Intrauterine growth restriction and prematurity influence regulatory T cell development in newborns

Dhriti Mukhopadhyay, Laura Weaver, Richard Tobin, Stephanie Henderson, Madhava Beeram, M. Karen Newell-Rogers, Lena Perger

Texas A&M University, Scott & White Memorial Hospital, Temple, TX, United States

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A B S T R A C T

Purpose: The aim of this study was to determine the relationship of birth weight and gestational age with regulatory T cells (Tregs) in cord blood of human newborns.

Methods: Cord blood mononuclear cells (CBMCs) of 210 newborns were analyzed using flow cytometry to identify Tregs (CD3+, CD4+, CD25high, FoxP3high) and measure FoxP3 mean fluorescence intensity (MFI). Suppressive index (SI) was calculated as FoxP3 MFI per Treg.

Results: Mode of delivery had no significant effect on Tregs at birth. Term babies with growth restriction had lower SI than their appropriate weight counterparts but equivalent SI. Preterm babies had higher percentages of Tregs, but lower SI than term controls. SI steadily increased through gestation.

Conclusions: Intrauterine growth restriction is correlated with fewer circulating Tregs, and prematurity with decreased functionality of Tregs compared to term appropriate weight infants. This may have implications in diseases such as necrotizing enterocolitis that disproportionately affect premature and lower birth weight infants.

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The immune system is a complex network of cellular and humoral checks and balances. CD4+ regulatory T cells (Tregs), characterized by the simultaneous expression of an IL-2 receptor γ-chain (CD25) and transcription factor forkhead box P3 (FoxP3), are a potent segment of the negative regulatory arm of the immune system in their ability to suppress both innate and adaptive immune responses [1,2]. Their importance as effectors of self-tolerance and regulators of overzealous immune activation to foreign antigens is well characterized for primary diseases of immunity such as autoimmunity and allergy [3,4]. Yet, much remains unknown about their diverse role in a variety of physiologic and pathologic situations. Specifically, Tregs have been implicated in the development of necrotizing enterocolitis (NEC) [5]. However, baseline values of circulating Tregs in infants at birth have not been established. Their dynamics in the early postnatal period and in newborn health and disease remain to be characterized. Tregs are typically defined as CD3+/CD4+/FoxP3high/CD25high cells by flow cytometry, a method of analysis that enables precise counting of blood cells by intracellular and extracellular markers. The first objective of this study, therefore, was to establish normal values of Treg frequency and activity in cord blood of healthy newborns using contemporary criteria.

Local and systemic injury in NEC is mediated by an overwhelming proinflammatory response of the immune system. Lack of suppression of this response may be attributed to decreased numbers or activity of Tregs. Previous studies utilizing flow cytometric analysis of Tregs suggest that numbers of CD25+CD4+ T cells and expression of FoxP3 are both inversely correlated with gestational age [2]. FoxP3 expression also appears to be attenuated in small for gestational age (SGA) infants compared to age-matched controls [6]. Because NEC disproportionately affects babies who are born prematurely or with intrauterine growth restriction (IUGR) in frequency and severity [7], our secondary objective was to determine whether such incomplete or restricted development during the fetal period translated to decreases in Treg populations or activity prior to development of disease.

1. Materials and methods

1.1. Selection of subjects

A prospective study was conducted between June 2012 and January 2013 with IRB approval (protocol 710737). All live births were included in the study with the exception of babies born to mothers with active infection or transfers from outside hospitals because of cord blood unavailability. Historical data was not available to power the study but a projected sample size of ten per study group was determined through a feasibility analysis. The study was closed once this sample size was achieved in each group.

Patients were assigned by gestational age and birth weight according to standard AAPG growth curves [8] into the following groups:

1. Term (>37 weeks) and appropriate for gestational age (AGA > 10th percentile on the growth curve).
Table 1
Demographics of study population.

