Supplementing Mesenchymal Stem Cells Improves the Therapeutic Effect of Hematopoietic Stem Cell Transplantation in the Treatment of Murine Systemic Lupus Erythematosus

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\textbf{ABSTRACT}

Transplantation of hematopoietic stem cells (HSCs) has been demonstrated to be a promising strategy in the treatment of systemic lupus erythematosus (SLE). Mesenchymal stem cells (MSCs) support hematopoiesis of HSCs and suppress immune response in a dose-dependent manner. Previous study showed that MSCs could alleviate the pathologic conditions of MRL/lpr mice (SLE animal model) when cotransplanted with bone marrow cells. Here, we investigated whether MSCs could improve the therapeutic effect of HSC transplantation in treating MRL/lpr mice in a dose-dependent manner. We found that lethally irradiated MRL/lpr mice were successfully reconstituted with HSCs alone or with various amounts of MSCs. Mice transplanted with HSCs and MSCs in the ratios of 5:1 (HSCs:MSCs) showed less transfusion-associated graft-versus-host reaction, steady body weight, and improved renal functions when compared with mice transplanted with HSCs only and those cotransplanted with MSCs in lower ratios. These results suggest that supplementing MSCs can improve the therapeutic effect of HSC transplantation in treatment of MRL/lpr mice in a dose-dependent manner.

\textbf{SYSTEMIC LUPUS ERYTHEMATOSUS (SLE)} is a common systemic autoimmune disease that affects many parts of body, including joints, heart, blood vessel, lung, brain, and kidney. SLE is characterized by widespread tissue damage and chronic inflammation. Immune cells such as T and B cells in SLE patients are abnormally activated and can produce antibodies that attack patients’ own cells and tissues [1,2]. SLE patients are usually treated with corticosteroid and immunosuppressants to alleviate symptoms. However, long-term use of these drugs may damage hepatic function and induce peptic ulcer, and they can cause osteoporosis, diabetes, infections, atherosclerosis, and other adverse effects. Currently, there is no cure for SLE.

Bone marrow transplantation (BMT) or hematopoietic stem cell transplantation (HSCT) has been used to treat various autoimmune diseases, including SLE [3,4]. In this regimen, the patient’s bone marrow system is replaced with allogenic cells, so that the defective immune cells are removed and a new immune system is established to avoid attacking the patient’s own cells. BMT or HSCT is a promising strategy to treat SLE. However, the patients receiving BMT or HSCT therapy usually have to be on immunosuppressive drugs for 6 to 12 months to avoid graft-versus-host disease (GVHD). Furthermore, GVHD resulting from the contamination with T cells from peripheral blood and rejection of grafted cells are common in patients treated with BMT or HSCT, which eventually contributes to low success rate of therapy [5].

The success of HSCT also relies on accurate and standardized HSC management in order to maintain the steady balance of various types of blood cells. This management includes controlling the survival, apoptosis, self-renewal, and differentiation of HSCs [6]. Furthermore, imbalance

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of hematopoietic process can be caused by gene defects, DNA damage of cells, and external factors [6].

In addition to HSCs, another type of stem cells, mesenchymal stem cells (MSCs) also exist in the bone marrow. MSCs are able to renew themselves and can differentiate into chondroblasts, osteoblasts, adipocytes, muscles, tendons, and other mesenchymal lineages under various differentiation conditions [7,8]. MSCs mainly maintain the properties of MSCs are dependent on the number of MSCs and are only detectable when a substantial number of MSCs are used [16].

Transplantation of MSCs only or with bone marrow into the MRL/lpr mice (SLE animal model) alleviates the pathologic conditions of mice, but with a high incidence of recurrence [17]. We propose that the inclusion of MSCs in HSCT in the management of SLE would be beneficial. To test the hypothesis, we reconstituted lethally irradiated MRL/lpr mice by transplanting HSCs with various amounts of MSCs and compared the therapeutic effects of the different conditions in MRL/lpr mice.

