody (n = 3).

As summarized in Table 1, the mean age of these patients at their first University of Pittsburgh visit was 47.7 years. The cohort was predominantly female (73%) and white (91%), which reflects racial and ethnic differences in disease distribution as well as regional demographics. At the time of initial evaluation, the mean duration of symptoms (not restricted to myositis) was 3.8 years. Organ system involvement and other extramuscular features that characterized this patient group are summarized in Table 1.

ELISA assessment of anti–Jo-1 antibody titer and correlation with measures of clinical disease activity. To determine potential associations between anti–Jo-1 antibody levels and disease activity in different organ systems, anti–Jo-1 antibody titers were initially measured using a commercially available ELISA kit. A review of the anti–Jo-1 antibody titer distribution indicated heavy skewing toward the high end of the scale, potentially confounding correlations with clinical indices of disease activity (Table 2). Yet, comparing antibody levels in the initial serum samples from all 81 patients with measures of clinical disease activity yielded statistically significant correlations between anti–Jo-1 antibody titers and serum CK levels, muscle as well as articular disease activity (recorded using both the MITAX and the MYOACT/VAS), and a composite index of global disease activity (global MITAX) (Figure 1 and Table 3). Of note, excluding patients with overlap diagnoses had no effect on the findings of this analysis (results not shown).

Although the overall distribution of anti–Jo-1 antibody titers determined with the commercial ELISA (substate antigen derived from calf thymus extract) appeared similar to that obtained with the alternative ELISA using rHuJo-1, the relatively modest correlation coefficient between these 2 systems (r = 0.59; P < 0.0001) suggests antibody recognition of species-specific Jo-1 epitopes that could be a key parameter influencing correlations with measures of myositis disease activity. In support of this conclusion, Table 3 shows greater correlation coefficients between the rHuJo-1 ELISA values and the MDAAT components such as global MITAX and global VAS.

Table 1. Demographic and clinical features of the study patients

<table>
<thead>
<tr>
<th>Clinical feature</th>
<th>no./no. assessed (%)</th>
<th>Age at initial evaluation, mean ± SD (range) years</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myositis</td>
<td>76/81 (94)</td>
<td>47.7 ± 12.5 (21.6–75.3)</td>
</tr>
<tr>
<td>Interstitial lung disease</td>
<td>45/65 (69)</td>
<td></td>
</tr>
<tr>
<td>Arthritis</td>
<td>46/81 (57)</td>
<td></td>
</tr>
<tr>
<td>Mechanic’s hands</td>
<td>14/81 (17)</td>
<td></td>
</tr>
<tr>
<td>Raynaud’s phenomenon</td>
<td>43/81 (53)</td>
<td></td>
</tr>
<tr>
<td>Gastrointestinal involvement/dysphagia</td>
<td>15/29 (52)</td>
<td></td>
</tr>
<tr>
<td>Sclerodactyl</td>
<td>8/65 (12)</td>
<td></td>
</tr>
</tbody>
</table>

* Units represent conversion derived from a standard curve of values at an optical density of 450 nm. ELISA = enzyme-linked immunosorbent assay.

Table 2. Results of a commercial ELISA for anti–Jo-1 antibody*  

<table>
<thead>
<tr>
<th>Anti–Jo-1 antibody level</th>
<th>No. of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative (&lt;20 units)</td>
<td>5</td>
</tr>
<tr>
<td>Weakly positive (20–39 units)</td>
<td>1</td>
</tr>
<tr>
<td>Moderately positive (40–80 units)</td>
<td>2</td>
</tr>
<tr>
<td>Strongly positive (&gt;80 units)</td>
<td>98</td>
</tr>
</tbody>
</table>
Longitudinal correlations between anti–Jo-1 antibody titer and clinical disease activity. In our cohort, 11 patients had 3–6 serum samples collected at different time points in their disease course. The interval between the first and the last sample ranged from 1.25 to 16 years. Separate analysis of these serial patient samples through mixed linear regression (after accounting for interpatient variability) showed significant correlations between the anti–Jo-1 antibody level and the following clinical parameters: muscle, joint, lung, and global disease activity (Table 4). Paralleling the results with the cross-sectional data set, use of the rHuJo-1 ELISA generated stronger associations with parameters of disease activity than did the commercial ELISA using calf thymus extract (Table 4). Further review of these serial data revealed that anti–Jo-1 antibody levels assessed by both ELISA methods became negative during periods of disease inactivity in 3 patients. As shown by

Table 3. Cross-sectional correlations with Jo-1 ELISA results*

<table>
<thead>
<tr>
<th>Clinical parameter</th>
<th>Commercial Jo-1 ELISA</th>
<th>Custom rHuJo-1 ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK (× the ULN)</td>
<td>0.38 0.002</td>
<td>0.39 0.001</td>
</tr>
<tr>
<td>Myositis VAS</td>
<td>0.36 0.002</td>
<td>0.31 0.009</td>
</tr>
<tr>
<td>Myositis MITAX</td>
<td>0.34 0.003</td>
<td>0.37 0.001</td>
</tr>
<tr>
<td>Arthritis VAS</td>
<td>0.40 0.001</td>
<td>0.42 0.0003</td>
</tr>
<tr>
<td>Arthritis MITAX</td>
<td>0.40 0.001</td>
<td>0.39 0.0006</td>
</tr>
<tr>
<td>Global VAS</td>
<td>0.28 0.02</td>
<td>0.30 0.01</td>
</tr>
<tr>
<td>Global MITAX</td>
<td>0.30 0.009</td>
<td>0.35 0.002</td>
</tr>
</tbody>
</table>

* Assessments were made in all 81 study patients. ELISA = enzyme-linked immunosorbent assay; rHuJo-1 = recombinant human Jo-1; CK = creatine kinase; ULN = upper limit of normal; VAS = visual analog scale; MITAX = Myositis Intention-to-Treat Index.

Table 4. Longitudinal correlations with Jo-1 ELISA results*

<table>
<thead>
<tr>
<th>Clinical parameter</th>
<th>Commercial Jo-1 ELISA</th>
<th>Custom rHuJo-1 ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK (× the ULN)</td>
<td>0.65 0.0002</td>
<td>0.78 0.0001</td>
</tr>
<tr>
<td>Myositis VAS</td>
<td>0.53 0.0008</td>
<td>0.69 0.0001</td>
</tr>
<tr>
<td>Myositis MITAX</td>
<td>0.63 0.06</td>
<td>0.68 0.0008</td>
</tr>
<tr>
<td>Arthritis VAS</td>
<td>0.53 0.006</td>
<td>0.73 0.002</td>
</tr>
<tr>
<td>Arthritis MITAX</td>
<td>0.62 0.04</td>
<td>0.75 0.02</td>
</tr>
<tr>
<td>Lung VAS</td>
<td>0.69 0.005</td>
<td>0.69 0.29</td>
</tr>
<tr>
<td>Lung MITAX</td>
<td>0.68 0.05</td>
<td>0.75 0.05</td>
</tr>
<tr>
<td>Global VAS</td>
<td>0.63 0.002</td>
<td>0.74 0.005</td>
</tr>
<tr>
<td>Global MITAX</td>
<td>0.64 0.0003</td>
<td>0.74 0.003</td>
</tr>
</tbody>
</table>

* See Table 3 for definitions.
† Based on a mixed linear regression model.
‡ Determined in 50 serial samples from 11 patients.
§ Determined in 35 serial samples from 9 patients.
data from 2 such patients (Figure 2), this temporal correlation further links anti–Jo-1 antibody titers with muscular as well as extramuscular features of myositis disease activity.

DISCUSSION

Taken together, these data demonstrate a moderate correlation between anti–Jo-1 antibody titers and clinical indicators of myositis disease activity that include elevation of CK levels, muscle dysfunction, and articular involvement. The strength of these associations is comparable to that between anti-dsDNA and renal disease in SLE (18), lending clinical and biologic significance to our findings. Importantly, several of the statistical correlations in this analysis are associated with increased \( r_s \) values when using a custom-designed ELISA based on rHuJo-1—a critical factor given variations in immunodominant epitopes between calf and human Jo-1 sequences that are reflected by the relatively modest correlation coefficient between the 2 detection methods \( (r_s = 0.59) \). Ultimately, the strength of association with measures of clinical disease activity may be enhanced through correlations with antibodies that recognize specific epitopes that are truly related to, or reflective of, pathogenic immune responses. This type of analysis may also establish connections between anti–Jo-1 antibody titers and features of extramuscular disease activity not captured by the current ELISA systems.

In contrast to this cross-sectional analysis, examination of associations between anti–Jo-1 antibody levels and parameters of clinical disease activity at different
time points (in individual patients) extends the relationship to other manifestations of the antisynthetase syndrome, including ILD. Although conclusions based on these statistical associations must be viewed with some caution given the limited numbers of serial specimens, the findings are provocative and suggest that following anti-Jo-1 antibody titers over time may be clinically useful considering the limitations in assessing active disease in extramuscular tissues such as lung. From a practical standpoint, therefore, interpreting serial anti-Jo-1 antibody levels may be analogous to the analysis of serum uric acid in gout, being most useful in longitudinal patient assessment.

Coupled with previous smaller case series that examined the relationship between anti-Jo-1 antibody levels and muscle involvement in idiopathic inflammatory myopathy (12,14,15), the power of this much larger analysis provides compelling evidence that the antibody titer parallels myositis disease activity. The lone study questioning this association reported serial assessment of antibody levels in only a single patient, which hampere[d] definitive conclusions (15).

Overall, the analysis presented here extends the findings of these earlier studies by demonstrating novel associations between anti-Jo-1 antibody levels and specific extramuscular manifestations such as articular and lung disease activity, particularly in longitudinal assessment. Although the retrospective application of this instrument is an inherent limitation that must be addressed in future prospective studies, comparative analysis of clinical evaluations in 34 patients (encompassing 237 disease subcategories) performed both retrospectively (by KBS) and prospectively (by CVO) shows an 85% concordance rate for the MITAX component of the MDAAT, with no significant alteration of statistical associations (data not shown). By extension, therefore, the results of this study provide additional validation of the MDAAT.

Perhaps more important than the implications for anti-Jo-1 antibody as a biomarker of clinical disease activity, this work lends potential insight regarding the immunopathogenesis of the antisynthetase syndrome. While histopathologic and immunohistochemical studies support T cell–mediated tissue damage rather than humoral mechanisms for myotoxicity (19–21), the association of class-switched anti–Jo-1 antibody with muscular as well as extramuscular disease activity suggests that underlying Jo-1–specific T cells fuel both anti–Jo-1 antibody formation and cell-mediated immune responses. In fact, we have previously demonstrated the existence of Jo-1–specific T cells in anti–Jo-1 antibody–positive patients (22). Such T cells also exist in the repertoire of nondiseased control subjects, but the absence of anti–Jo-1 antibody in these individuals indicates that Jo-1–specific T cells have not been activated in vivo.

Regardless of the precise mechanism, the relationship between anti–Jo-1 antibody levels and different clinical aspects of the antisynthetase syndrome supports a pathogenic link between Jo-1 antigen and inflammation in tissues as disparate as muscle and lung. Ultimately, however, establishing this etiologic relationship more firmly and elucidating factors that govern organ specificity will require additional in vitro and in vivo models based on Jo-1–specific T cells.

**AUTHOR CONTRIBUTIONS**

Dr. Stone had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

**Study design.** Stone, Oddis, Ascherman.

**Acquisition of data.** Stone, Fertig, Katsumata, Lucas.

**Analysis and interpretation of data.** Stone, Oddis, Katsumata, Vogt, Ascherman.

**Manuscript preparation.** Stone, Ascherman.

**Statistical analysis.** Stone, Vogt, Domsic.

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Identification of a Novel Autoantibody Directed Against Small Ubiquitin-like Modifier Activating Enzyme in Dermatomyositis

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Objective. Myositis-specific autoantibodies (MSAs) are directed against cell machinery proteins such as aminoacyl–transfer RNA synthetases, signal recognition particle, Mi-2, and CADM-140. Because serologic subsets can define patients with specific clinical manifestations, the identification of further MSAs may help to identify additional disease subsets within the myositis spectrum.

Methods. Sera from 20 adult patients with dermatomyositis (DM) were screened for autoantibodies. Two patients were further characterized due to the presence of the same novel immunoprecipitation (IP) pattern on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and similar clinical manifestations. Both patients presented with cutaneous disease, followed by proximal myositis 6 months later. Both patients had associated nonspecific interstitial pneumonia but no signs of malignancy. The novel targets were identified using a combination of IP, SDS-PAGE, and matrix-assisted laser desorption ionization–time-of-flight mass spectrometry.

Results. Indirect HEp-2 immunofluorescence on sera from both patients displayed a diffuse, coarse, speckled, nucleolar-sparing pattern. IP revealed the presence of previously uncharacterized bands at ~40 kd and ~90 kd in both patients. The novel targets were identified as the small ubiquitin-like modifier 1 (SUMO-1) activating enzyme A subunit and SUMO-1 activating enzyme B subunit.

Conclusion. These findings reveal previously uncharacterized autoantibodies directed against a protein involved in posttranslational modification, the SUMO activating enzyme, in 2 patients with DM who had similar clinical features, including severe skin disease and interstitial pneumonia.

Myositis-specific autoantibodies (MSAs) are observed in up to 50% of patients with polymyositis (PM) or dermatomyositis (DM) and are directed against cytoplasmic or nuclear cellular components. Serologic subsets can define patients with specific clinical manifestations, including patients with autoantibodies to transfer RNA synthetases, signal recognition particle, Mi-2, and CADM-140 (1,2).

Herein, we report 2 patients who presented with cutaneous features of DM and in whom myositis with limited interstitial lung disease developed several months later; neither of these patients had a history of malignancy. Serologic testing revealed the presence of a novel autoantibody against the small ubiquitin-like modifier 1 (SUMO-1) activating enzyme A subunit (SAE1) and the SUMO-1 activating enzyme B subunit (SAE2) in both patients. SUMOs are involved in posttranslational modification of specific proteins mediated by a cascade of enzymes that include the activating enzyme (SAE) (3,4). The finding of autoantibodies to enzymes involved in sumoylation may identify a further discrete subset of patients with DM and have implications in terms of the pathogenesis of myositis.

PATIENTS AND METHODS

Serum samples were obtained from 44 adult patients with inflammatory myopathies (20 with DM and 24 with PM),
all of whom were seen at the Royal National Hospital for Rheumatic Diseases, Bath, UK. The diagnosis of probable or definite DM or PM was based on the criteria described by Bohan and Peter (5,6). Sera from disease control subjects (150 with systemic sclerosis [SSc] and 40 with systemic lupus erythematosus [SLE]) and 40 normal control subjects were also analyzed. All patients with SSc or SLE fulfilled recognized published criteria (7,8). The study was approved by the Bath Local Ethics Committee, and informed written consent was provided by all patients.

**Patients.** Two patients (a 52-year-old white woman and a 62-year-old white man) presented with clinically amyopathic DM, with features of significant cutaneous involvement including Gottron’s papules, periungal erythema, heliotrope rash, V sign rash, and shawl sign rash. Approximately 6 months later, clinical myopathy associated with elevated levels of creatinine kinase developed in both patients, and examination revealed limited peripheral lung involvement (nonspecific interstitial pneumonia) but no signs of underlying malignancy.

**Indirect immunofluorescence (IIF) and immunodiffusion.** IIF was performed using HEP-2 cells and fluorescent carbocyanin-labeled anti-human immunoglobulin (Sigma, Poole, UK). Immunodiffusion was performed using either 70 μl rabbit thymus extract (Pel-Freez Biologicals, Rogers, AR) or 70 μl Polyex-tract II (Bio-Diagnostics, Worcestershire, UK) and 35 μl of serum.

**Immunoprecipitation (IP) using 35S-methionine.** IP from K562 cell extracts was performed as previously described (9). Briefly, 10 μl serum was mixed with 2 mg protein A–Sepharose beads (Sigma) in IP buffer (10 mM Tris HCl [pH 8.0], 500 mM NaCl, 0.1% [volume/volume] Igepal) at room temperature for 30 minutes. Beads were washed in IP buffer prior to the addition of 120 μl 35S-methionine–labeled K562 cell extract. Samples were washed for 2 hours at 4°C. Beads were washed in IP buffer followed by Tris buffered saline buffer (10 mM Tris HCl [pH 7.4], 150 mM NaCl) before being resuspended in 50 μl sodium dodecyl sulfate (SDS) sample buffer (10 mM Tris HCl, 150 mM NaCl, 0.05% v/v Igepal) and 300 μl phenyl:chloroform:isoamyl alcohol (50:50:1 ratio). RNA was phenol extracted and ethanol precipitated. The resultant RNA pellet was suspended in 20 μl of 30 μM sodium acetate, 30 μl 10% (weight/volume) SDS, 2 μg oyster type XI glycogen, and 300 μl phenylchloroform:isoamyl alcohol (50:50:1 ratio). RNA was then transferred to a fresh tube (tube C) and stored at −80°C. IPs using serum from patient 1 or patient 2 and either 150 μl control 35S-methionine–labeled cell extract or the immunodepleted supernatants (tube C) were completed as described for IP using 35S-methionine.