<table>
<thead>
<tr>
<th></th>
<th>Term AGA</th>
<th>Term SGA</th>
<th>Preterm AGA</th>
<th>Early preterm AGA</th>
<th>Preterm SGA</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample size (n)</td>
<td>80</td>
<td>18</td>
<td>33</td>
<td>13</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Gestational age</td>
<td>39 (39–40)</td>
<td>39 (38–40)</td>
<td>35 (35–36)</td>
<td>29 (27–30)</td>
<td>33 (28–36)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Birth weight</td>
<td>3440 (3150–3724)</td>
<td>2546 (2289–2723)</td>
<td>2573 (2306–2794)</td>
<td>1250 (970–1455)</td>
<td>1347 (993–1916)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Gender (% male)</td>
<td>41%</td>
<td>44%</td>
<td>61%</td>
<td>62%</td>
<td>40%</td>
<td>0.3137</td>
</tr>
<tr>
<td>Mode of delivery (% vaginal)</td>
<td>46%</td>
<td>56%</td>
<td>45%</td>
<td>85%</td>
<td>60%</td>
<td>0.1263</td>
</tr>
<tr>
<td>Ethnicity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Black</td>
<td>15%</td>
<td>28%</td>
<td>21%</td>
<td>23%</td>
<td>10%</td>
<td>0.2998</td>
</tr>
<tr>
<td>% White</td>
<td>50%</td>
<td>39%</td>
<td>52%</td>
<td>62%</td>
<td>60%</td>
<td>0.8852</td>
</tr>
<tr>
<td>% Hispanic</td>
<td>28%</td>
<td>17%</td>
<td>9%</td>
<td>15%</td>
<td>20%</td>
<td>0.1536</td>
</tr>
<tr>
<td>Appgar at 1 minute</td>
<td>9 (8–9)</td>
<td>9 (8–9)</td>
<td>8 (7–9)</td>
<td>7 (4–8)</td>
<td>9 (7–9)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Appgar at 5 minutes</td>
<td>9 (9–9)</td>
<td>9 (9–9)</td>
<td>9 (9–9)</td>
<td>7 (5–8)</td>
<td>9 (9–9)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Maternal gravidity (% primigravida)</td>
<td>21%</td>
<td>50%</td>
<td>33%</td>
<td>31%</td>
<td>80%</td>
<td>0.005</td>
</tr>
<tr>
<td>Maternal parity (% nulliparous)</td>
<td>28%</td>
<td>50%</td>
<td>33%</td>
<td>38%</td>
<td>80%</td>
<td>0.0145</td>
</tr>
</tbody>
</table>

AGA: appropriate for gestational age.
SGA: small for gestational age.
Early preterm: 23–32 weeks gestational age.
Late preterm: 33–36 weeks gestational age.
Kruskal–Wallis ANOVA conducted for continuous and χ² test for categorical variables with significance defined as p < 0.05. Continuous data are reported as median (interquartile range) and categorical as percentages.

2. Term and small for gestational age (SGA ≤ 10th percentile),
3. Preterm (<37 weeks EGA) AGA
   a. Late (born between 33 and 36 weeks),
   b. Early (born between 23 and 32 weeks),
4. Preterm SGA.

1.2. Samples

Cord blood routinely collected in EDTA-coated vacutainers (BD, Franklin Lakes, NJ) following birth was obtained from the institutional blood bank within three days of birth. Two hundred and ten total samples were collected and processed until all study groups were filled. Two to three milliliters of blood per patient was processed to isolate cord blood mononuclear cells (CBMCs) using cellular density gradient centrifugation according to the manufacturer’s instructions (Lymphoprep, Accurate Chemicals, Westbury, NY). Samples processed not according to protocol or with technical issues were excluded from analysis.

1.3. Flow cytometry

Isolated CBMCs were stained for cell-surface markers using fluorescent conjugated primary mouse anti-human antibodies, anti-CD3 (Pacific Blue), anti-CD4 (PE-Cy7), and anti-CD25 (APC) as well as intracellular anti-FoxP3 (PE) (all antibodies from BD Biosciences, San Jose, CA), with a PE-anti-mouse/rat FoxP3 staining set (e-Biosciences, San Diego, CA). Cells were sorted using flow cytometry (BD FACS Canto II Flow Cytometer, Franklin Lakes, NJ).

1.4. Analysis

Samples were analyzed by comparison to isotype controls. Using standard gating for all samples, we determined CD4 + T cell numbers (defined as CD3+, CD4+) and within this population, T_{reg} were counted (defined as CD25^{high}, FoxP3^{high}). Final analyses were performed by determining cell ratios rather than absolute numbers because of significant variability in number of cells per sample. Flow cytometry data was used to calculate the percentage of CD4 + T cells of all CBMCs and the percentage of T_{reg} of all CD4 + T cells. Mean fluorescence intensity (MFI) was calculated as the geometric mean of FoxP3 in CD4 + T-cell populations per sample (FlowJo 10.0.0 software), which measures the expression of FoxP3 and has been validated in the literature as proportionate to T_{reg} suppressive ability [2,6]. In order to normalize suppressive ability, we defined an index for the comparison of MFI per T_{reg} (total FoxP3 MFI/absolute number of T_{reg}) as the suppressive index (SI) of each sample to determine suppressive capability of individual T_{reg} in each patient.

GraphPad Prism 6.0c software was utilized for statistical analysis using Mann Whitney test, Kruskal–Wallis and linear regression in order to compare groups with nonparametric distributions. Significance was defined as p < 0.05.