MATERIALS AND METHODS
Animals and Reagents
Female MRL/lpr mice were purchased from the Shanghai Institute of Materia Medica (Shanghai, China, SCXX [Shanghai] 2008–0017) and maintained under specific pathogen-free conditions at Sun Yat-Sen University Medical School. Male BALB/c mice were obtained from an animal facility at the Southern Medical University (SCXX [Guangdong province] 2006–0015).

The reagents used in this study include OriCell Cynomologus Monkey MSC Growth Medium (Cyagen Biosciences, Guangzhou, China), phycoerythrin-Cy5 (PE-Cy5) anti-mouse CD3e (eBioscience, United States), anti-mouse CD4 (LJT4; eBioscience), fluorescein isothiocyanate (FITC) anti-mouse CD8a (Ly-2; eBioscience), Mouse Lineage Cell Depletion Kit (Miltenyi Biotec, Germany), anti-mouse CD117 MicroBeads (Miltenyi Biotec), MRL/lpr Mice

Monitoring of MRL/lpr Mice
The index of acute GVHD was evaluated by recording the following parameters in experimental mice: mental changes, weight, arched posture, depilation, proteinuria, serum creatinine, leukocyte, erythrocyte, and hemoglobin. Proteinuria was determined by using the Micro BCA Protein Assay Kit (Thermo scientific) before and after transplantation every 2 weeks according to the manufacturer’s instruction. Serum creatinine was examined at 8 weeks after transplantation. Leukocyte, erythrocyte, and hemoglobin levels were measured in peripheral blood collected by cutting the tip of the mouse’s tail. MRL/lpr mice were sacrificed for histologic analysis at 8 weeks after transplantation.

Flow Cytometry
Lymphocyte populations in the peripheral blood samples from mice before and 8 weeks after cell transplantation were collected and analyzed by flow cytometry. About 100 μL peripheral blood from each mouse was collected into an EDTA blood collection tube (Improvacuter, Chiny) from its tail. Of this, blood cells (50 μL) were stained at room temperature for 10 minutes with PE-Cy5 conjugated anti-mouse mononodal CD3e antibody (1 μg), PE conjugated anti-mouse CD4 (1 μg), and FITC conjugated anti-mouse CD8a (1 μg; eBioscience). RBCs were lysed for a minute using 10 μL hemolytic buffer. Stained cells were then washed with
of HSCs and MSCs. Only 2 mice, 1 from group A (1:5) and another from group D (1:0), died at 4 weeks after transplantation.

To evaluate whether transplantation of HSCs with various amounts of MSCs could reconstitute the hematopoietic system of lethally irradiated MRL/lpr mice, we measured the number of leukocytes and erythrocytes and measured the level of hemoglobin in peripheral blood harvested before cell transplantation and at 8 weeks post-cell transplantation. No significant differences were observed in the concentrations of leukocytes and erythrocytes, and the levels of hemoglobin measured between the groups before and 8 weeks after transplantation (Fig 1).

![Fig 1. Successful reconstitution of the hematopoietic system in lethally irradiated MRL/lpr mice following transplantation of hematopoietic stem cells (HSCs) with various amount of mesenchymal stem cells (MSCs).](image)

Table 1. List of Primers and Probes Used to Detect SRY and β-Actin Genes

<table>
<thead>
<tr>
<th></th>
<th>β-Actin (Chrom:5)</th>
<th>SRY (Chrom:Y)</th>
</tr>
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<tbody>
<tr>
<td>Forward primer</td>
<td>5'-CCCTAAACCTAGAGGTTGCTCTG-3'</td>
<td>5'-GTCACCTGACAGGGTGTACG-3'</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>5'-CATGAAGATCAAGGTGCTACATC-3'</td>
<td>5'-CAGGAAAGGTGCTAAAGTGTCAC-3'</td>
</tr>
<tr>
<td>TaqMan probe</td>
<td>HEX-AACCAAGGGCCCA</td>
<td>FAM-CTGGAGGGCTGTTAAA</td>
</tr>
<tr>
<td></td>
<td>CCCTCA-BHQ1</td>
<td>ATGCCACTCCT-BHQ1</td>
</tr>
<tr>
<td>Fragment length</td>
<td>84bp</td>
<td>88bp</td>
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</table>
indicating that lethally irradiated MRL/lpr mice were reconstituted successfully by HSCs, both with and without varying amounts of MSCs.