**Preparative IP for protein isolation and mass spectrometry (MS).** Samples were prepared for MS as previously described (9). IP was completed using 40 μl sera, 2 mg protein A–Sepharose beads, and 5 mM bis(sulphosuccinimidyldi) substrate crosslinker (Perbio Science, Tattenhall, UK). Beads were incubated in 2 ml K562 cell extract for a total of 2 hours at 4°C. Samples were resuspended in 80 μl SDS sample buffer and heated. Proteins were fractionated by 10% SDS-PAGE and stained with Imperial protein stain (Perbio Science). Unique bands were prepared for matrix-assisted laser desorption ionization–time-of-flight (MALDI-TOF) MS, at the University of the West of England. Database matching using ProteinLynx software (Micromass, Manchester, UK) required peptide coverage of >20%, with matching of the major theoretical and experimental peptide peaks.

**IP with rabbit polyclonal anti-SAE2.** Ten microliters of serum or 50 μl of commercial rabbit antibody to SAE2 (Abgent, San Diego, CA) was mixed with 100 μl of prewashed protein G Dynabeads (Dynal, Liverpool, UK) in sodium phosphate at room temperature for 30 minutes. Samples were washed with sodium phosphate, and antigens were immunoprecipitated as described for IP, using 35S-methionine.

**IP blotting.** IP and SDS-PAGE were completed as described above (preparative IP for protein isolation and MS), using a polyclonal sheep anti-SAE1 antibody (Axxora, Nottingham, UK), patient 1 or patient 2 serum, normal control serum, or serum containing known autoantibodies. Immunoprecipitates were transferred to nitrocellulose by Western blotting and probed with the anti-SAE1 antibody (1:3,000 dilution) for 90 minutes. Bands were detected using an alkaline phosphatase–conjugated donkey anti-sheep IgG antibody (Sigma) and a BCIP/nitroblue tetrazolium liquid substrate solution (Sigma, Irvine, UK).

**HLA genotyping.** DNA was prepared from EDTA-preserved whole blood samples using a saltting-out procedure. HLA–DRB1 and HLA–DOB1 alleles were identified using polymerase chain reaction, by the Histocompatibility and Immunogenetics Department at the National Blood Transfusion Center, Bristol, UK (10,11).

**RESULTS**

**Identification of novel DM-related autoantibodies.** IP screening was completed on 20 patients with DM. The results showed the presence of the same novel IP pattern in patient 1 and patient 2. Sera from both of these patients showed a coarse, speckled, nucleolar-sparing staining pattern on HEP-2 IIF (results not
shown). Both patient sera were shown to immunoprecipitate proteins of ~40 kd and ~90 kd (Figure 1, lanes 2 and 3) that did not correspond to any known autoantigen profile. This same profile was not seen in other disease and normal control sera. Both patient 1 and patient 2 had negative immunodiffusion results, and
results of RNA IP were negative using serum from both patients (results not shown).

Immunodepletion studies were completed to demonstrate that the IP pattern seen with sera from patients 1 and 2 was attributable to precipitation of the same autoantigens. When the cell extracts were predepleted with normal serum, the 40-kd and 90-kd antigens were still visible after IP with each patient’s serum (Figure 2, lanes 2 and 6). However, after predepletion of cell extracts with either patient 1 serum or patient 2 serum, the 40-kd and 90-kd targets were absent in all subsequent IPs (Figure 2, lanes 3, 4, 7, and 8). These results, therefore, provide good evidence that the sera from patients 1 and 2 both had the same autoantibody specificity.

Identification of the SAE antigens. Nonradiolabeled IP and Coomassie-stained SDS-PAGE demonstrated the presence of 40-kd and 90-kd bands, using both patient 1 and patient 2 sera. MALDI-TOF MS and Swiss-Prot analysis (www.expasy.org) of the peptide fingerprints from these bands corresponded to SAE1 (38-kd protein) for the 40-kd band and SAE2 (71-kd protein) for the 90-kd band. The results were consistent when the experiments were repeated on 3 separate occasions using targets immunoprecipitated by both patient 1 and patient 2 sera.

These results were further confirmed by the use of a commercial antibody raised against SAE2. IP resulted in the precipitation of a 90-kd band, using both patient 1 and patient 2 sera (Figure 1, lanes 13 and 14). In addition, this band was also present when IPs were completed using an anti-SAE2 rabbit antibody (Figure 1, lane 15). The band obtained with the commercial antibody was weaker than the bands seen with the patient sera, probably due to the fact that the rabbit antibody was raised only against the N-terminal of the protein. However, the band was present at the same molecular weight as that observed in serum from patients 1 and 2, implying IP of the same antigen. Additionally, a faint band was also visible at 40 kd, corresponding to the SAE1 subunit. This was probably attributable to slight cross-reactivity of the commercial antibody between the 2 SAE subunits.

The identity of the novel autoantibodies was further confirmed by IP blotting. IPs were completed using a commercial antibody to SAE1, patient 1 serum, patient 2 serum, normal control serum, and serum containing autoantibodies to other known myositis-
related autoantigens. The results (Figure 3) displayed strong bands of an appropriate molecular weight for patient 1 and patient 2 sera, along with a weaker band for the commercial antibody. Overall, these results showed that the novel precipitation pattern seen in the sera of the 2 patients with DM is attributable to autoantibodies directed against SAE.

**HLA typing.** HLA typing was completed on DNA from patients 1 and 2. The HLA types found in patient 1 were HLA–A1, A2, B8, and B62, DRB1*0301 and *0401, and DQB1*0201 and *0302. The HLA types found in patient 2 were DRB1*0301 and *1101, and DQB1*0201 and *0301.

**DISCUSSION**

MSAs appear to define specific homogeneous clinical subsets within the myositis disease spectrum, and a good example of this is patients with the antisynthetase syndrome, in whom clinical features include myositis, interstitial pneumonia, and characteristic skin changes (1,2,9). Therefore, the identification of novel MSAs is of interest and may define further specific clinical groups. This is the first report to describe autoantibodies directed against SAE in 2 patients with DM, both of whom had specific clinical features of widespread skin involvement, dysphagia, and limited nonspecific interstitial pneumonia. This combination of clinical manifestations was not observed in the other patients with DM, who were negative for anti-SAE autoantibodies. In addition, when comparing patients with anti–Mi-2 autoantibodies, the type of skin lesions were similar, but the anti-SAE–positive patients also had dilated nailfold capillaries. Furthermore, the anti-SAE–positive patients had both dysphagia and lung disease, whereas patients with anti–Mi-2 did not. However, this number of patients is too small to allow a direct comparison.

SUMOs play a key role in the posttranslational modification of specific proteins. The SUMO family consists of 4 members, of which SUMO-1 is the best characterized. Sumoylation leads to the formation of stable conjugates of target proteins including protein kinases and transcription factors. Previous studies have suggested a role for sumoylation in nucleocytoplasmic transport and signal transduction (3,4). SUMO has been previously associated with other inflammatory diseases. In a study by Franz et al (12), high levels of SUMO-1 messenger RNA expression were demonstrated in rheumatoid synovial tissue compared with control tissue. In addition, Janka et al (13) reported the presence of autoantibodies against SUMO-1 and SUMO-2 autoantigens in a subset of patients with primary biliary cirrhosis (PBC). Although PBC has been described in association with DM (14), our index patients did not have PBC.

SAE is a heterodimer composed of the subunits SAE1 and SAE2. Studies by Desterro et al (15) have shown that the 2 polypeptide species, SAE1 and SAE2, have apparent molecular weights of 40 kd and 90 kd, respectively, with predicted molecular masses of 38 kd and 72 kd. These results are consistent with the data in our study, in which SAE2 was shown to migrate at a higher molecular weight than predicted on SDS-PAGE (15). IIF staining using the SAE subunits has shown that SAE is distributed throughout the nuclei but is excluded from the nucleoli (16). These results are consistent with the IIF results for sera from patients 1 and 2, in which a coarse, speckled, nucleolar-sparing staining pattern was seen.

We have described anti-SAE autoantibodies in only 2 patients, and therefore it is difficult to draw definite conclusions. However, the clinical homogeneity of these patients suggests that this finding is important. We believe that it highlights how the use of proteomic techniques can identify novel autoantigens that may associate with specific clinical subsets of patients with inflammatory diseases, including myositis.

**ACKNOWLEDGMENTS**

We thank Juliet Dunphy and Pat Owen for their technical assistance. We also thank Charlotte Carmichael for her assistance in collecting informed consent from all patients in this study.

**AUTHOR CONTRIBUTIONS**

Dr. McHugh had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

**Study design.** Betteridge, Gunawardena, North, McHugh.

**Acquisition of data.** Betteridge, Gunawardena, Slinn, McHugh.

**Analysis and interpretation of data.** Betteridge, Gunawardena, North, Slinn, McHugh.

**Manuscript preparation.** Betteridge, Gunawardena, McHugh.

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Pattern of Interleukin-1β Secretion in Response to Lipopolysaccharide and ATP Before and After Interleukin-1 Blockade in Patients With CIAS1 Mutations

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Objective. To examine the synthesis, processing, and secretion of interleukin-1β (IL-1β), as well as the clinical and biologic effects of IL-1 blockade, in patients with chronic infantile neurologic, cutaneous, articular (CINCA) syndrome and Muckle-Wells syndrome (MWS), in an effort to understand the molecular mechanisms linking mutations of the CIAS1 gene and IL-1β hypersecretion, and the underlying response to IL-1 receptor antagonist (IL-1Ra).

Methods. Six patients with CINCA syndrome or MWS were treated with IL-1Ra and followed up longitudinally. Monocytes obtained from the patients and from 24 healthy donors were activated with lipopolysaccharide (LPS) for 3 hours, and intracellular and secreted IL-1β levels were determined by Western blotting and enzyme-linked immunosorbent assay before and after exposure to exogenous ATP.

Results. LPS-induced IL-1β secretion was markedly increased in monocytes from patients with CIAS1 mutations. However, unlike in healthy subjects, secretion of IL-1β was not induced by exogenous ATP. Treatment with IL-1Ra resulted in a dramatic clinical improvement, which was paralleled by an early and strong down-regulation of LPS-induced IL-1β secretion by the patients’ cells in vitro.

Conclusion. Our results showed that the requirements of ATP stimulation for IL-1β release observed in healthy individuals are bypassed in patients bearing CIAS1 mutations. This indicates that cryopyrin is the direct target of ATP and that the mutations release the protein from the requirement of ATP for activation. In addition, the dramatic amelioration induced by IL-1Ra treatment is at least partly due to the strong decrease in IL-1β secretion that follows the first injections of the antagonist. These findings may have implications for other chronic inflammatory conditions characterized by increased IL-1β.

Interleukin-1β (IL-1β) plays a pivotal role in the pathogenesis of autoinflammatory diseases and represents a potential target of therapeutic intervention in both monogenic and multifactorial inflammatory diseases (1). Unlike most cytokines, IL-1β lacks a secretory signal peptide and is externalized by monocytic cells through a nonclassic pathway, arranged in 2 steps (2,3). First, Toll-like receptor ligands, such as lipopolysaccharide (LPS), induce gene expression and synthesis of the inactive IL-1β precursor (proIL-1β). Monocytes stimulated with LPS alone release only ~20% of the IL-1β over 24–48 hours (4). A second stimulus, such as exogenous ATP, strongly enhances the proteolytic maturation and secretion of IL-1β (5,6). ATP-triggered
IL-1β secretion is mediated by P2X7 receptors expressed on the surface of monocytes (7) and involves a series of events that have only been partly clarified (8,9).

A crucial role in IL-1β processing is played by the inflammasome, a multiprotein complex responsible for the activation of caspase 1, which, in turn, converts proIL-1β to the mature, active 17-kd form (10). A key protein of the inflammasome is cryopyrin (also known as NALP3 or cold-induced autoinflammatory syndrome 1 [CIAS-1]) (11,12). Experimental mouse models have recently revealed that monocytes from knockout mice deficient in cryopyrin cannot activate caspase 1 upon stimulation with LPS and ATP, resulting in a lack of IL-1β secretion (13–15). In contrast, mutations in the CIAS1 gene in humans are associated with diseases characterized by excessive production of IL-1β synthesis, processing, and secretion in vivo target of anakinra remains to be elucidated. In each of these diseases, blocking the IL-1 receptors by daily injections of the recombinant human IL-1 receptor antagonist (IL-1Ra) anakinra returns the patients to normalcy, thus indicating the strong IL-1 dependency of the pathogenesis of these syndromes (18,25–27). Despite these clear clinical results, the in vivo target of anakinra remains to be elucidated.

The present study was aimed at investigating the mode of IL-1 synthesis, processing, and secretion in patients with CIAS1 mutations. Our findings indicate excessive IL-1 secretion by monocytes from these patients. Strikingly, treatment of patients with IL-1Ra resulted in normalization of this in vitro hypersecretion. Both findings have value in elucidating the pathogenesis of CINCA syndrome and MWS and help in understanding the therapeutic effects of treatment with IL-1Ra.

### PATIENTS AND METHODS

**CINCA syndrome and MWS patients.** Six patients, 5 with CINCA syndrome and 1 with MWS (2 females and 4 males; mean age 13.8 years [range 3.1–33.8 years]), were enrolled in the study. The clinical characteristics of the study patients are shown in Table 1. The CINCA syndrome patients have been described in part elsewhere (28). Disease onset occurred within the first month of life in most of the study patients. The disease course in the CINCA syndrome patients was characterized by daily urticarial rashes, persistent elevation of levels of acute-phase reactants, and neurosensory, ocular, and bone involvement. In the MWS patient, symptoms began at the age of 16 months, with recurrent episodes of fever, urticarial rash, and arthritis/arthralgia, with persistent mild elevations of acute-phase reactant levels, consistent with a clinical diagnosis of MWS. Five of the 6 study patients had a mutation in exon 3 of the CIAS1 gene (Table 1).

At baseline, the patients were experiencing daily urticarial rashes (5 of 6 patients), arthritis (4 of 6 patients), and

### Table 1

<table>
<thead>
<tr>
<th>Patient/age (years)/sex</th>
<th>Age at onset of CINCA/MWS</th>
<th>Fever</th>
<th>Rash</th>
<th>Arthritis</th>
<th>Hearing loss</th>
<th>Uveitis/papilledema</th>
<th>Facies/bone dysplasia</th>
<th>Developmental delay</th>
<th>Chronic meningitis</th>
<th>CIAS1 mutation</th>
<th>Treatment at the time of study</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1/8.5/M</td>
<td>1 week</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>N477K</td>
<td>NSAID</td>
</tr>
<tr>
<td>C2/5.9/F</td>
<td>Birth</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>D303N</td>
<td>NSAID</td>
</tr>
<tr>
<td>C3/18.5/M</td>
<td>2 weeks</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>T48M</td>
<td>NSAID</td>
</tr>
<tr>
<td>C4/33.8/M</td>
<td>3 weeks</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>–</td>
<td>++</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Negative</td>
<td>NSAID</td>
</tr>
<tr>
<td>C5/3.1/M</td>
<td>6 months</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>M406I</td>
<td>NSAID</td>
</tr>
<tr>
<td>MWS1/12.8/F</td>
<td>16 months</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>E525K</td>
<td>NSAID</td>
</tr>
</tbody>
</table>

*The intensity of the clinical manifestations was scored as follows: − = absent, + = present, ++ = severe, and +++ = very severe. Patients C1–CS have been described in part elsewhere (28). CINCA syndrome = chronic infantile neurologic, cutaneous, articular syndrome; MWS = Muckle-Wells syndrome; NSAID = nonsteroidal antiinflammatory drug.
headaches (2 of 6 patients). Elevation of acute-phase reactants and severe leukocytosis were observed in all patients. Hypochromic anemia was present in 4 of the 6 patients. Patients were treated with IL-1Ra (anakinra) at a starting dosage of 1–2 mg/kg/day, delivered subcutaneously (maximum daily dose 100 mg).

Informed consent was obtained from all patients and healthy donors. The study was approved by the Board of Ethics of G. Gaslini Institute.

**Cell cultures.** Monocytes from buffy coats obtained from the blood of 24 healthy adult donors matched for sex and ethnicity or from heparinized blood obtained from the study patients were enriched by adherence in RPMI 1640 medium containing 10% fetal bovine serum, and then activated with 1

**Figure 1.** Production, processing, and secretion of interleukin-1β (IL-1β) by monocytes from healthy adult donors (HD). a, Normal monocytes were cultured for 3 hours in the absence (top) and presence (bottom) of 1 μg/ml of lipopolysaccharide (LPS). Aliquots from cell lysates were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and analyzed by Western blotting with anti–IL-1β. Migration of the 35-kd and 29-kd proIL-1β molecular forms is indicated. The lower IL-1β bands were not blocked by caspase 1 inhibitors (results not shown) and are probably nonspecific endoproteases that were activated during the preparation of the samples (3). b, Supernatants from normal monocytes cultured for 3 hours with LPS (top) or for 3 hours with LPS followed by 15 minutes with ATP (bottom) were concentrated and analyzed as in a. Shown are 6 representative controls displaying different levels of basal secretion and different responses to ATP: high (donors D and B), low (donors I and O), and intermediate (donors E and F). Migration of the 17-kd mature IL-1β is indicated. Detection of secreted IL-1β was not associated with the release of lactate dehydrogenase (results not shown), which rules out a role of cell lysis in the externalization of the cytokine. c, IL-1β secreted by monocytes from 24 healthy donors during 3 hours of incubation with LPS or during 15 minutes of exposure to ATP following LPS stimulation was quantified by enzyme-linked immunosorbent assay. Results are expressed as ng/ml of IL-1β secreted per 10⁶ cells. Values are the mean of at least 3 different experiments.
μg/ml of LPS (all from Sigma-Aldrich, Milan, Italy) for 3 hours at 37°C in RPMI 1640 medium supplemented with 1% Nutridoma-HU (Roche Applied Science, Monza, Italy) as described elsewhere (3,8,9). Supernatants were collected and replaced with RPMI 1640/1% Nutridoma-HU in the presence or absence of 1 mM ATP (Sigma-Aldrich) for 15 minutes. After the addition of ATP, supernatants were collected and cells were lysed in 1% Triton X-100 lysis buffer.

**Determination of lactate dehydrogenase (LDH) release.** The release of LDH into supernatants as a marker of cell lysis during cocultures of dendritic cells and natural killer cells was determined by standard methods, using an LDH colorimetric assay (Sigma-Aldrich) (3).

**Enzyme-linked immunosorbent assay (ELISA).** IL-1β content in supernatants was determined by ELISA (R&D Systems, Minneapolis, MN).

**Western blot analysis.** Cell lysates and trichloroacetic acid–concentrated supernatants were prepared as described previously (8), resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 12% gels, and then transferred to nitrocellulose membranes. Membranes were incubated with rabbit anti–caspase 1 antiserum R105 (kind gift of Dr. D. K. Miller, Merck Research Laboratories, Rahway, NJ) followed by the relevant horseradish peroxidase–conjugated secondary antibody (Dako, Glostrup, Denmark) and developed by enhanced chemiluminescence using ECL Plus (Amersham Pharmacia Biotech, Milan, Italy).

**Mutational screening of the CIAS1 gene and detection of P2RX7 polymorphisms.** DNA was extracted from peripheral blood samples by standard methods. All coding regions and intronic flanking sequences of the CIAS1 gene were amplified by polymerase chain reaction (PCR) using specific primers (available upon request from the authors) designed with Primer Express 2.0 software (Applied Biosystems, Foster City, CA). Four primer pairs were used to selectively amplify exons 5, 9, 10, 11, and 13 of the P2RX7 gene, as previously described (29).

PCR fragments were purified with enzymatic mix Sap 1–Exo III by incubation for 40 minutes at 37°C and for 15 minutes at 80°C, and then analyzed for mutations by direct DNA sequencing using the BigDye Terminator cycle sequencing kit v3.1 and an ABI 3100 automated DNA sequencer (both from Applied Biosystems). DNA analysis of each exon was also performed in 50 adult individuals attending our blood transfusion center.

**Statistical analysis.** Comparisons among clinical and laboratory parameters before and after treatment with anakinra were performed using Wilcoxon’s matched pairs test for continuous variables and McNemar’s chi-square test for categorical variables. All statistical tests were 2-tailed with an alpha level of 5%.

**RESULTS**

**IL-1β production and secretion by monocytes from CINCA syndrome and MWS patients with CIAS1 gene mutations.** The production and secretion of IL-1β by monocytes from 4 patients with CINCA syndrome (patients C1–C4, Table 1), 1 patient with MWS (patient MWS1, Table 1), and 24 healthy individuals (Figure 1) were compared under resting conditions and after activation with LPS alone or with LPS followed by a brief exposure to exogenous ATP.

In the absence of LPS, adherent control monocytes synthesized little or no proIL-1β (Figure 1a, top, showing 6 representative subjects). Similarly, monocytes from the CINCA syndrome and MWS patients spontaneously produced small or moderate amounts of proIL-1β (Figure 2a, lanes 1–4 and Figure 2c, lane 1). Neither control monocytes (results not shown) nor patient monocytes (Figure 2b, lanes 1, 4, 7, and 10 and Figure 2c, lane 3) secreted detectable IL-1β under these conditions. LPS stimulation drove the synthesis and intracellular accumulation of proIL-1β to a similar extent in monocytes from normal donors (Figure 1a, bottom) and patients (Figure 2a, lanes 5–8 and Figure 2C, lane 2). Notably, the lower IL-1β bands shown in Figure 1a were not blocked by treatment with caspase 1 inhibitors (results not shown) and are probably due to nonspecific endoproteases being activated during the sample preparation (3).

Control monocytes activated with LPS for 3 hours secreted variable amounts of IL-1β, which was detected as a faint 17-kd band (Figure 1b, top). As previously reported (6–9), brief exposure (15 minutes) to extracellular ATP strongly induces the secretion of mature IL-1β (Figure 1b, bottom). Quantification by ELISA (Figure 1c) confirmed the variable secretion of IL-1β during 3 hours of LPS stimulation and the different, but consistent, increases after exposure to ATP in the 24 normal donors. The presence of variable amounts of proIL-1β in supernatants was erratic and was not paralleled by a release of the cytosolic enzyme LDH (results not shown), ruling out a role of cell lysis in the externalization of the cytokine (2,3).

In contrast, the LPS-induced secretion of mature 17-kd IL-1β by monocytes from the 3 CINCA syndrome patients bearing a CIAS1 mutation who were tested (patients C1–C3, Table 1) was dramatically high (Figure 2b, lanes 2, 5, and 8). Remarkably, the addition of exogenous ATP failed to stimulate further IL-1β secretion in these patients (Figure 2b, lanes 3, 6, and 9). Conversely, monocytes from the single CINCA syndrome patient who lacked CIAS1 mutations (patient C4, Table 1) displayed a pattern of IL-1β secretion similar to that of healthy donors, with low secretion of IL-1β during the 3 hours of stimulation with LPS, which increased following induction with ATP (Figure 2b, lanes 11 and 12). Monocytes from the MWS patient
carrying the E525K mutation of the CIAS1 gene (patient MWS1, Table 1) secreted in response to LPS an IL-1β band that was weaker than that observed in the CINCA syndrome patients (Figure 2c, lane 2; compare with Figure 1b, top). However, similar to patients with CIAS1 mutations, ATP was unable to further induce IL-1β secretion (Figure 2c, lane 5).

The results of the Western blot analyses were confirmed and quantified by ELISA (Figure 2d).

**Induction of caspase 1 secretion by LPS-stimulated monocytes from CINCA syndrome patients.** ATP-induced IL-1β secretion by monocytes from healthy individuals was accompanied by the conversion of procaspase 1 to active caspase 1, followed by secretion of the enzyme (8,30,31). Comparison of caspase 1 production and secretion in healthy individuals and CINCA syndrome patients revealed that both normal and mutated monocytes constitutively produced procaspase 1 (p46), with only a little, if any, increase being detected after LPS stimulation (Figure 3a, lanes 1 and 2). However, while control monocytes did not secrete mature 20-kd caspase 1 unless they were exposed to LPS plus ATP treatment (Figure 3a, top blot, lanes 3–5), in monocytes from patients C1 and C3 (Figure 3a, middle and bottom blots) caspase 1 activation and secretion of abundant p20 active caspase 1 occurred following activation with LPS alone (Figure 3a, lane 4). ATP was unable to drive caspase 1 secretion in cells from CINCA syndrome patients (Figure 3a, lane 5), a finding similar to that for the secretion of IL-1β.

**Figure 2.** Production, processing, and secretion of interleukin-1β (IL-1β) by monocytes from 4 patients with chronic infantile neurologic, cutaneous, articular (CINCA) syndrome and 1 patient with Muckle-Wells syndrome (MWS). Peripheral blood monocytes were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and analyzed by Western blotting with anti–IL-1β. Migration of the 35-kd and 29-kd proIL-1β molecular forms in cell lysates and the 17-kd mature IL-1β form in supernatants is indicated. **a,** Aliquots of cell lysates from untreated or lipopolysaccharide (LPS)-treated monocytes from CINCA syndrome patients were analyzed by Western blotting. **b,** Supernatants from CINCA syndrome patients were incubated for 3 hours without LPS, lanes 1, 4, 7, and 10, with LPS, lanes 2, 5, 8, and 11, or with LPS followed by 15 minutes with ATP, lanes 3, 6, 9, and 12, and analyzed by Western blotting. Detection of secreted IL-1β was not associated with the release of lactate dehydrogenase (results not shown). **c,** Aliquots of cell lysates from untreated or LPS-treated monocytes from an MWS patient were analyzed by Western blotting. Supernatants from the MWS patient monocytes were incubated for 3 hours without LPS, lanes 3, and with LPS, lanes 4, or with LPS followed by 15 minutes with ATP, lanes 5, and analyzed by Western blotting. **d,** IL-1β secreted by patient monocytes during 3 hours of incubation with LPS or during 15 minutes of exposure to ATP following LPS stimulation was quantified by enzyme-linked immunosorbent assay. Values are the mean of at least 3 different experiments. The mean and SD values in the 24 healthy donors (HD) shown in Figure 1d are provided for comparison. Results are expressed as ng/ml of IL-1β secreted per 10⁶ cells.
Lack of relationship between resistance to ATP stimulation in CINCA syndrome or MWS patients and mutations of the gene for the P2X7 receptor.

P2RX7, the gene for the purinergic receptor responsible for the ATP-dependent release of IL-1β (7), is highly polymorphic, and a number of loss-of-function mutations have been characterized (29). The presence of functionally relevant P2RX7 polymorphisms in CINCA syndrome and MWS patients was investigated (Table 2). Patients C1, C2, and C3, whose monocytes secreted little IL-1β following ATP triggering, expressed the wild-type P2RX7, ruling out a role of this receptor in the lack of response to ATP. In contrast, patient C4, who lacked CIAS1 mutations and whose monocytes secreted IL-1β in response to ATP at levels similar to those in healthy individuals, was heterozygous for the 1513 A>C nucleotide mutation; this mutation is associated with a complete loss of function only when 2 copies are present (32,33). The P2RX7 gene in patient MWS1 carried on both alleles the nucleotide substitution 489 C>T. This variant is present in ~50% of the population, and although it was proposed to increase the function of P2X7 (34), its functional relevance with respect to IL-1β secretion is uncertain (29).

Clinical and biologic effects of anakinra treatment in patients with CINCA syndrome or MWS. The clinical response to anakinra treatment in the study patients is summarized in Figures 4a and b. After the first dose of anakinra was administered, all patients displayed dramatic improvement, with complete resolution of urticarial rash, arthritis, and fever within 1 week from the beginning of the treatment (Figure 4a). A rapid decrease in the levels of acute-phase reactants was also observed in the first weeks of treatment, with complete normalization in the majority of the patients (Figure 4b). Monitoring of the patients during anakinra treatment

Table 2. Functionally relevant polymorphisms of P2RX7 in patients with CINCA syndrome or MWS

<table>
<thead>
<tr>
<th>Patient</th>
<th>P2RX7 genotype</th>
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<td></td>
<td>nt 489 C&gt;T</td>
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<tr>
<td>C1</td>
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<tr>
<td>C4</td>
<td>C/C</td>
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<tr>
<td>MWS1</td>
<td>T/T†</td>
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* CINCA syndrome = chronic infantile neurologic, cutaneous, articular syndrome; MWS = Muckle-Wells syndrome; nt = nucleotide.
† The effect of His155Tyr substitution is a subject of controversy. Shemon and coworkers (29) reported this mutation as nonfunctional, whereas Cabrini and coworkers (34) proposed that it increases the activity of P2RX7.
‡ Arg307Gln and Ile568Asn substitutions have also been reported to confer complete loss of function when 2 copies were present (29).
§ The most common substitutions at amino acid residues 357 and 496 of P2RX7 were demonstrated to induce a complete loss of receptor function when 2 copies were present or in combination with another loss-of-function polymorphism (32).
¶ Mutation.
(mean 12.6 months [range 5–15 months]) revealed that all of them continued to experience complete control of their symptoms of inflammation, with nearly complete amelioration of their general conditions (Figures 4a and b). However, no significant improvement in the visual and/or acoustic impairments has so far been observed in these patients.

Monocytes were obtained from the patients at different time intervals following the initiation of anakinra therapy and analyzed for IL-1β/H9252 production and secretion. Whereas LPS-induced IL-1β/H9252 synthesis remained unchanged in individual patients as compared with the findings before anakinra treatment (results not shown), the amount of IL-1β secreted in response to LPS activation by monocytes from the 3 CINCA syndrome patients with CLASI mutations (patients C1–C3) was dramatically decreased (Figure 5a, lanes 2, 5, and 8). Interestingly, the decrease in IL-1β secretion induced by LPS was not accompanied by reestablishment of the responsiveness to ATP, which again failed to induce IL-1β secretion (Figure 5a, lanes 3, 6, and 9). Notably, in patients C1 and C3, secretion of caspase 1 was also strongly reduced after anakinra treatment (Figure 3b), indicating that the secretion of the 2 proteins is tightly associated.

In contrast, in the CINCA syndrome patient who lacked a CLASI mutation (patient C4) and who displayed a pattern of IL-1β secretion comparable to that in normal individuals before treatment (see Figure 2b), the amount of IL-1β secreted following either LPS activation or ATP stimulation did not change significantly (Figure 5a, lanes 10–12), despite a very good clinical response to anakinra. In addition, cells from patient MWS1, who similarly displayed a good clinical response to anakinra, did not present significant variations in the amount of IL-1β secreted over 3 hours of LPS stimulation and remained insensitive to ATP stimulation (Figure 5b).

Figure 5c shows the quantification of IL-1β present in cell supernatants, as determined by ELISA. Close monitoring of IL-1β secretion by monocytes from CINCA syndrome patients during anakinra treatment...
showed that, although some fluctuations occurred during the year of therapy, IL-1β secretion was still low after 12 months (data not shown).

**DISCUSSION**

In this study, we analyzed the synthesis, processing, and secretion of IL-1β in patients with CINCA syndrome and MWS, both before and after treatment with anakinra, and compared the in vitro data with the clinical responses. Our study has generated 2 major new findings. First, monocytes from CINCA syndrome patients following LPS stimulation secrete impressively greater amounts of mature IL-1β despite a cytoplasmic accumulation of the precursor, comparable to that of healthy individuals, but fail to increase IL-1β secretion in response to ATP. These peculiarities are likely to be dependent on the mutated cryopyrin, since they were absent in the single CINCA syndrome patient who lacked known mutations. Interestingly, the MWS patient, who had a CIAS1 mutation and displayed milder clinical manifestations, failed to respond to ATP despite having levels of LPS-induced IL-1β secretion that were comparable to those secreted by normal individuals. The analysis of the sequence coding for the ATP receptor P2X_7 revealed that none of the patients expressed nonfunctional P2RX7 variants, which ruled out the pos-
sibility that the failure of ATP to induce IL-1β secretion was due to a loss-of-function mutation of the highly polymorphic P2RX7 gene (7,29).

The second main finding was that the treatment with anakinra in CINCA syndrome or MWS patients, which was associated with a dramatic clinical response, was paralleled by a sharp decrease in LPS-induced IL-1β secretion, but did not restore the ability of the patients’ monocytes to secrete IL-1β in response to ATP.

We found that 2 signals are required for inflammasome activation. The dramatic secretion of IL-1β following LPS stimulation of CINCA syndrome monocytes could be dependent upon the induction of proIL-1β synthesis in a cell that, bearing a CIAS1 mutation, displays a constitutively activated inflammasome (11,12). However, if this were the case, caspase 1, which, unlike IL-1β, is also synthesized by resting monocytes (8), would be processed and secreted even in the absence of LPS stimulation. Our study ruled out this possibility by showing that caspase 1, although normally produced by resting CINCA syndrome monocytes, was processed and secreted only after exposure to LPS.

The different behavior of normal and CIAS1-mutated monocytes (showing low versus high IL-1β and caspase 1 secretion in response to LPS and different responsiveness to ATP) suggests that in normal monocytes, 2 stimuli (LPS and ATP) are required to achieve a rapid activation of the inflammasome, whereas in patients carrying a CIAS1 mutation, LPS alone is sufficient to overactivate procaspase 1 and proIL-1β processing and secretion. In principle, the dramatic increase in IL-1β secretion following LPS stimulation of monocytes from CINCA syndrome patients could account for a “secretory exhaustion” of a cell that is no longer responsive to ATP triggering. However, “secretory exhaustion” cannot account for the lack of response to ATP of MWS monocytes that secreted IL-1β in physiologic amounts following LPS stimulation. Furthermore, the lack of response to ATP persisted in monocytes from CINCA syndrome patients after treatment with anakinra, which dramatically decreased the LPS-induced IL-1β secretion.

Recent data obtained from studies of cryopyrin-deficient mice indicate that cryopyrin is essential for assembly of the inflammasome in response to components of the signaling pathway mediated by Toll-like receptor and ATP (13,14). Our present findings in humans support the hypothesis that cryopyrin could be the direct target of ATP (most probably mediated by the low intracellular levels of K+ induced by P2X7 receptor triggering [5]), and when mutated, cryopyrin is freed from the requirement of ATP for its activation. LPS would act upstream, possibly inducing the expression and/or activation of a different (known or unknown) inflammasome component.

In the present study, unstimulated monocytes from CINCA syndrome or MWS patients secreted little, if any, IL-1β. This result differs partially from data obtained by other groups of investigators, which showed an increased synthesis (19) and secretion (11,20,25) of IL-1β by purified monocytes or mononuclear cells from CINCA syndrome or MWS patients, even in the absence of LPS stimulation. This discrepancy is likely explained by the methods used. The cell adherence time we used in this study before testing proIL-1β content and IL-1β secretion is much shorter than that used by the other groups. In our experience, a longer adherence time also results in the activation of normal monocytes in the absence of LPS, although to different extents in different individuals.