Analysis of Chimerism

Chimerism analysis was used to evaluate the success of transplantation. The analysis was performed using dual fluorescence-quantitative PCR method. Thirteen of 16 female MRL/lpr mice receiving cell transplantation showed chimerism at 8 weeks after transplantation. The average chimerism rates were 0.29%, 0.74%, 10.94%, and 0.91% in group A (1:5), B (1:1), C (1:5), and D (1:0), respectively. No statistically significant difference in the chimerism was observed between the groups.

Graft-Versus-Host Reaction

Mice in all the 4 experimental groups did not show any symptom of acute GVHD, such as listlessness, weight loss, arched posture, and depilation in the first 4 weeks after cell transplantation. At 8 weeks post-cell transplantation, these symptoms were observed in mice from each experimental groups and it was more obvious in groups A (1:5), B (1:1), and D (1:0) than in group C (5:1). A small nodule was observed in the right eye of 1 mouse in group A (1:5). In group B (1:1), a mouse was found to have severely arched posture with severe weight loss (decreased by about 39.8%) and unable to move with its left leg in group B (1:1). In group D (1:0), 3 mice were observed to have severely arched posture.

Effect of Cell Transplantation on Weight of MRL/lpr Mice

MRL/lpr mice tend to lose weight over time. The weight loss was still observed in groups A (1:5), B (1:1), and D (1:0) with no obvious difference between mice from these 3 experimental groups. However, mice in Group C (5:1) showed significantly less weight loss compared to groups A, B, or D (Fig 2).

Effect of Cell Transplantation on Renal Function of MRL/lpr mice

The renal function of MRL/lpr mice is compromised due to lupus nephritis. To evaluate the therapeutic effect of cell transplantation on the renal function of mice, we compared the levels of serum creatinine and proteins in urine from the mice in the various experimental and control groups. We observed that mice in groups B, C, and D had significantly lower levels of serum creatinine and proteinuria compared to mice in control group (Fig 3). Furthermore, the level of both serum creatinine and proteinuria in group C was

Fig 2. Change of body weight of MRL/lpr mice following bone marrow reconstitution. The body weight of mice from each group was measured and reported as average with standard error of the mean along the time following cell transplantation. For brief experimental description, see the legend in Fig 1.*P < .05 vs group A, B, or D, respectively.

Fig 3. Levels of proteinuria and serum creatinine in mice at 8 weeks post-transplantation. For brief description of experiments, see the legend in Fig 1. (A) *P < .05 vs control group; **P < .05 vs group A. (B) *P < .05 vs control group; $P < .05$ vs all other groups; *P < .05 vs group A.

Fig 3.
significantly lower than the mice in other experimental groups (Fig 3).

Histopathology of Kidneys

Prior to cell transplantation, MRL/lpr mouse kidney showed proliferating glomerular parietal epithelial cells, thickening of basement membrane, moderate hyperplasia of mesangial cells and mesangial matrix, tubular and interstitial infiltrates of leukocytes and monocytes, a typical histopathology of SLE kidney with pathologic score of grade II to III (Fig 4A). This histopathology was also observed in kidney sections from mice in the control group (Fig 4B). The histopathology of kidneys from all experimental groups showed improved pathologic scores of grade I (Fig 4C to F).
Increased T Lymphocytes in Peripheral Blood of MRL/lpr Mice Receiving Cell Transplantation