How does anakinra render CIAS1-mutated monocytes refractory to LPS-induced IL-1β secretion? All 6 of our CINCA syndrome or MWS patients experienced complete clinical remission following treatment with anakinra, including the patient who lacked CIAS1 mutations; this supports the results recently reported (25) in a different cohort of patients. In addition, while Goldbach-Mansky et al (25) observed a decrease in IL1B gene expression after 3 months of treatment, we found an early (after 48 hours) and marked down-modulation of LPS-induced IL-1β secretion in all of our patients with CIAS1 mutations, independently of proIL-1β synthesis, that remained substantially unchanged as compared with pretreatment levels in each patient. This observation is consistent with the prompt and dramatic clinical response observed in all CINCA syndrome patients soon after the introduction of anakinra. In contrast, no significant decrease in IL-1β secretion was observed in the CINCA syndrome patient lacking a CIAS1 mutation or in the MWS patient. Whether this is due to a low level of LPS-induced secretion by cells from these 2 patients, which could make the decrease less evident, or whether it is due to other mechanisms remains to be clarified.

The mechanisms related to the dramatic effects of anakinra in patients with autoinflammatory syndromes are still largely unclear. Our study suggests that anakinra could play roles other than a simple competition with free circulating IL-1β for IL-1 receptor type I. In fact, the rapid clinical remission obtained in our patients was associated with a prompt decrease in IL-1β secretion by LPS-stimulated peripheral blood mono-
cytes, which strongly suggests that the monocytes themselves represent a major target of anakinra. This observation is consistent with previous studies highlighting the capacity of IL-1 to induce IL-1 itself (35–37). Anakinra can block this positive feedback, as confirmed by the observation that normal monocytes activated in vitro with LPS in the presence of recombinant IL-1Ra decrease IL-1β secretion (data not shown).

However, the simple occupancy of monocyte IL-1 receptors by anakinra in vivo is unlikely to be responsible for the decreased IL-1β secretion observed in vitro in monocytes from CINCA syndrome patients, since the procedures used to purify and wash the monocytes should greatly reduce the amount of bound recombinant IL-1Ra. If a consistent proportion of LPS-induced IL-1β secretion occurs as a result of IL-1β itself, one could speculate that in monocytes from CINCA syndrome patients treated with anakinra, IL-1 receptor type I is down-regulated, resulting in a reduced secretion of IL-1β. It remains to be elucidated whether interference by anakinra in the vicious circle of IL-1 inducing IL-1 is the major cause of the decreased secretion or whether other mechanisms are involved.

In conclusion, our findings confirm the pivotal role of IL-1β in the pathogenesis of CINCA syndrome or MWS and shed new light on the underlying molecular mechanisms. ATP as a secretory trigger is not operative in monocytes from patients with CINCA syndrome or with MWS who carry mutated cryopyrin genes. This suggests that while in normal monocytes, 2 signals are required to activate the inflammasome, thus tightly controlling IL-1β secretion, in patients with a CLASI mutation, a single stimulus, even one that in low amounts (such as danger signals released by injured cells or bacterial products) would be unable to trigger IL-1β secretion in healthy individuals, is sufficient to drive a dramatic inflammatory cascade.

The in vivo encounter with anakinra not only blocks the effects of IL-1 on target cells, but also inhibits the production of IL-1β by monocytes, resulting in an impressive amelioration of the clinical manifestations. Our findings may also have implications for other chronic inflammatory conditions characterized by an increased expression of IL-1β and responsive to IL-1 blockade, such as systemic-onset juvenile idiopathic arthritis and adult Still’s disease (38–41).

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

Drs. Gattorno and Rubartelli had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Gattorno, Martini, Rubartelli.

Acquisition of data. Gattorno, Tassi, Carta, Delfino, Ferlito, Pelagatti, D’Ossolando, Buoncompagni, Alpigiani, Alessio.

Analysis and interpretation of data. Gattorno, Tassi, Carta, Delfino, Ferlito, Pelagatti, Buoncompagni, Rubartelli.

Manuscript preparation. Gattorno, Martini, Rubartelli.

Statistical analysis. Gattorno.

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cryopyrin in innate and adaptive immunity through its regulation of caspase-1. Immunity 2006;24:317–27.
Febrile Temperatures Control Antineutrophil Cytoplasmic Autoantibody–Induced Neutrophil Activation via Inhibition of Phosphatidylinositol 3-Kinase/Akt

Sibylle von Vietinghoff, Mira Choi, Susanne Rolle, Friedrich C. Luft, and Ralph Kettritz

Objective. Neutrophil activation by antineutrophil cytoplasmic autoantibodies (ANCAs) is central to the pathogenesis of the ANCA-associated vasculitides. Febrile infections occur frequently during these diseases, often in the context of immunosuppressive treatment. Heat exposure may affect the underlying pathophysiologic processes of the vasculitis. In this study we tested the hypothesis that short-term exposure to heat inhibits ANCA-induced neutrophil activation.

Methods. After exposure to temperatures from 37°C to 42°C, human neutrophils were primed with either tumor necrosis factor α (TNFα) or granulocyte–macrophage colony-stimulating factor (GM-CSF) and stimulated with monoclonal antibodies to myeloperoxidase or to proteinase 3. Respiratory burst activity was assayed using rhodamine and a nitroblue tetrazolium reduction assay. Specific inhibition experiments against p38 MAPK, ERK, and phosphatidylinositol 3-kinase (PI 3-kinase)/Akt, and Western blotting with phospho-specific antibodies were used to identify key components in the antibody-induced respiratory burst.

Results. A temperature-dependent reduction in ANCA-induced respiratory burst was observed over a range of heat exposures from 37°C to 42°C. Inhibition of human ANCA–induced neutrophil stimulation was significant at 40°C (after priming with 2 ng/ml TNFα, mean [± SEM] fluorescence intensity [MFI] 114 ± 12 at 37°C versus 53 ± 6 at 40°C; after priming with 20 ng/ml GM-CSF, MFI 92 ± 16 at 37°C versus 35 ± 6 at 40°C; both P < 0.01). In the priming phase, the transient activation of the p38 MAPK, ERK, and PI 3-kinase/Akt pathways by TNFα was blocked by prior exposure of the neutrophils to heat, but GM-CSF–induced activation was unaltered by heat. However, in the second, antibody-induced wave of kinase activation, exposure to heat inhibited only the PI 3-kinase/Akt pathway, and these effects were independent of the priming agent used.

Conclusion. Short-term spikes of modest heat abrogate ANCA-induced activation of neutrophils via inhibition of PI 3-kinase/Akt signaling. Febrile responses in ANCA-mediated diseases may therefore have a physiologic purpose.

Antineutrophil cytoplasmic antibodies (ANCAs) are found in a group of diseases now termed the ANCA-associated vasculitides (1,2). Neutrophil activation by ANCAs has been demonstrated in vitro in several studies. Proinflammatory mediators prime neutrophils for the subsequent ANCA-mediated activation. The ANCA-activated neutrophils generate reactive oxygen species and release tissue-toxic granule proteins, both of which are functions that mediate endothelial cell damage and ultimately lead to vasculitis (3–11). Recently, the central role of neutrophils in the induction of ANCA-mediated vasculitis was shown by neutrophil depletion in a mouse model of glomerulonephritis (12). Investigations of the underlying signal transduction pathways that control this cytokine-mediated priming and ANCA-induced activation of neutrophils identified...
the participation of p38 MAPK, ERK, protein kinase C, Syk, p21\(^\text{as}\), and phosphatidylinositol 3-kinase (PI 3-Kinase)/Akt (9,10,13–16).

Neutrophils are frequently exposed to local and systemic temperature increases during inflammation. The signaling and functions of neutrophils are modulated by short-term exposures to heat. We demonstrated previously that moderate, short-term increases in temperature can block activation of NF-κB in neutrophils, and thereby abrogate antiapoptotic signals (17,18). The inhibitory effect of heat on neutrophil signaling was also detected in mice that were exposed to a short-term fever spike of 40.5°C (18), indicating that the effects of heat are relevant in vivo. Results from other studies have suggested that fever plays a beneficial role in host defense reactions, and that antipyretic treatment interferes with this effect (19–21).

Moderate temperature increases can occur in patients with ANCA-associated vasculitis, particularly during infections that are a frequent complication of immunosuppressive treatment (22,23). We tested the hypothesis that short-term heat exposure inhibits the signal transduction pathways that control activation of respiratory burst activity in cytokine-primed neutrophils that had been activated by ANCA. We found that febrile-range temperatures have potentially clinically relevant effects on ANCA-induced neutrophil activation.

**PATIENTS AND METHODS**

**Materials.** Granulocyte–monocyte colony-stimulating factor (GM-CSF) and tumor necrosis factor α (TNFα) were obtained from R&D Systems (Wiesbaden-Nordenstedt, Germany). Phorbol 12-myristate 13-acetate (PMA), nitroblue tetrazolium (NBT), dihydrodithionamide (DHR), and Ficoll-Hypaque were from Sigma (Deisenhofen, Germany). Hanks’ balanced salt solution (HBSS), phosphate buffered saline (PBS), and trypan blue dye were from Biochrom (Berlin, Germany), and dextran was purchased from GE Healthcare (Amsterdam, The Netherlands). The monoclonal antibody (mAb) to proteinase 3 (anti-PR3), 4A5, was from Wieslab (Lund, Sweden), and the mAb to myeloperoxidase (anti-MPO) was from Dako (Hamburg, Germany). Other antibodies were from Acris (Herford, Germany), the horseradish peroxidase–labeled donkey anti-rabbit IgG was from GE Healthcare, and phosho-specific antibodies to p38 MAPK, ERK, and Akt (S473) were obtained from Cell Signaling Technology (Bevpehr, MA). The inhibitors LY294002, SB202190, and PD98059 were purchased from Calbiochem (Bad Soden, Germany). Endotoxin-free reagents and plastic disposable were used in all experiments.

**Preparation of human neutrophils and human IgG.** Neutrophils were isolated from the heparinized whole blood of healthy human donors as described previously (16). Cell viability was detected in every cell preparation by trypan blue dye exclusion. The viability of the cells was found to be >99%, and the percentage of neutrophils present after isolation was >95%, as shown by Wright-Giemsa staining and light microscopy.

Normal IgG and ANCA-containing IgG were prepared from the blood of normal healthy volunteers and patients with MPO- and PR3-specific ANCA’s, respectively, and samples were analyzed by clinical enzyme-linked immunosorbent assay using a High-Trap protein G column in an Akta fast-performance liquid chromatography system (both from GE Healthcare). A single band was detected on Coomassie blue–stained gels.

The study was carried out according to the principles of the Declaration of Helsinki. Written informed consent was obtained from all subjects prior to initiation of the study, and the institutional ethics committee provided approval for the study.

**In vitro heat exposure.** Isolated neutrophils at 10⁷ cells/ml HBSS were incubated for 60 minutes at 37°C or were exposed to 38°C, 39°C, 40°C, 41°C, or 42°C. Eppendorf Thermo blocks (Hamburg, Germany) were used for heat exposures, and the temperature was checked with a digital thermometer (Testo 900). Thereafter, cells were adjusted for 15 minutes at 37°C before further testing. Cell viability was always higher than 95% as determined by trypan blue dye exclusion. Staining with annexin V was carried out to detect apoptosis, using an ApoAlert–annexin V–fluorescin isothio-cyanate cell apoptosis kit (BD Biosciences, Heidelberg, Germany) according to the manufacturer’s instructions, and results were quantified by flow cytometry.

**Measurement of respiratory burst by oxidation of DHR to rhodamine.** The generation of reactive oxygen radicals was assessed using DHR, as described previously (14). Briefly, neutrophils (1 x 10⁶/ml HBSS) were loaded with DHR (1 μM) for 10 minutes at 37°C. After 40 minutes of priming with 2 ng/ml TNFα or 20 ng/ml GM-CSF, the cells were divided and 5 x 10⁵ cells were incubated with each of the stimuli in a total assay volume of 100 μl. Antibodies were added and the reactions were stopped after another 30 minutes by adding 400 μl of ice-cold PBS/1% bovine serum albumin. Samples were analyzed using a FACScan (Becton Dickinson, Heidelberg, Germany), and data were collected from 10,000 cells per sample. The shift of green fluorescence in the FL-1 mode was determined. For each condition, the results were determined as the mean fluorescence intensity, representing the amount of hydrogen peroxide generated.

**NBT reduction test.** As an alternative method of analyzing respiratory burst activity, superoxide generation was measured by reduction of NBT to formazan (24). Neutrophils were incubated at a concentration of 5 x 10⁷/ml in 0.05% NBT and with priming agents for 15 minutes at 37°C. Antibodies were added and the reaction was allowed to proceed for 30 minutes at 37°C. Cells were spun down and resuspended at 10⁶/ml in 5% sodium dodecyl sulfate (SDS)–50 mM HCl, and the absorbance was read at 590 nm after 10 minutes at room temperature.

**Western blotting.** After stimulation for various time periods, cells were lysed on ice in 20 mM Tris HCl, pH 8.8, 138 mM NaCl, 10% glycerol, 2 mM EDTA, 1% Triton X-100, and 1% Nonidet P40, which was supplemented with protease
inhibitors (10 μg/ml quercetin, 10 μg/ml leupeptin, 0.1 mM aprotinin, 5 mM iodoacetamide, 0.2 mM Na,VO₄, 20 mM NaF, 1 mM 4-[2-aminophenyl]benzenesulfonyl fluoride, 1 μg/ml peptatin A, 0.5 mM benzamidine, 1 mM dithiothreitol, 2 mM diisopropylfluorophosphate, or 1 mM phenylmethylsulfonyl fluoride). For Western blot analyses, samples were incubated for 5 minutes at 95°C in loading buffer (250 mM Tris HCl, pH 6.8, with 4% SDS, 20% glycerol, 0.01% bromphenol blue, and 10% β-mercaptoethanol). Five to twenty micrograms of protein was electrophoresed on a 10% SDS–polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was blocked with Tris buffered saline–Tween/5% skim milk for 1 hour and incubated overnight with the primary antibodies, followed by a horseradish peroxidase–labeled secondary antibody. The blot was developed in an enhanced chemiluminescence substrate (Pierce, Rockford, IL) and exposed to radiographic film.

**Statistical analysis.** Results are expressed as the mean ± SEM. Comparisons between 2 groups were carried out using paired t-tests. Comparisons between multiple groups were performed using one-way or two-way analysis of variance, as appropriate. Specific differences between multiple groups were then determined using the Bonferroni post-hoc test. P values less than 0.05 were considered significant.

**RESULTS**

**Abrogation of respiratory burst activity by short-term heat exposure in TNFα- and GM-CSF–primed neutrophils stimulated with anti-PR3 mAb, anti-MPO mAb, and human ANCAAs.** We exposed neutrophils for 60 minutes to increasing temperatures over a range of 37°C to 42°C. Thereafter, the cells were cytokine primed and subsequently stimulated with mAb to PR3 or mAb to MPO. As a result, a dose-dependent inhibition of hydrogen peroxide generation was observed (Figures 1A and B). In contrast, no such inhibitory effect of heat was observed in PMA-stimulated neutrophils, which confirms that NADPH oxidase was still functioning after exposure to heat. This heat-dose–dependent inhibition occurred independent of the priming cytokine, namely TNFα and GM-CSF.

We then measured respiratory burst activity with a second independent method, the NBT reduction test, which assessed the generation of superoxide. After short-term exposure of neutrophils to a modest temperature of 40°C and incubation with the anti-MPO mAb, the results confirmed the inhibition of respiratory burst by heat (Figure 1C). In addition, we stimulated TNFα- and GM-CSF–primed neutrophils with human PR3- and MPO-specific ANCA preparations, with the results revealing a significant inhibition after prior exposure of the neutrophils to 40°C (Figure 1D). Together, these findings indicate that short-term exposure of neutrophils to moderate heat inhibits ANCA-induced respiratory burst activity in both TNFα- and GM-CSF–primed neutrophils.

Assessment of cell viability and analyses of cell apoptosis revealed no differences between neutrophils exposed to 37°C for 60 minutes and those exposed to 40°C for 60 minutes, with both subsequently incubated at 37°C for the total period of 130 minutes. Assessment by trypan blue dye exclusion yielded a cell viability rate of 96 ± 1% at 37°C and 95 ± 0% at 40°C. In parallel experiments annexin V staining demonstrated that 95 ± 1% of cells were nonapoptotic at 37°C and 93 ± 3% of cells were nonapoptotic at 40°C (n = 3 independent experiments; P not significant for each).

**Regulatory role of PI 3-kinase/Akt and p38 MAPK in respiratory burst activity in TNFα- and GM-CSF–primed, mAb-stimulated neutrophils.** To assess whether the PI 3-kinase/Akt, p38 MAPK, and ERK signaling pathways are necessary for respiratory burst to occur in response to anti-PR3 and anti-MPO mAb, cells were incubated, prior to cytokine priming, with the respective inhibitory compounds for 20 minutes and then were activated with each of the mAb (Figures 2A and B). The results demonstrated a significant inhibition of respiratory burst activity following pharmacologic blockade of PI 3-kinase/Akt (with 10 μM LY294002) and p38 MAPK (with 10 μM SB202190), but a rather weak effect following blockade of ERK (with 25 μM PD98059). Nevertheless, Western blot analyses showed that 25 μM PD98059 was sufficient to block ERK phosphorylation (results not shown; see supplementary Figure 1, available upon request from the corresponding author). Similar results were obtained between cells primed with TNFα (Figure 2A) and those primed with GM-CSF (Figure 2B). The results of these experiments confirm the functional importance of PI 3-kinase/Akt and p38 MAPK in ANCA-induced activation of respiratory burst activity in TNFα-primed neutrophils. In addition, our results are the first to demonstrate a similar process in GM-CSF–primed cells.

**Inhibition by heat exposure of p38 MAPK, ERK, and PI 3-kinase/Akt activation during TNFα priming of neutrophils, but not during GM-CSF priming.** Since we found that PI 3-kinase/Akt and p38 MAPK controlled neutrophil burst, we next investigated the effects of short-term heat exposure on the transient activation of these pathways during the priming phase; the effects of heat on activation of ERK were also evaluated, since we could not, as yet, completely reject the role of this pathway. We found that exposure to heat almost completely abrogated the activation of PI 3-kinase/Akt, p38 MAPK, and ERK in neutrophils primed with 2 ng/ml

**Statistical analysis.** Results are expressed as the mean ± SEM. Comparisons between 2 groups were carried out using paired t-tests. Comparisons between multiple groups were performed using one-way or two-way analysis of variance, as appropriate. Specific differences between multiple groups were then determined using the Bonferroni post-hoc test. P values less than 0.05 were considered significant.

**RESULTS**

**Abrogation of respiratory burst activity by short-term heat exposure in TNFα- and GM-CSF–primed neutrophils stimulated with anti-PR3 mAb, anti-MPO mAb, and human ANCAs.** We exposed neutrophils for 60 minutes to increasing temperatures over a range of 37°C to 42°C. Thereafter, the cells were cytokine primed and subsequently stimulated with mAb to PR3 or mAb to MPO. As a result, a dose-dependent inhibition of hydrogen peroxide generation was observed (Figures 1A and B). In contrast, no such inhibitory effect of heat was observed in PMA-stimulated neutrophils, which confirms that NADPH oxidase was still functioning after exposure to heat. This heat-dose–dependent inhibition occurred independent of the priming cytokine, namely TNFα and GM-CSF.

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Figure 1. Effect of heat exposure on respiratory burst activity. After 60 minutes of exposure to temperatures from 37°C to 42°C, neutrophils were primed with 2 ng/ml tumor necrosis factor α (TNFα) (A) or 20 ng/ml granulocyte–macrophage colony-stimulating factor (GM-CSF) (B) and stimulated with 5 µg/ml monoclonal antibodies (mAb) to proteinase 3 (anti-PR3) (triangles), 5 µg/ml mAb to myeloperoxidase (anti-MPO) (circles), or 5 µg/ml control IgG (diamonds). Hydrogen peroxide production was measured using the rhodamine test (n = 4) (A and B). As an independent estimation method of respiratory burst, the nitroblue tetrazolium test was used to compare the extent of superoxide generation after exposure to 37°C and after exposure to 40°C in cells stimulated with anti-MPO mAb (αMPO) (n = 3) (C). Cells stimulated with phorbol 12-myristate 13-acetate (PMA) (25 ng/ml) (squares in A and B) served as controls in these tests (A–C). Hydrogen peroxide production was also assayed using rhodamine in human antineutrophil cytoplasmic antibody (ANCA)–stimulated cells (D), involving 3 separate experiments with 2 different PR3-specific ANCAs, 2 different MPO-specific ANCAs, or 2 different healthy control (CTRL) preparations (each at 150 µg/ml). Results are the mean and SEM mean fluorescence intensity (MFI). * = P < 0.05; ** = P < 0.01 versus exposure at 37°C or versus PMA control.

Figure 2. Effect of inhibition of p38 MAPK, ERK, and phosphatidylinositol 3-kinase (PI 3-kinase)/Akt on respiratory burst activity. Neutrophils were pretreated with the p38 MAPK inhibitor SB202190 (SB), the ERK inhibitor PD98059 (PD), and the PI 3-kinase/Akt inhibitor LY294002 (LY) or buffer control (Bu) prior to priming with 2 ng/ml TNFα (A) or 20 ng/ml GM-CSF (B). Cells were then stimulated with mAb to MPO or to PR3 (αPR3) (each at 5 µg/ml). Results are the mean and SEM MFI determined by rhodamine assay in 3 independent experiments. * = P < 0.05; ** = P < 0.01 versus buffer control. See Figure 1 for other definitions.
TNFα (Figure 3A) but had basically no effect on the activation of these pathways after priming of neutrophils with 20 ng/ml GM-CSF (Figure 3B).

Blockade of ANCA-induced activation of PI 3-kinase/Akt, but not of p38 MAPK and ERK, by heat. Priming with TNFα and GM-CSF resulted in a transient activation of p38 MAPK, ERK, and PI 3-kinase/Akt that returned to basal values after 30–40 minutes. Stimulation of neutrophils with the mAb to MPO or the mAb to PR3 at 40 minutes after cytokine priming resulted in a second wave of activation of all 3 pathways, which was induced by the specific mAb but not by the control IgG. Prior exposure of neutrophils to 40°C for 60 minutes significantly inhibited the activation of PI 3-kinase/Akt but had no effect on the activation of p38 MAPK and ERK (Figure 4). The fact that the phosphorylation of p38 MAPK and ERK was unaffected by this exposure underscores the notion that heat lacks nonspecific toxic effects on signaling. These results together with the findings from the inhibition studies (shown in Figure 2) collectively suggest that the inhibition by heat of ANCA-induced respiratory burst activity is mediated by the inhibitory effects of heat on PI 3-kinase/Akt activation.

Selective inhibition of the second ANCA-induced, but not cytokine-induced, wave of PI 3-kinase/Akt activation, and subsequent control of respiratory burst. We next evaluated whether the observed effect of heat was specific to ANCA-induced signals. In parallel experiments we subjected TNFα-primed cells, with or without prior heat exposure, to a second stimulation with either the anti-MPO mAb or the priming agents TNFα or GM-CSF (Figure 5A). These experiments again demonstrated that prior heat exposure markedly reduced the phosphorylation of Akt in response to stimulation with the anti-MPO mAb. In contrast, exposure to heat neither inhibited the effects of GM-CSF nor inhibited the second round of TNFα-induced activation. Interestingly, the second TNFα challenge resulted in much less Akt phosphorylation than was observed with the first TNFα challenge. Nevertheless, the extent of this phosphorylation was similar regardless of whether the cells had undergone prior heat exposure. Thus, these findings

Figure 3. Effect of heat exposure on PI 3-kinase/Akt, ERK, and p38 MAPK activation as assessed by Western blotting. Cells were kept at either 37°C or 40°C for 60 minutes before incubation with 2 ng/ml TNFα (A) or 20 ng/ml GM-CSF (B) for the indicated time periods. Kinase activation was assessed by Western blotting with phospho-specific antibodies (n = 4). Optical densitometry results are the mean and SEM of 4 independent experiments. * = P < 0.05; ** = P < 0.01 versus exposure to 37°C. See Figures 1 and 2 for definitions.
suggest that the observed effect of heat on ANCA-induced activation has at least some specificity.

We further dissected the functional role of Akt phosphorylation in respiratory burst activity by assessing neutrophil responses during priming and during ANCA stimulation. Figure 2 shows the results obtained following preincubation of the neutrophils with the PI 3-kinase/Akt blocker LY294002 before priming. We subsequently applied the PI 3-kinase blocker LY294002 in parallel experiments either before or after priming but prior to the incubation with the anti-MPO mAb. As shown in Figure 5B, blockade of PI 3-kinase/Akt abrogated the ANCA-induced respiratory burst activity even if the pathway was blocked only after priming. Thus, these findings establish the importance of PI 3-kinase/Akt in ANCA-activated respiratory burst. In Western blot experiments, the results (not shown) confirmed that the addition of LY294002 after cytokine priming blocked the phosphorylation of Akt in response to the anti-MPO mAb.

**Importance of human ANCA-induced PI 3-kinase/Akt activation for respiratory burst.** We then investigated whether heat exposure would also abrogate the activation of PI 3-kinase/Akt in neutrophils that had been stimulated by human ANCA. Neutrophils were primed with TNFα or GM-CSF and subsequently stimulated with different human PR3- and MPO-specific ANCA preparations. The results from Western blot and
statistical analyses indicated that a short-term exposure to heat significantly inhibited the activation of PI 3-kinase/Akt in response to human ANCA (Figure 6).

**DISCUSSION**

The novel finding from this study is that ANCA-induced activation of cytokine-primed human neutrophils is down-regulated by prior short-term exposure to heat. The inhibitory effects of heat occur in a dose-dependent manner and are independent of the priming cytokines used. We identified these inhibitory effects on PI 3-kinase/Akt activation as a mechanism by which heat abrogates ANCA-induced neutrophil activation. We were impressed by the fact that the effect appeared to be linear and not merely a function of the highest temperatures.

Binding of ANCA to cytokine-primed neutrophils and the subsequent activation of these neutrophils comprise a key event in ANCA-associated vasculitis. The activation of neutrophils by ANCA induces respiratory burst activity and the release of toxic granule proteins (3). Recently, the passive transfer of anti-MPO mAb from MPO-deficient mice into wild-type mice resulted in systemic vasculitis, and the clinical manifestations included necrotizing pauciimmune glomerulonephritis (25). Moreover, neutrophil depletion completely prevented this disease in another mouse model, thus firmly establishing the role of ANCA and neutrophils in the induction of vasculitis (12). The findings from animal models regarding PR3-specific ANCA are currently less clear, although in one mouse model, anti-PR3 antibodies accelerated TNFα-mediated dermal inflammation (26).
Low concentrations of proinflammatory cytokines function by priming and augmenting ANCA-neutrophil interactions under in vitro and in vivo conditions, as has been shown in experiments with TNFα, GM-CSF, and interleukin-18 (3,27,28). In the present study we used 2 primers, namely TNFα and GM-CSF. In our experiments with these agents we observed similar effects of neutrophil priming in terms of the resulting ANCA-induced respiratory burst. Of note, we found that exposure of the neutrophils to heat for 60 minutes significantly abrogated this activation, independent of the primer used. We observed similar results when neutrophils were primed with the synthetic bacterial compound fMLP (results not shown). Thus, our findings indicate that this novel heat-mediated, inhibitory effect on ANCA-induced neutrophil activation occurs under various proinflammatory-cytokine–priming conditions.

Several signal transduction studies have been performed to better understand how ANCAs activate neutrophils. Thus far, no differences in the signaling pathways between PR3- and MPO-specific ANCAs have been revealed. Previously, the findings from a study by Ben-Smith et al and those from our group were able to demonstrate the importance of the PI 3-kinase/Akt pathway in controlling ANCA-induced activation (15,16). Herein we show that PI 3-kinase/Akt is also needed for ANCA-induced activation of GM-CSF–primed neutrophils. Of note, we demonstrated that short-term exposure to 40°C inhibited the PI 3-kinase/Akt activation that was induced with mAb to PR3, mAb to MPO, and human ANCA preparations.

The inhibitory effect of heat occurred in a dose-dependent manner over a range of temperatures from 37°C to 42°C. Whereas a body temperature of 42°C is rarely observed in patients with ANCA-associated conditions, moderate temperature spikes may occur. Measuring body temperature systemically provides little information regarding the local temperature that is present in areas where neutrophils are the most active. In any event, the heat values used in our experiments were clearly no lower than those that would be determined systemically.

In addition to elevated body temperatures that are associated with constitutive symptoms of generalized vasculitis, infections are common in patients with ANCA-associated vasculitis, as has been shown in the 2 largest treatment trials of patients with ANCA-associated vasculitis (22,23). Our results suggest that heat has beneficial effects by limiting ANCA-induced inflammation. However, it is not clear from our in vitro studies whether the down-regulation of inflammation during infections would also be beneficial. Conceivably, heat may impair the efficacy of the host defense during the initial neutrophilic inflammatory response, whereas it may limit the collateral damage at later stages. These issues need to be addressed in future in vivo studies.

We observed no toxic effects on the neutrophils over the range of temperatures studied, and the fact that NADPH oxidase activity in response to PMA was not affected further excludes the possibility that the effects of heat are nonspecific. Interestingly, our data suggest that short-term heat exposure has differential effects on different stimuli as well as on different signal transduction pathways in response to a given stimulus. A shorter...
duration of heat exposure did not prevent the ANCA-induced activation of p38 MAPK and ERK, whereas ANCA-induced PI 3-kinase/Akt activation was diminished. Exposure to heat resulted in blockade of the p38 MAPK and ERK activation stimulated by TNFα, but no such response was observed in cells primed with GM-CSF or stimulated with ANCA. These observations imply that the cytokine profile of a given inflammatory stimulus determines whether or not heat can down-regulate inflammatory signaling. Further investigations are needed to address these differential effects of heat.

We reported earlier that NF-κB controls neutrophil survival, and that moderate heat in vitro abrogates the NF-κB–dependent signals in neutrophils after challenge with TNFα and lipopolysaccharide (LPS) (17,18,29). We observed the same NF-κB inhibition in neutrophils isolated from mice that were exposed to 40.5°C for 30 minutes (18), suggesting that these effects also occur under in vivo conditions. NF-κB blockade by heat was found to abrogate antiapoptotic signals after 20 hours of LPS treatment, to inhibit cytokine-induced up-regulation of ANCA antigens, and to prevent the NF-κB–induced production of proinflammatory genes, such as TNFα. The results from the present study point in the same direction, suggesting that short-term heat exposure abrogates proinflammatory pathways that could transduce ANCA-induced activation signals in neutrophils. Collectively, these results support the contention that short-term heat is antiinflammatory.

It is, however, conceivable that additional heat effects need to be considered in vivo. For example, heat-accelerated apoptosis could result in proinflammatory removal of apoptotic neutrophils, particularly when these apoptotic cells become opsonized with ANCA (30–32). However, other investigators have found that, based on observations in animal and human studies, fever is beneficial in host defense reactions, and also that antipyretic treatment interferes with this effect (19–21,33).

In summary, our results show that short-term exposure of neutrophils to moderate heat modifies the signal transduction in these neutrophils as they are responding to priming cytokines as well as to ANCA. Inhibition of PI 3-kinase/Akt by heat has an important functional consequence, in that, as our results indicated, it blocks ANCA-induced respiratory burst independent of the priming cytokine. Thus, fever and increased local body temperatures may function as a part of the anti-inflammatory arsenal of the immune system.

AUTHOR CONTRIBUTIONS

Dr. Kettritz had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Von Vietinghoff, Choi, Luft, Kettritz.

Acquisition of data. Von Vietinghoff, Choi, Rolle.

Analysis and interpretation of data. Von Vietinghoff, Choi, Rolle, Kettritz.


Statistical analysis. Von Vietinghoff.
HLA–DRB4 as a Genetic Risk Factor for Churg-Strauss Syndrome

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Objective. To explore the association between HLA alleles and Churg-Strauss syndrome (CSS), and to investigate the potential influence of HLA alleles on the clinical spectrum of the disease.

Methods. Low-resolution genotyping of HLA–A, HLA–B, and HLA–DR loci and genotyping of TNFA−238A/G and TNFA−308A/G single-nucleotide polymorphisms were performed in 48 consecutive CSS patients and 350 healthy controls.

Results. The frequency of the HLA–DRB1*07 allele was higher in the CSS patients than in controls (27.1% versus 13.3%; χ² = 12.64, P = 0.0003, corrected P [Pcorr] = 0.0042, odds ratio [OR] 2.42, 95% confidence interval [95% CI] 1.47–3.99). The HLA–DRB4 gene, present in subjects carrying either HLA–DRB1*04, HLA–DRB1*07, or HLA–DRB1*09 alleles, was also far more frequent in patients than in controls (38.5% versus 20.1%; χ² = 16.46, P = 0.000058, Pcorr = 0.000232, OR 2.49, 95% CI 1.58–3.09). Conversely, the frequency of the HLA–DRB3 gene was lower in patients than in controls (35.4% versus 50.4%; χ² = 7.62, P = 0.0057, Pcorr = 0.0228, OR 0.54, 95% CI 0.35–0.84). CSS has 2 major clinical subsets, antineutrophil cytoplasmic antibody (ANCA)–positive, with features of small-vessel vasculitis, and ANCA-negative, in which organ damage is mainly mediated by tissue eosinophilic infiltration; analysis of HLA–DRB4 in patients categorized by different numbers of vasculitic manifestations (purpura, alveolar hemorrhage, mononeuritis multiplex, rapidly progressive glomerulonephritis, and constitutional symptoms) showed that its frequency strongly correlated with the number of vasculitis symptoms (P for trend = 0.001).

Conclusion. These findings indicate that HLA–DRB4 is a genetic risk factor for the development of CSS and increases the likelihood of development of vasculitic manifestations of the disease.

Churg-Strauss syndrome (CSS) is a rare vasculitic disease characterized by granulomatous and eosinophilic inflammation and systemic necrotizing vasculitis affecting small and medium-sized vessels (1,2). It usually occurs in patients with asthma and eosinophilia, and has a heterogeneous clinical spectrum that includes constitutional symptoms, sinusitis, pulmonary infiltration, peripheral neuropathy, and skin (e.g., purpura, nodules), renal (e.g., isolated urinary abnormalities, rapidly progressive glomerulonephritis [RPGN]), and gastrointestinal manifestations (3–6). Antineutrophil cytoplasmic antibodies (ANCAs) are present in ~40% of patients, usually in those developing clinical features resulting from active small-vessel vasculitis (e.g., RPGN, purpura) (7,8).

The pathogenesis of CSS has not been clearly elucidated. Eosinophils probably directly mediate organ damage, but T cells may also play a role, since serum interleukin-2 (IL-2) receptor levels are persistently high.