The number of CD3<sup>+</sup>CD4<sup>+</sup> and CD3<sup>+</sup>CD4<sup>+</sup> T lymphocytes decreased progressively in the peripheral blood of MRL/lpr mice. We examined whether transplantation of HSCs and various amounts of MSCs could reverse the observed decrease in both types of T lymphocytes in MRL/lpr mice by comparing the percentages of both T-lymphocyte populations before transplantation and at 8 weeks post-transplantation. In the control group of MRL/lpr mice, the percentage of CD3<sup>+</sup>CD4<sup>+</sup> or CD3<sup>+</sup>CD8<sup>+</sup> decreased significantly along the time, while a significant increase was observed in both types of T lymphocytes in all the experimental groups of MRL/lpr mice receiving cell transplantation (Fig 5), suggesting reconstitution of bone marrow reverted the tendency of decrease of CD3<sup>+</sup>CD4<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup> T-lymphocyte populations. We also observed significantly higher numbers of CD3<sup>+</sup>CD4<sup>+</sup> population in group A than in groups B, C, and D.

**DISCUSSION**

The success of HSCT to treat SLE has been hampered by the high incidence of GVHD and difficulty in HSC management [22]. In this study, we investigated the potential benefit of MSCs in HSCT to treat SLE and found that transplantation of HSCs alone or with various amounts of MSCs could be used to fully reconstitute the completely wiped bone marrow system of mice to treat MRL/lpr mice (SLE animal model). In our study, one million HSCs were used in each experimental group supplementing with the ratio (HSCs:MSCs) of 1:5, 1:1, 5:1, or 1:0 of MSCs. All mice had normal concentrations of leukocytes and erythrocytes and levels of hemoglobin at 8 weeks post-transplantation (Fig 1). Chimerism analysis showed that mice from each experimental group had similar chimerism rates. We also found that mice from each experimental group had similar improved kidneys on histopathology when compared to the mice from the no treatment control group (Fig 4). Finally, mice from each experimental group had increased CD3<sup>+</sup>CD4<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup> lymphocytes (Fig 5).

We also found that supplementing MSCs improved the therapeutic effect of HSCT in treating MRL/lpr mice in a cell number–dependent manner. This conclusion was supported by our observations that the mice transplanted with HSCs and MSCs at the ratio of 5:1 (HSCs:MSCs) had less GVHD, no weight loss, and more highly improved renal functions than the mice from the other experimental groups. A previous report that showed that MSCs inhibited the proliferation of T lymphocytes isolated from MRL/lpr mice in a dose-dependent manner [9] supports our current observation and conclusion.

GVHD is a major cause of the failure of HSCT [1]. In our study, GVHD was not observed in all mice from experimental group with MSCs at 4 weeks post-HSCT. After this time point, the immune system of the recipient mice gradually recovered, thus the severe symptoms of GVHD began to be observed in groups A, B, and D, while mice in group C, which received HSCs and MSCs at a ratio of 5:1, showed mild GVHD.

Chimerism rate was used to monitor the degree of reconstitution [23,24]. Supplementing MSCs in BMT promoted the proliferation and differentiation of transplanted HSCs via improving the abnormal bone marrow hematopoietic microenvironment [25,26] and suppressing the immune response [14]. In our study, we observed similar chimerism in all mice in experimental groups, including mice receiving HSCs alone. The quality of HSCs may have led to this observation. It has been reported that HSCs isolated from older mice have a lower ability to reconstitute bone marrow system of lethally irradiated mice [27]. However, the HSCs used in our study were from 6-week-old BALB/c mice and were pure and viable. It is possible that the number of MSCs used in this study may not have been enough. Usually, by 2 to 3 weeks after cell transplantation, the host immune system has recovered enough to reject the transplanted allogenic cells [28,29]. If the number of MSCs is not enough to suppress the host immune system, transplanted cells will be rejected. Finally, the effect of MSCs on HSC proliferation and differentiation may be observed at later time (8 weeks after cell transplantation).

In summary, our study demonstrated that supplementing MSCs along with HSCs improved HSCT-based treatment in lupus-prone MRL/lpr mice.
REFERENCES