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in CSS patients (suggestive of T cell activation) (9), and CD4+ and CD8+ cells are abundant in CSS vasculitic lesions (10). Both Th1 and Th2 responses occur in CSS (11); the former may lead to granulomatous and vasculitic lesions as a result of interferon-γ production, whereas the latter may contribute to cosinophilia mediated by IL-4 and IL-13. T cell responses are usually due to an antigen-driven process; in CSS, as in other autoimmune diseases, several antigens potentially trigger the disease in a susceptible host (12).

HLA molecules are critically involved in antigen presentation and thymic deletion of autoreactive T cells, and this is basically why they are thought to contribute to the pathogenesis of immunologically mediated diseases (13). Additionally, some HLA alleles confer susceptibility to numerous immunopathologic conditions, such as giant cell arteritis, Behçet’s syndrome, and rheumatoid arthritis (RA) (14–16).

Several studies have explored the role of HLA molecules in ANCA-associated vasculitides. Increased frequencies of HLA–B8 (17), HLA–DR2 (18), and HLA–DQ7 (19) and a decreased frequency of HLA–DR13 (20) have been found in Wegener’s granulomatosis, and positive associations with HLA–DQ7 (19) and with the HLA–DRB1*0901;DQB1*0303 haplotype have been demonstrated in microscopic polyangiitis (21). However, subsequent studies have failed to confirm most of these associations (22). To our knowledge, the role of HLA in CSS has been investigated in only 2 studies, in which patients with CSS, Wegener’s granulomatosis, and polyarteritis nodosa were pooled. Those studies, which included 14 CSS patients (18) and 7 CSS patients (23), respectively, did not show an association between HLA alleles and CSS, except for a (non-statistically significant) lower frequency of the HLA–DRB1*03 allele in CSS patients (23); however, the lack of significant associations might be explained by the inadequacy of the sample size and the genotyping methods.

The aim of the present study was to assess whether there is an association between HLA alleles and CSS, and whether this association differs according to the clinical pattern of the disease. We examined the alleles of genes belonging to HLA class I (HLA–A and B) and class II (HLA–DR); HLA class III also contains several polymorphic genes, such as TNFA (the gene for tumor necrosis factor α). We evaluated 2 single-nucleotide polymorphisms (SNPs) located in the promoter of the TNFA gene (TNFA −238A/G and TNFA −308A/G), since they have been demonstrated to modulate tumor necrosis factor α expression and increase susceptibility to autoimmune diseases (24).

**PATIENTS AND METHODS**

**Patients and controls.** We recruited 48 consecutive patients with CSS (30 women and 18 men, with a median age of 48 years [range 18–78]). The patients had been diagnosed at internal medicine departments (nephrology, clinical immunology, rheumatology, pulmonary medicine, and others) of general hospitals in Northern Italy. CSS was diagnosed based on the presence of asthma, hypereosinophilia (>10%, or >1,500 cells/mm³), and clinical manifestations consistent with systemic vasculitis, with or without histologic confirmation (4). The absence of hypereosinophilia was not considered an exclusion criterion in patients receiving steroids for asthma if histologic evidence of vasculitis or extravascular eosinophils was available (8). In all patients, the diagnosis of CSS satisfied the American College of Rheumatology classification criteria (25) or the Chapel Hill Consensus Conference nomenclature (26). All patients underwent a physical examination, routine laboratory testing, and appropriate imaging studies. The presence of ANCA was determined at the time of diagnosis, using indirect immunofluorescence on ethanol-fixed granulocytes, and antigen-specific proteinase 3 and myeloperoxidase (MPO) enzyme-linked immunosorbent assays (ELISAs). The different immunofluorescence patterns were characterized as previously reported (8). ANCAs were first tested in each local participating center, and the results were subsequently rechecked at the laboratory of San Carlo Borromeo Hospital, a participating institution in the European Commission/Community Bureau of Reference study for ANCA assay standardization (27).

Three hundred fifty healthy subjects (176 men and 174 women, with a median age of 32 years [range 20–55]) with no history of autoimmune/inflammatory disease served as controls. All of the cases and controls were white Italians; subjects from genetic isolates were not included. The frequencies of the analyzed alleles in our control subjects were concordant with those in other Italian control populations (28).

Written informed consent was obtained from all study participants. The study protocol was approved by the Ethics Committee of the University of Parma.

**HLA genotyping.** Genomic DNA was extracted from EDTA (5 ml)-treated peripheral blood samples using the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA, CA) and stored at −20°C until use. Low-resolution genotyping for HLA–A, HLA–B, and HLA–DR loci was performed by polymerase chain reaction (PCR) using sequence-specific primers (One Lambda, Canoga Park, CA); 21 alleles in HLA–A genes, 35 in HLA–B genes, and 13 in HLA–DR genes were investigated. High-resolution genotyping for the HLA–DRB4 and HLA–DRB3 alleles was carried out, using the dyeoxy chain termination method, on an automated sequencer (CEQ 2000XL DNA Analysis System; Beckman Coulter, Fullerton, CA). Sequence data were aligned using Seqman, version II (DNAStar, Madison, WI). The primer sequences have been described elsewhere (29).

TNFA −238A/G and TNFA −308A/G SNPs were genotyped using sequence-specific primers and a Cyclerplate Cytokines 2 System, according to the protocol recommended...
by the manufacturer (Protrans, Ketsch, Germany). The PCR products were resolved on 2.5% (weight/volume) agarose gels stained with ethidium bromide. Quality measures were adopted as recommended by consensus conferences of the Centers for Disease Control and Prevention and the National Institutes of Health (30,31). Each patient and healthy subject was assigned a code. The samples were handled by 2 investigators, who were unaware of the subjects’ clinical status. The degree of reproducibility between quality control replicates was 100%. The laboratory in which the HLA genotyping was performed is certified by the European Foundation for Immunogenetics.

**Statistical analysis.** Differences in allele frequencies between CSS patients and controls were analyzed by Pearson’s chi-square test. The direction and strength of these differences were assessed by calculating odds ratios (ORs). Two-sided P values less than 0.05 were considered significant. Nominal P values for allele associations were corrected by the Bonferroni method to allow for multiple testing. The power of these tests was estimated using the Power for Association With Errors program (32), with the test allele as the first allele and the other alleles as the second allele, under the assumption of an error rate of 1%. The patient and control groups were in Hardy-Weinberg equilibrium for all alleles analyzed.

Because CSS has different disease patterns, we used 2 approaches to explore the clinical manifestations associated with the HLA–DRB4 gene. First, we examined whether the prevalence of each clinical manifestation differed between HLA–DRB4–positive and HLA–DRB4–negative patients, using Fisher’s exact test and the Mann–Whitney U test. Second, in order to achieve a more powerful analysis, we performed trend tests of the proportion of DRB4-positive patients across different categories defined by increasing numbers of symptoms characterizing the “vasculitic subset” of CSS (33), i.e., purpura, alveolar hemorrhage, mononeuritis multiplex, RPGN, and constitutional symptoms (7,8,33). The chi-square test for trend in proportions was used for this analysis.

**RESULTS**

**Clinical characteristics and laboratory findings.**

Clinical and laboratory characteristics of the CSS patients are shown in Table 1. All of the patients had bronchial asthma. Peripheral neuropathy was another major clinical manifestation, affecting 32 patients (67%), 17 of whom had mononeuritis multiplex. Constitutional symptoms (e.g., fatigue, fever, anorexia, weight loss, diffuse myalgias, and arthralgias) were found in 30 patients (63%). Among the otolaryngologic manifestations, sinusitis occurred in 27 patients (56%), but other features were also found, such as nasal polyps (23 cases [48%]) and sensorineural hearing loss (8 cases [17%]). Fifty percent of the patients exhibited lung involvement: pulmonary infiltrates were common (13 cases [27%]), but some patients also presented with nodules (6 cases [13%]), pleural effusions (4 cases [8%]), and alveolar hemorrhage (4 cases [8%]). Skin manifestations were found in 21 patients (44%), with purpura (6 cases [13%]), maculopapular rash (5 cases [10%]), and nodules (4 cases [8%]) being the most frequent lesions.

Twenty-two patients (46%) exhibited renal involvement: isolated urinary abnormalities (i.e., microscopic hematuria and proteinuria) were found in 17 cases (36%); 5 patients (10%) had renal insufficiency (serum creatinine >1.4 mg/dl), 3 of whom had RPGN. Gastrointestinal manifestations (e.g., abdominal pain, gastrointestinal bleeding), cardiac manifestations (e.g., pericarditis, acute coronary syndrome), and central nervous system manifestations (e.g., stroke, meningitis) occurred in 13 patients (27%), 7 patients (15%), and 3 patients (6%), respectively.

Eosinophilia was found in all but 5 patients (who were receiving oral corticosteroids for asthma). ANCA-positive perinuclear pattern was positive by immunofluorescence in 21 patients (44%), negative in 23 (48%), and undetermined in 4 (8%); this rate of ANCA positivity was similar to that reported by other investigators (4,7,8). Sixteen of the 21 ANCA-positive patients (76%) had a perinuclear ANCA pattern; ELISA findings were specific for MPO in 14 of these cases but negative in the remaining 2. A cytoplasmic ANCA (cANCA) pattern was found in 2 of 21 patients (10%); 1 was negative by ELISA whereas the other, whose immunofluorescence pattern was “cANCA atypical” (8), tested positive for MPO. Finally, 3 of 21 cases (14%) had an atypical immunofluorescence pattern (cytoplasmic plus perinuclear), and all of them were positive for MPO.

In accordance with previous findings (7,8), the

| Table 1. Demographic, clinical, and laboratory characteristics of the 48 patients with CSS* |
|---------------------------------|----------------|
| No. male/no. female             | 18/30          |
| Age, median (range) years       | 48 (18–78)     |
| Asthma                          | 48 (100)       |
| Peripheral neuropathy           | 32 (66.7)      |
| Constitutional symptoms†       | 30 (62.5)      |
| Sinusitis                       | 27 (56.2)      |
| Lung involvement                | 24 (50.0)      |
| Renal involvement               | 22 (45.8)      |
| Skin involvement                | 21 (43.7)      |
| Gastrointestinal involvement    | 13 (27.1)      |
| Cardiac involvement             | 7 (14.6)       |
| CNS involvement                 | 3 (6.2)        |
| Eosinophilia, median (range) cells/mm³ | 2,913 (63–28,815) |
| ANCA positivity‡                | 21 (47.7)      |

* Except where indicated otherwise, values are the number (%) of patients. CSS = Churg-Strauss syndrome; CNS = central nervous system; ANCA = antineutrophil cytoplasmic antibody.
† Fatigue, fever, anorexia, weight loss, diffuse myalgias, and arthralgias.
‡ Data available on 44 patients.
ANCA-positive patients showed a higher frequency of manifestations of small-vessel vasculitis, such as alveolar hemorrhage and purpura, whereas the ANCA-negative patients more frequently exhibited cardiac, gastrointestinal, or lung involvement (other than alveolar hemorrhage) (data not shown). One or more tissue biopsies were performed in 29 patients (60.4%), and in each case, findings in at least 1 biopsy sample were suggestive of CSS.

**HLA findings.** No statistically significant differences were found in the frequencies of HLA–A and HLA–B alleles between CSS patients and controls (data not shown). Table 2 illustrates the frequencies of the genes or alleles that showed a significant (positive or negative) association with CSS, as well as the frequencies of other alleles of the HLA–DRB4 haplotype.

The HLA–DRB1*07 allele was present in 24 of 48 CSS patients (50.0%) and in 87 of 350 controls (24.9%), and its allele frequency in the 2 groups, respectively, was 26 of 96 (27.1%) and 93 of 700 (13.3%) \((P = 0.0003, \text{OR corrected for multiple comparisons } [P_{corr} = 0.0042, \text{OR} 2.42, \text{uncorrected 95\% confidence interval [95\% CI 1.47–3.99]}])\). We also examined the allele frequencies of HLA–DRB1*04 and HLA–DRB1*09 (which, like DRB1*07, belong to the HLA–DRB4 haplotype), but they were not found to differ significantly between CSS patients and controls \((P = 0.13 \text{ and } P = 0.58, \text{respectively})\).

The HLA–DRB4 gene, which encodes the super-typic HLA–DR53 antigen, is in strong linkage disequilibrium with the HLA–DRB1*04, *07, and *09 alleles; therefore, we calculated the HLA–DRB4 gene frequency by summing the frequencies of the 3 alleles and then confirmed these findings using high-resolution genotyping by HLA–DRB4 gene sequencing. The DRB4 gene was present in 31 of 48 patients (64.6%) and in 124 of 350 controls (35.4%), whereas its allelic frequency was 37 of 96 (38.5%) and 141 of 700 (20.1%) in patients and controls, respectively \((P = 0.000058, P_{corr} = 0.000232, \text{OR} 2.49, \text{uncorrected 95\% CI 1.58–3.09})\). The power value for allelic tests for the HLA–DRB4 gene was 98.2%.

High-resolution genotyping by sequencing was also performed to investigate whether a particular HLA–DRB4 allele was associated with CSS. Only 2 alleles (HLA–DRB4*0101 and HLA–DRB4*0103) were present with similar frequencies \((P = 0.62)\) in the CSS patients and controls.

Unlike the HLA–DRB1*07 allele and the HLA–DRB4 gene, the HLA–DRB1*13 allele was less frequent in the CSS patients (allelic frequency 3 of 96 [3.1%]) than in controls (87 of 700 [12.4%]) \((P = 0.0069)\), although the significance was lost after correction for multiple comparisons \((P_{corr} = 0.0966)\). In addition, the frequency of the HLA–DRB1*03 allele was lower in the CSS patients (4 of 96 [4.2%]) than in controls (64 of 700 [9.1%]), but, in accordance with previous findings (23), this difference did not reach statistical significance \((P = 0.10)\).

Because the HLA–DRB3 gene, which encodes the HLA–DR52 antigen, is in strong linkage disequilibrium with the HLA–DRB1*03, *11, *12, *13, and *14 alleles, the frequencies of these alleles were summed to estimate HLA–DRB3 gene frequency. The HLA–DRB3 gene was significantly less frequent in the CSS patients (27 of 48 [56.2%]) than in controls (258 of 350 [73.7%]); its allelic frequency was 34 of 96 (35.4%) in the patients and 353 of 700 (50.4%) in the controls \((P = 0.0057, P_{corr} = 0.0228, \text{OR} 0.54, \text{uncorrected 95\% CI 0.35–0.84})\).

High-resolution genotyping with HLA–DRB3 gene sequencing confirmed these results. The power value for allelic tests for the HLA–DRB3 gene was 78%.

The frequencies of HLA–DRB1*07, HLA–DRB4, and HLA–DRB3 in the subgroups of patients with and those without biopsy confirmation of the diagnosis of CSS were not significantly different (data not shown). Finally, we found no difference in TNFA–238A and TNFA–308A allele frequencies between the CSS patients and controls \((P = 0.48 \text{ and } P = 0.13, \text{respectively})\).

### Table 2. Main HLA findings in the 48 CSS patients and 350 healthy controls*

<table>
<thead>
<tr>
<th>Allele/gene</th>
<th>Patients</th>
<th>Controls</th>
<th>(\chi^2)</th>
<th>(P)</th>
<th>(P_{corr})</th>
<th>OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>DRB1*04</td>
<td>10/96 (10.4)</td>
<td>44/700 (6.3)</td>
<td>2.278</td>
<td>0.1312</td>
<td>–</td>
<td>1.74</td>
<td>0.84–3.57</td>
</tr>
<tr>
<td>DRB1*07</td>
<td>26/96 (27.1)</td>
<td>93/700 (13.3)</td>
<td>12.64</td>
<td>0.0003</td>
<td>0.0042</td>
<td>2.42</td>
<td>1.47–3.99</td>
</tr>
<tr>
<td>DRB1*09</td>
<td>1/96 (1.0)</td>
<td>4/700 (0.6)</td>
<td>0.299</td>
<td>0.5844</td>
<td>–</td>
<td>3.78</td>
<td>0.20–16.56</td>
</tr>
<tr>
<td>DRB1*13</td>
<td>3/96 (3.1)</td>
<td>87/700 (12.4)</td>
<td>7.287</td>
<td>0.0069</td>
<td>0.0966</td>
<td>0.23</td>
<td>0.07–0.73</td>
</tr>
<tr>
<td>DRB4</td>
<td>37/96 (38.5)</td>
<td>141/700 (20.1)</td>
<td>16.461</td>
<td>0.000058</td>
<td>0.000232</td>
<td>2.49</td>
<td>1.58–3.09</td>
</tr>
<tr>
<td>DRB3</td>
<td>34/96 (35.4)</td>
<td>353/700 (50.4)</td>
<td>7.616</td>
<td>0.0057</td>
<td>0.0228</td>
<td>0.54</td>
<td>0.35–0.84</td>
</tr>
</tbody>
</table>

* Values are the number positive/number of subjects (%). CSS = Churg-Strauss syndrome; \(P_{corr}\) = corrected \(P\) (after Bonferroni adjustment for multiple testing); OR = odds ratio; 95% CI = 95% confidence interval.
HLA–DRB4 and vasculitic manifestations of CSS. Comparison of the main clinical and laboratory findings in the HLA–DRB4–positive versus the HLA–DRB4–negative CSS patients showed that the former group had significantly more frequent constitutional symptoms (Table 3). In addition, they exhibited a trend, though not statistically significant, toward a higher prevalence of “vasculitis symptoms” that identify the ANCA-positive subset of CSS, i.e., purpura, alveolar hemorrhage, and mononeuritis multiplex (7,8,33).

This phenomenon could be better distinguished after the patients were divided into categories defined on the basis of the number of vasculitis symptoms and the proportion of HLA–DRB4–positive patients was computed for each of these categories (Figure 1). This analysis showed that the higher the number of vasculitis symptoms, the greater the probability that the CSS patients carried the HLA–DRB4 gene. In fact, HLA–DRB4 was found in 3 of 10 patients with no vasculitic manifestations (30.0%), 13 of 22 patients with 1 vasculitic manifestation (59.1%), 10 of 11 patients with 2 vasculitic manifestations (90.9%), and 5 of 5 patients with ≥3 vasculitic manifestations (100%) (P for trend = 0.001) (Figure 1A). When ANCA positivity was included among the vasculitic manifestations, we found that HLA–DRB4 was positive in 2 of 8 patients with no vasculitic manifestations (25.0%), 10 of 17 with 1 vasculitic manifestation (58.8%), 7 of 11 with 2 vasculitic manifestations (63.6%), and 12 of 12 with ≥3 vasculitic manifestations (100%) (P for trend = 0.0015) (Figure 1B).

**DISCUSSION**

The results of this study provide evidence that there is an association between HLA genotype and CSS. In comparison with healthy controls, the frequencies of

---

**Table 3.** Comparison of the main clinical and laboratory characteristics of the HLA–DRB4–positive and HLA–DRB4–negative CSS patients*

<table>
<thead>
<tr>
<th></th>
<th>DRB4-positive (n = 31)</th>
<th>DRB4-negative (n = 17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mononeuritis multiplex</td>
<td>13 (41.9)</td>
<td>4 (23.5)</td>
</tr>
<tr>
<td>Constitutional symptoms†</td>
<td>24 (77.4)‡</td>
<td>6 (35.3)</td>
</tr>
<tr>
<td>Sinusitis</td>
<td>20 (64.5)</td>
<td>7 (41.2)</td>
</tr>
<tr>
<td>Lung involvement (all kinds)</td>
<td>14 (45.2)</td>
<td>10 (58.8)</td>
</tr>
<tr>
<td>Alveolar hemorrhage</td>
<td>4 (12.9)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Kidney involvement (all kinds)</td>
<td>16 (51.6)</td>
<td>6 (35.3)</td>
</tr>
<tr>
<td>RPGN</td>
<td>2 (6.5)</td>
<td>1 (5.9)</td>
</tr>
<tr>
<td>Purpura</td>
<td>6 (19.4)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Gastrointestinal involvement</td>
<td>6 (19.4)</td>
<td>7 (41.2)</td>
</tr>
<tr>
<td>Cardiac involvement</td>
<td>5 (16.1)</td>
<td>2 (11.8)</td>
</tr>
<tr>
<td>Eosinophilia, median (range) cells/mm³</td>
<td>3,168 (291–28,815)</td>
<td>2,399 (63–22,903)</td>
</tr>
<tr>
<td>ANCA positivity§</td>
<td>17 (58.6)</td>
<td>4 (26.7)</td>
</tr>
</tbody>
</table>

* Except where indicated otherwise, values are the number (%) of patients. CSS = Churg-Strauss syndrome; RPGN = rapidly progressive glomerulonephritis; ANCA = antineutrophil cytoplasmic antibody.

† Fatigue, fever, anorexia, weight loss, diffuse myalgias, and arthralgias.

‡ P = 0.006 versus DRB4-negative patients, by Fisher’s exact test.

§ Data available on 44 patients.
the HLA–DRB4 gene and the HLA–DRB1*07 allele were significantly higher in our CSS patients, and the frequency of the HLA–DRB3 gene was lower. Furthermore, the association between HLA–DRB4 and CSS was stronger in patients with full-blown vasculitic manifestations.

However, the study has some limitations. As in the case of all association studies, the findings do not necessarily indicate causation, but might also be explained by the presence of another susceptibility locus within or near the HLA region, or by an artifact caused by population admixture. They should therefore be confirmed by replication studies in other populations. Additionally, our study of the HLA class III alleles assessed only 2 SNPs of the TNFA gene, but a number of other candidate genes located in this region warrant analysis. Finally, some associations might have been missed, as a result of use of the Bonferroni multiple comparison adjustment procedure, which has the disadvantage of being very conservative.

Nevertheless, the apparent close association between HLA and CSS has potential implications with regard to pathogenesis. HLA molecules are critical in the dialogue between T cells and antigen-presenting cells, since the former recognize antigenic epitopes only when they are displayed by antigen-presenting cells in association with HLA molecules (14). The finding that CSS patients have a restricted HLA repertoire supports the hypothesis that only selected antigenic determinants may be involved in CSS, and simultaneously highlights the pathogenetic role of T cells. In accordance with this view, clonal T cell expansion has been demonstrated in CSS patients, and, because the T cell clones had similarly specific T cell receptors, they could recognize only a limited number of antigens (34). This finding, together with the present results, supports the notion that CSS is an antigen-driven disease.

The HLA–DRB4 gene encodes the supertypical HLA–DR53 antigen and exists only on haplotypes possessing the HLA–DRB1*04, DRB1*07, and DRB1*09 alleles. A number of diseases are linked to these specificities, some of which also have clinical and pathogenetic features relevant to CSS. Among autoimmune conditions, RA is associated with HLA–DRB1*04 (35), and HLA–DRB7 has been found to correlate with the occurrence of antcardiolipin antibodies in systemic lupus erythematosus (36). Some vasculitides are also associated with such HLA alleles: giant cell arteritis is significantly associated with HLA–DRB1*04 (14). HLA–DRB1*07 and (to a lesser extent) HLA–DRB1*07 have been found to be associated with atopy (37,38), but these results were not corroborated in other investigations (39). Finally, an HLA profile strikingly similar to that observed in CSS (i.e., increased HLA–DRB4 and reduced HLA–DRB3 frequency) has been found in childhood acute lymphoblastic leukemia (40).

The observation that CSS and lymphoproliferative diseases may have a common immunogenetic background suggests that lymphocyte clones may play a role in CSS. Abnormal T cell clones producing high levels of IL-5 are involved in the pathogenesis of idiopathic hypereosinophilic syndrome (HES) (41), a disease whose clinical manifestations often overlap those of CSS since the latter also has hallmark features of idiopathic eosinophilia and cosmetic tissue infiltration (33).

In some conditions, not only does HLA play a role in disease susceptibility, but it also influences the spectrum of clinical characteristics. For instance, the HLA–DRB1*0401 allele is closely associated with extrarticular manifestations in RA (42), and HLA–C3 is associated with rheumatoid vasculitis (16).

CSS has traditionally been regarded as a single disease entity, but its clinical manifestations are heterogeneous. Recently, 2 independent studies showed that ANCA positivity in CSS patients was strongly associated with renal involvement and, in particular, RPGN; furthermore, ANCs were more frequent in patients with alveolar hemorrhage, mononeuritis multiplex, and purpura, which are considered to be features of small-vessel vasculitis (7,8). In addition, constitutional symptoms suggesting systemic rather than localized vasculitic disease were far more frequent in the ANCA-positive group (8), whereas the ANCA-negative group was characterized by a higher prevalence of cardiac and lung involvement (other than alveolar hemorrhage), which are more often due to eosinophilic tissue infiltration and subsequent fibrotic organ damage (7,8,33). On the basis of these findings, it has been postulated that there are 2 distinct subsets of CSS: one that is ANCA positive and has the features of systemic necrotizing vasculitis, and one that is ANCA negative and more related to eosinophilic tissue infiltration, and may also follow pathways comparable with those underlying other eosinophilic disorders such as HES (33).

After identifying the association between HLA–DRB4 and CSS, we compared the main clinical and laboratory findings in HLA–DRB4–positive and HLA–DRB4–negative patients and observed that the former not only had constitutional symptoms significantly more frequently, but also more frequently exhibited vasculitic features, including purpura, alveolar hemorrhage, mononeuritis multiplex, and ANCs. These latter dif-
ferences were not statistically significant, possibly because of the sample size and, in some cases, the relative infrequency of the clinical features (e.g., alveolar hemorrhage), but they prompted us to evaluate the prevalence of HLA–DRB4 across groups of patients with different degrees of vasculitic manifestations. There was a statistically significant trend in the association between the “vasculitic phenotype” and HLA–DRB4: in particular, all of the patients with 3 or more vasculitis symptoms were positive for HLA–DRB4.

Our data therefore not only reinforce the view that CSS has 2 separate forms, but also provide preliminary evidence that immunogenetic factors play a role in determining this clinical dichotomy. These findings require confirmation in larger populations of CSS patients, and mechanistic studies to clarify the underlying pathophysiologic mechanisms are warranted.

In conclusion, the results of the present study show that HLA–DRB4 can be a genetic risk factor for the development of CSS. Furthermore, they indicate that DRB4 positivity may be associated with a disease subset that is characterized by features of small-vessel vasculitis.

AUTHOR CONTRIBUTIONS

Dr. Vaglio had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Vaglio, Martorana, Neri.

Acquisition of data. Martorana, Grasselli, Zanetti, Pesci, Garini, Manganelli, Bottero, Tumati, Sinico, Savi, Buzio.

Analysis and interpretation of data. Vaglio, Martorana, Maggiore, Savi, Buzio, Neri.


Statistical analysis. Martorana, Maggiore, Neri.

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REFERENCES


B cell infiltration in systemic sclerosis–associated interstitial lung disease

Rituximab has shown efficacy for the treatment of rheumatoid arthritis (RA) and is currently under study in systemic lupus erythematosus and a variety of other rheumatic diseases (1–3). This monoclonal antibody to CD20 depletes peripheral B cells, but the mechanism of its therapeutic effect is uncertain (4,5). Findings of several studies have suggested the potential importance of B cells in systemic sclerosis (SSc). Levels of circulating naive B cells are increased in SSc patients, while memory B cells, although reduced in number, show markers of activation (6). Both B cell populations exhibit increased expression of CD19, an important regulator of B cell maturation (6). Findings in studies of mice have complemented these observations (7), suggesting that B cells might play an important role in fibrotic disease. In addition, microarray results demonstrating a prominent immunoglobulin signature in the skin of patients with SSc (8) and the results of studies of rituximab treatment in other rheumatic diseases have led to increased interest in these cells. The presence of infiltrating B cells in the skin of patients with SSc (8) provided additional motivation for our current, ongoing open-label study of rituximab for SSc skin disease.

Interstitial lung disease (ILD) is a grave complication of SSc, leading to significant morbidity and mortality (9). Treatment options are extremely limited, with cyclophosphamide as the only therapy that has shown some, albeit modest, efficacy (10). ILD occasionally complicates RA, and recent histopathologic studies of affected lung tissue from RA patients have revealed variable degrees of B cell infiltration (11). To better understand the potential importance of B cells in SSc-associated pulmonary disease, we studied B cell infiltration in stored tissue specimens from patients with SSc-associated ILD.

Pulmonary tissue samples from 11 patients with SSc-associated ILD (4 with nonspecific interstitial pneumonitis [NSIP], 7 with usual interstitial pneumonitis [UIP]) were stained for CD20 (a marker of mature B cells and the target of the monoclonal antibody rituximab), CD3 (a marker of T cells), and CD68 (a marker of macrophages). B cell infiltration was a prominent finding in many of the specimens. B cells were frequently found arranged in lymphoid aggregates (Figures 1a, h, and i), but were also seen in a more diffuse pattern (Figure 1d). T cells were also found, in both lymphocyte aggregates and more diffuse lymphocyte infiltrates (Figures 1b and e). Most lymphocyte aggregates lacked macrophages (results not shown), but macrophages were commonly seen in intraalveolar and interstitial spaces (Figures 1c and f).

B cell infiltration in the specimens was assessed by counting the number of B cells/high-power field (400× total magnification), with the observer blinded with regard to the biopsy classification. Biopsy samples were not counterstained, to lessen the likelihood of the reader being influenced by the histopathologic diagnosis. Examples of staining are shown in Figures 1h and i. Specimens from patients with each of the two primary pulmonary pathologic subtypes (UIP and NSIP) showed variable, but some striking, degrees of B cell staining.
(Figures 1h and i). Staining tended to be more intense in specimens from patients with UIP, but the degree of staining did not correlate statistically with histopathologic subtype in this relatively small sample size.

The presence of B cells in the lungs of patients with SSc-associated ILD suggests that these cells might contribute to disease pathogenesis and provides additional rationale for clinical investigation into B cell depletion as a therapeutic strategy for SSc-associated ILD. B cell infiltration in idiopathic pulmonary fibrosis has also been reported recently (12), reinforcing the notion that these diseases might share common pathogenetic mechanisms. The considerable variability of B cell infiltration indicates that if B cells localized in pulmonary tissue play a key role in pathogenesis, they may be important in only a subset of patients. Alternatively, they might disappear from tissue during inactive stages of disease. Possible mechanisms of B cell effector function in localized tissue sites have been postulated to include antigen-presenting cell function and cytokine secretion (4,5). Our ongoing open-label trials of rituximab in SSC dermal fibrosis (RL) and rheumatoid lung disease (EM) should provide further data to guide development of this agent for the treatment of SSc-associated ILD.

AUTHOR CONTRIBUTIONS

Dr. Lafyatis had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Lafyatis.

Acquisition of data. Lafyatis, O’Hara, Matteson.

Analysis and interpretation of data. Lafyatis, O’Hara.

Manuscript preparation. Lafyatis, Feghali-Bostwick, Matteson.

Statistical analysis. Lafyatis.

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Meta-analysis evidence of a differential risk of the FCRL3 –169T→C polymorphism in white and East Asian rheumatoid arthritis patients

Association between a functional promoter polymorphism (rs7528684) in the Fc receptor–like gene, FCRL3, and rheumatoid arthritis (RA) has been observed in 3 independent Japanese case–control sample sets (1,2). Studies examining the role of this polymorphism in risk of RA in 9 independent white sample sets, however, have yielded conflicting results (3–8). Further, a large study of Korean subjects failed to demonstrate association of this single-nucleotide polymorphism (SNP) with RA (9). Although the precise function of FCRL3, which has strong structural homology with the classic Fcγ receptors, is unknown, the existing data are consistent with the hypothesis that it may influence the fate of B cells and augment the emergence of self-reactive cells in the germinal center (for review, see ref. 1), making it an excellent candidate gene for autoimmune disease. Consequently, we undertook a meta-analysis of the data from these 13 study samples, analyzing East Asians (3,172 cases and 2,916 controls) and whites (5,645 cases and 5,592 controls) separately. Our findings indicate that, although the data support the notion that rs7528684 has a role in risk of RA among East Asians, there is no reliable evidence of association of this SNP with RA in whites of European descent.

The polymorphism –169T→C in FCRL3, which has been shown to alter the binding affinity of NF-κB and to regulate FCRL3 expression, was first demonstrated to be associated with RA in 2 independent Japanese case–control studies (1); sample 1 included 824 cases and 649 controls (allelic odds ratio [OR] 1.37, Pexact = 3.85 × 10−5) and sample 2 included 540 cases and 636 controls (allelic OR 1.19, Pexact = 0.041) (Table 1). (To permit comparisons across all of the individual sample sets, allelic and genotypic P values were
Table 1. Allele and genotype frequencies of the Fc receptor–like 3 (FCRL3) promoter polymorphism, rs7528684, in rheumatoid arthritis cases and controls

<table>
<thead>
<tr>
<th>Author, year (ref.)</th>
<th>No. of subjects</th>
<th>Genotype†</th>
<th>Minor allele frequency</th>
<th>Allele analysis (C vs. T)</th>
<th>Genotype analysis (CC vs. CT + TT)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TT</td>
<td>TC</td>
<td>CC</td>
<td>OR (95% CI)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>East Asian subjects</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kochi et al, 2005 (1) (set 1, Japan)</td>
<td>Cases (n = 824)</td>
<td>291</td>
<td>374</td>
<td>159</td>
<td>0.420</td>
</tr>
<tr>
<td></td>
<td>Controls (n = 649)</td>
<td>266</td>
<td>318</td>
<td>65</td>
<td>0.345</td>
</tr>
<tr>
<td>Kochi et al, 2005 (1) (set 2, Japan)</td>
<td>Cases (n = 540)</td>
<td>182</td>
<td>281</td>
<td>77</td>
<td>0.403</td>
</tr>
<tr>
<td></td>
<td>Controls (n = 636)</td>
<td>251</td>
<td>310</td>
<td>75</td>
<td>0.362</td>
</tr>
<tr>
<td>Ikari et al, 2006 (2) (Japan)</td>
<td>Cases (n = 748)</td>
<td>238</td>
<td>377</td>
<td>133</td>
<td>0.430</td>
</tr>
<tr>
<td></td>
<td>Controls (n = 934)</td>
<td>333</td>
<td>472</td>
<td>129</td>
<td>0.391</td>
</tr>
<tr>
<td>Choi et al, 2006 (9) (Korea)</td>
<td>Cases (n = 1,060)</td>
<td>343</td>
<td>521</td>
<td>196</td>
<td>0.431</td>
</tr>
<tr>
<td></td>
<td>Controls (n = 697)</td>
<td>255</td>
<td>317</td>
<td>125</td>
<td>0.362</td>
</tr>
<tr>
<td><strong>Meta-analysis of all studies of East Asians</strong></td>
<td>Cases (n = 3,172)</td>
<td>1,054</td>
<td>1,553</td>
<td>565</td>
<td>0.423</td>
</tr>
<tr>
<td></td>
<td>Controls (n = 2,916)</td>
<td>1,105</td>
<td>1,417</td>
<td>394</td>
<td>0.378</td>
</tr>
<tr>
<td><strong>White subjects</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hu et al, 2006 (3) (set 1, North America)</td>
<td>Cases (n = 467)</td>
<td>149</td>
<td>231</td>
<td>87</td>
<td>0.434</td>
</tr>
<tr>
<td></td>
<td>Controls (n = 473)</td>
<td>148</td>
<td>226</td>
<td>99</td>
<td>0.448</td>
</tr>
<tr>
<td>Hu et al, 2006 (3) (set 2, North America)</td>
<td>Cases (n = 565)</td>
<td>164</td>
<td>286</td>
<td>115</td>
<td>0.457</td>
</tr>
<tr>
<td></td>
<td>Controls (n = 743)</td>
<td>218</td>
<td>354</td>
<td>171</td>
<td>0.468</td>
</tr>
<tr>
<td>Eyre et al, 2006 (4) (UK)</td>
<td>Cases (n = 1,065)</td>
<td>324</td>
<td>524</td>
<td>217</td>
<td>0.450</td>
</tr>
<tr>
<td></td>
<td>Controls (n = 2,073)</td>
<td>595</td>
<td>1,055</td>
<td>423</td>
<td>0.459</td>
</tr>
<tr>
<td>Owen et al, 2007 (5) (New Zealand)</td>
<td>Cases (n = 761)</td>
<td>207</td>
<td>386</td>
<td>168</td>
<td>0.474</td>
</tr>
<tr>
<td></td>
<td>Controls (n = 553)</td>
<td>153</td>
<td>275</td>
<td>125</td>
<td>0.475</td>
</tr>
<tr>
<td>Martinez et al, 2006 (6) (Madrid, Spain)</td>
<td>Cases (n = 448)</td>
<td>117</td>
<td>229</td>
<td>102</td>
<td>0.483</td>
</tr>
<tr>
<td></td>
<td>Controls (n = 229)</td>
<td>75</td>
<td>113</td>
<td>41</td>
<td>0.426</td>
</tr>
<tr>
<td>Martinez et al, 2006 (6) (Granada, Spain)</td>
<td>Cases (n = 221)</td>
<td>61</td>
<td>122</td>
<td>38</td>
<td>0.448</td>
</tr>
<tr>
<td></td>
<td>Controls (n = 489)</td>
<td>153</td>
<td>233</td>
<td>103</td>
<td>0.449</td>
</tr>
<tr>
<td>Newman et al, 2006 (7) (Toronto, Canada)</td>
<td>Cases (n = 855)</td>
<td>263</td>
<td>401</td>
<td>191</td>
<td>0.458</td>
</tr>
<tr>
<td></td>
<td>Controls (n = 303)</td>
<td>111</td>
<td>141</td>
<td>51</td>
<td>0.401</td>
</tr>
<tr>
<td>Newman et al, 2006 (7) (Halifax, Canada)</td>
<td>Cases (n = 332)</td>
<td>96</td>
<td>162</td>
<td>74</td>
<td>0.467</td>
</tr>
<tr>
<td></td>
<td>Controls (n = 159)</td>
<td>52</td>
<td>72</td>
<td>35</td>
<td>0.447</td>
</tr>
<tr>
<td>Thabet et al, 2007 (8) (The Netherlands)</td>
<td>Cases (n = 931)</td>
<td>288</td>
<td>428</td>
<td>215</td>
<td>0.461</td>
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<tr>
<td></td>
<td>Controls (n = 570)</td>
<td>177</td>
<td>287</td>
<td>106</td>
<td>0.438</td>
</tr>
<tr>
<td>Meta-analysis of all studies of whites</td>
<td>Cases (n = 5,645)</td>
<td>1,669</td>
<td>2,769</td>
<td>1,207</td>
<td>0.459</td>
</tr>
<tr>
<td></td>
<td>Controls (n = 5,592)</td>
<td>1,682</td>
<td>2,756</td>
<td>1,154</td>
<td>0.453</td>
</tr>
</tbody>
</table>

* Odds ratios (ORs), 95% confidence intervals (95% CIs), and P values were recalculated for each individual sample set using the published genotype counts. Because these P values were calculated using Fisher’s exact test, they may differ from those cited in the original publications. For the meta-analyses, allelic and genotypic Mantel-Haenszel common ORs were calculated separately for the studies of East Asian subjects and the studies of white subjects. Genotypic common ORs were calculated under a recessive model; 95% CIs were obtained from Monte Carlo simulations with 30,000 replicates. Combined P values were calculated by summing William’s corrected G-statistics from each study and testing the sum, which was approximately chi-squared distributed, against the null chi-squared distribution.

† Values are the number of individuals with each genotype.
recalculated for each study using Fisher’s exact test [referred to as $P_{\text{exact}}$ throughout] A genotypic analysis of the first sample set suggested that the predisposing effect was most significant in individuals harboring 2 copies of the minor allele, $–169\text{C}$ (recessive model [CC versus CT + TT] OR 2.15, $P_{\text{exact}} = 6.06 \times 10^{-7}$); however, statistical analysis did not reveal a “recessive-like” mode of inheritance in the second sample set (OR 1.24, $P_{\text{exact}} = 0.223$) (1). Data from subphenotype analyses in sample set 1 also suggested that the $–169\text{C}$ risk allele was positively correlated with rheumatoid factor (RF) titer, the presence of anti–cyclic citrullinated peptide (anti-CCP), and the number of copies of the HLA–DRB1–encoded shared epitope (SE); this information was not presented for the second sample set.

Ikari and colleagues replicated this association in a third independent Japanese sample set of 748 unrelated cases and 954 controls and, although the effect size was smaller (allelic OR 1.18, $P_{\text{exact}} = 0.028$), they confirmed the observation that individuals carrying 2 copies of the risk allele were at increased risk for RA relative to the other 2 genotypes combined (OR 1.35, $P_{\text{exact}} = 0.030$) (2). However, they failed to confirm the positive correlation between serum RF levels and FCRL3 genotype (information on anti-CCP and SE status was not reported). Of interest, in a recent large Korean case–control study with 1,060 patients and 697 controls, the FCRL3 polymorphism was not significantly associated with RA (allelic OR 1.10, $P_{\text{exact}} = 0.163$; recessive model OR 1.04, $P_{\text{exact}} = 0.801$), even after stratification by RF and SE status (9).

Results of attempts to replicate association of this SNP with RA in white patients, all fulfilling the American College of Rheumatology (ACR; formerly, the American Rheumatism Association) criteria for the disease (10), have been inconsistent. Studies of 2 independent North American RA sample sets (3), a large UK sample set (4), and a sample set from New Zealand (5) showed no evidence of association of the SNP with RA (Table 1). A similar finding was obtained in a study of whites of Spanish origin, although, when the authors combined their 2 sample sets and stratified patients and controls by genotype at a putative NFKB1 promoter polymorphism, $–94\text{ins}/\text{del}\text{ATTG}$, the distribution of FCRL3 $–169\text{T} \rightarrow \text{C}$ genotypes was significantly different between patients and controls who were heterozygous for the NFKB1 polymorphism ($P = 0.001$) (6).

A Canadian study showed a statistically significant, albeit modest, association of the $–169\text{T} \rightarrow \text{C}$ SNP with RA in a combined analysis of 2 sample sets, one from Toronto and the other from Halifax (allelic OR 1.19, $P_{\text{exact}} = 0.024$; CC versus TT OR 1.41, $P_{\text{exact}} = 0.032$), although the findings regarding a recessive mode of inheritance were not significant (OR 1.26, $P_{\text{exact}} = 0.107$) (7). Analysis of these 2 sample sets independently showed statistically significant association in the Toronto data set (allelic OR 1.26, $P_{\text{exact}} = 0.017$; recessive model OR 1.42, $P_{\text{exact}} = 0.048$); however, data from the Halifax study were not significant (allelic OR 1.09, $P_{\text{exact}} = 0.584$; recessive model OR 1.02, $P_{\text{exact}} = 1$). Interestingly, the results obtained when the 2 sample sets were combined and stratified by the known RA risk allele in PTPN22, 188ST (11), suggested that association of FCRL3 with RA was stronger in the patient subgroup lacking the PTPN22 risk allele (CC versus TT OR 1.65, $P = 0.004$) than in the PTPN22 risk group (CC versus TT OR 0.79, $P = 0.52$) (7). Finally, Thabet et al compared the frequency of the FCRL3 promoter SNP in 931 Dutch RA cases and 570 Dutch controls and reported that individuals homozygous for the minor allele at this SNP were at increased risk of RA relative to individuals carrying the other 2 genotypes (OR 1.31, $P_{\text{exact}} = 0.044$) (8). Results of an allelic test of association, however, were not significant (OR 1.10, $P_{\text{exact}} = 0.227$). It should be noted that in the subset of studies that analyzed the correlation of this SNP with autoantibody and/or SE status (3–5, 7, 8), no association was observed.

Of interest is the observation that the frequency of the rs7528684 minor allele varied as much within the 2 ethnic groups (East Asians and whites) as it did between them. The $–169\text{C}$ allele frequency was lowest in Japanese controls (34.5–39.1%), higher in Korean controls (40.7%), and within the white sample sets, varied from 40.1% in the Toronto control population to 47.5% in the New Zealand control population.

In order to better understand the role of the FCRL3 promoter polymorphism in risk of RA, we performed a meta-analysis. To avoid potential confounding effects due to population substructure, we analyzed the data from the 4 East Asian sample sets (3 Japanese and 1 Korean; 3,172 cases and 2,916 controls) separately from the 9 white sample sets (2 North American, 1 UK, 1 New Zealand, 2 Spanish, 2 Canadian, and 1 Dutch; 5,645 cases and 5,592 controls). First we assessed the level of heterogeneity of the effect size across sample sets, using the Mantel-Haenszel procedure for determining homogeneity of ORs (12) and performing the analysis separately for the white subjects and the East Asian subjects. The test was applied to both allelic and genotypic data, and to assess the statistical significance of the results, a Monte Carlo simulation was performed. The simulation yielded nonsignificant $P$ values in both analyses in the studies of white subjects ($P_{\text{allelic}} = 0.24, P_{\text{genotypic}} = 0.25$). The same effect-size homogeneity analysis in the East Asian studies also revealed a nonsignificant $P$ value for the allelic data, but showed a significant $P$ value in the genotypic analysis ($P_{\text{allelic}} = 0.20, P_{\text{genotypic}} = 0.0038$). Hence, only the genotype data across the East Asian samples exhibited significant effect variability across studies.

Next we calculated a common OR across all 9 white sample sets and tested whether the $–169\text{T} \rightarrow \text{C}$ polymorphism was significantly associated with risk of RA. The results of this analysis provided no evidence that this polymorphism is associated with risk of RA in whites (allelic OR$_{\text{common}}$ 1.03 [95% confidence interval 0.98–1.09], $P = 0.234$; recessive model OR$_{\text{common}}$ 1.03 [95% confidence interval 0.94–1.14], $P = 0.266$) (Table 1). Power calculations, in which we systematically evaluated different ORs given the existing sample sizes using Monte Carlo simulations, showed that the combined study of the 9 white sample sets had $\sim$90% power to detect an allelic OR of 1.09 and, assuming a recessive model, a genotypic OR of 1.16 at the 5% significance level.

Similar calculations performed for the East Asian studies provided evidence that rs7528684 has a role in RA susceptibility in individuals of East Asian ancestry (allelic OR$_{\text{common}}$ 1.20 [95% confidence interval 1.12–1.29], $P = 1.20 \times 10^{-5}$; recessive model OR$_{\text{common}}$ 1.36 [95% confidence interval 1.18–1.56], $P = 2.87 \times 10^{-6}$); this appeared to be driven primarily by the data from the Japanese sample sets (Table 1). However, given the substantial level of genotypic OR heterogeneity across these sample sets as noted above.
(P = 0.0038), additional studies may be needed for full understanding of these results.

Since results of the Canadian study suggested that the FCRL3 risk variant was more strongly associated with RA in patients lacking the PTPN22 1858T risk allele (7), we reanalyzed one of the North American sample sets (467 cases and 473 controls) with stratification for the PTPN22 1858T risk allele, but we did not detect an association (CC versus TT OR 0.80, $P_{\text{exact}} = 0.335$ in the PTPN22 non-risk group; OR 1.04, $P_{\text{exact}} = 1$ in the PTPN22 risk group), consistent with findings in the British (4) and Dutch (8) studies.

In conclusion, the results of these meta-analyses provide no evidence that the FCRL3 $-169 T \rightarrow C$ polymorphism plays a significant role in determining RA risk in whites of European descent. Although all of these patients met the ACR 1987 criteria for the diagnosis of RA, it is still possible that clinical heterogeneity could explain the differing results across studies. Information on autoantibody and SE status was available for a subset of these 9 studies; however, the data did not support the notion of association between the SNP and these RA phenotypes (3–5,7,8). An alternative explanation is that the results of the single study showing significant allelic association and the 2 studies showing marginally significant genotypic association may be due to stochastic effects not related to disease status. This is not entirely unexpected given that the most significant result had a nominal false-positive rate of >15% as determined by the method of Sellke et al (13). These findings do not preclude the possibility that other SNPs within this gene or in the neighboring Fc receptor-like genes are associated with RA risk in whites (5). In contrast, the results from the East Asian studies, driven primarily by data from the Japanese sample sets, appear compelling and suggest that this promoter polymorphism may represent another example of a genetic variant that displays differential risk in different ethnic groups (e.g., PADH4 and PTPN22) (summarized in refs. 3 and 4). Finally, these results highlight the challenges inherent in studies of common complex diseases in which individual causal genetic variants are thought to make relatively small contributions to overall heritability, and reiterate the importance of large, well-powered study designs in which replication in independent sample sets is incorporated and population history considered, before conclusions are drawn about the role of genetic variants in these diseases.

Dr. Begovich, Ms Chang, and Dr. Schrodi hold stock or stock options in Celera.

AUTHOR CONTRIBUTIONS

Dr. Begovich had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Begovich.

Acquisition of data. Begovich, Chang.

Analysis and interpretation of data. Begovich, Chang, Schrodi.


Statistical analysis. Begovich, Schrodi.

Suppressive treatment of calcium pyrophosphate deposition disease

To the Editor:

In a recent article in Arthritis & Rheumatism (1), Chollet-Janin et al stated that no currently available drug is known to prevent the progression of calcium pyrophosphate crystal deposition with gradual joint deterioration (calcium pyrophosphate deposition disease [CPDD]) and suggested that methotrexate might have a therapeutic role. However, methotrexate does have side effects. One particular side effect, though rare, is cancer. Thus, it is perhaps more reasonable to consider safer alternatives.

Previous approaches to CPDD treatment have included nonsteroidal antiinflammatory agents, magnesium (by lavage or orally), intraarticular injections of yttrium-90, glycosaminoglycan polysulfate, and corticosteroids, heat, cold, ultrasound, diathermy, and even radiotherapy (2–6). Ryan and McCarty (7) incidentally noted improvement of CPDD in patients treated with suppressive agents for what was initially mistaken as rheumatoid arthritis, and my group documented effectiveness of hydroxychloroquine for the treatment of CPDD in a double-blind controlled study (8). The inflammatory arthritis in CPDD shows a remarkably rapid response to hydroxychloroquine, often at quite low dosage (8). The empirical observation of reduction (by at least 30%) of both tender and swollen joint counts within 1 week of treatment initiation in 15% of treated patients and within 1 month in 60% of treated patients seems unparalleled among the other forms of inflammatory arthritis.

It is clear that CPDD responds to suppressive treatment, as Ryan and McCarty suggested (7). Given the relative toxicity of various suppressive medications, safer alternatives would seem appropriate to consider before methotrexate.

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Erratum

In the title-page footnotes of the article by Soyfoo et al published in the August 2007 issue of Arthritis & Rheumatism (pp. 2566–2574), ARC grant 05/10-328 to one of the authors, Dr. Olivier Devuyst, was incorrectly shown as being from the Arthritis Research Campaign. In addition, the academic degrees of three of the authors, Chantal Mathieu, Olivier Devuyst, and Serge Steinfeld, were incorrectly shown as PhD rather than MD, PhD.

We regret the errors.
ACR ANNOUNCEMENTS
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Annual Meetings
November 6–11, 2007, Boston
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ACR/ARHP Annual Scientific Meeting

Come join more than 10,000 rheumatologists and rheumatology health professionals in Boston for the 71st ACR Annual Scientific Meeting and the 41st ARHP Annual Scientific Meeting! The meeting will be held Wednesday, November 7–Sunday, November 11 at the new Boston Convention and Exhibition Center. Preconference courses begin Tuesday, November 6.

Keynote speaker David Blumenthal, MD, MPP will discuss Medicine In silico: The Information Age Comes to Medicine, in the ACR’s opening lecture on Wednesday evening. Dr. Blumenthal is Director of the Institute for Health Policy and is a physician at The Massachusetts General Hospital/Partners HealthCare System in Boston.

The ACR/ARHP Annual Scientific Meeting is the premier event for specialists in the field of rheumatology, and we are continuing to enhance our meeting to bring you the best. Here’s what’s new for the 2007 annual meeting:

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The advance meeting and housing registration deadline is September 28th. If received by that date, the registration fee for the Annual Scientific Meeting is $350 for ACR members, $175 for member trainees and emeritus members, $450 for nonmembers, and $300 for nonmember trainees. There are separate fees for preconference courses and Meet the Professor and workshop sessions. After September 28th, the registration fees are significantly higher. Complete program and registration information is available on the ACR Web site at www.rheumatology.org/annual.