2. Results

2.1. Demographics

Study populations were similar to the control group in terms of gender and ethnic distributions as well as method of birth (Table 1). Median Appgar scores at 1 and 5 minutes were significantly lower with younger gestational age (9/9 in term infants, 8/9 in late preterm and 7/7 in early preterm). SGA babies were significantly more often born to nulliparous women than AGA babies, regardless of gestational age (nulliparous: 28% controls, 33% late preterm, 38% early preterm vs. 50% term SGA, 80% preterm SGA).

Table 2
Comparison of CD4 + T cells and T_{reg} proportions, mean fluorescence intensity, and suppressive index in babies delivered vaginally with those delivered by Cesarean section.

<table>
<thead>
<tr>
<th>Vaginal delivery (n = 40)</th>
<th>Cesarean section (n = 29)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>% CD4 T cells of total cells counted</td>
<td>10 (4–19)</td>
<td>9 (6–15)</td>
</tr>
<tr>
<td>% T_{reg} of CD4 + T cells</td>
<td>6 (4–9)</td>
<td>7 (6–11)</td>
</tr>
<tr>
<td>T_{reg} mean fluorescence intensity</td>
<td>3772 (3032–4785)</td>
<td>3312 (2553–5137)</td>
</tr>
<tr>
<td>T_{reg} suppressive index</td>
<td>80 (25–202)</td>
<td>65 (41–140)</td>
</tr>
</tbody>
</table>

Mann–Whitney test conducted with significance defined as p < 0.05. Values reported as median (interquartile range).
2.2. Mode of delivery

Comparisons of babies born by uncomplicated spontaneous vaginal delivery (SVD) and elective Cesarean section (C-section) were conducted in the term AGA group. No significant differences were found in CD4+ T lymphocyte or T_{reg} percentage, MFI or SI between the two groups. The mode of delivery did not appear to have a significant immediate effect on regulatory T-cell populations or activity in newborn peripheral blood, so babies born by both methods were included as the control group for all subsequent analyses (Table 2).
2.3. Intrauterine growth restriction

Term appropriate-for-gestational-age babies (AGA, n = 80) were compared to term small-for-gestational-age babies (SGA, less than 10th percentile birth weight, n = 18) to determine the relationship between intrauterine growth restriction and regulatory T cells. Results are summarized in Fig. 1. Median Treg percentage of CD4+ T cells was found to be significantly lower in the SGA group (7% in AGA vs. 6% in SGA, p = 0.0121). The SGA group also had FoxP3 mean fluorescence intensity that was significantly lower than in AGA babies (3602 in AGA vs. 2686 in SGA, p = 0.0021). However, when the Treg suppressive index of the two groups was compared (67 in AGA vs. 81 in SGA, p = 0.6905), no significant difference was found. These findings are depicted in Fig. 1C.

2.4. Gestational age

AGA babies born at 37 weeks estimated gestational age (EGA) or later (term, n = 80) were compared to AGA babies born between 33–36 weeks EGA (late preterm, n = 33) and 23–32 weeks EGA (early preterm, n = 13) to investigate the changes in Treg through gestation. Results are summarized in Table 3 and Fig. 2. A significant decline in median CD4+ T cell percentages was observed early in the third trimester, between the early preterm and late preterm groups (16% in early vs. 12% in late preterm, p = 0.0218), then remained steady through the completion of gestation (10% in the term group). Treg numbers also declined through gestation, but this occurred later in the third trimester, upon completion of gestation. Early preterm and late preterm groups had no significant difference in Treg percentages (10% in early and 11% in late) but there was a significant decline in babies who were born at term (7%, p = 0.0025).

Though mean fluorescence intensity remained steady throughout gestation (4124 in early preterm, 4133 in late preterm, and 3602 in term, p = 0.4332), the suppressive index rose steadily through gestation, which was statistically significant (25 in early preterm vs. 35 in late preterm vs. 67 in term, p = 0.0037).

3. Discussion

The interplay of intrinsic and environmental factors in a developing infant is so complex that no single insult has yet been identified that reproducibly leads to the development of NEC. Infant characteristics such as prematurity and growth restriction, as well as environmental factors like microbial colonization and formula feeding have all been implicated as risk factors. It is clear from the extensive body of literature that a multitude of derangements at any point during uterine life can predispose a baby to this potentially devastating disease [9–13]. The stage for its development is likely set prenatally in a genetically susceptible infant who then sustains a postnatal environmental insult. Bacterial translocation through damaged epithelium combined with a proinflammatory immune state leads to intestinal necrosis, systemic sepsis and the short- and long-term manifestations of NEC [7]. An overwhelming systemic and local immune response is an important etiologic factor in its pathogenesis [14–16], and the role of Tregs as powerful regulators of the immune system needs to be defined in order to target them as a potential prophylactic or therapeutic option in NEC.

This study thus establishes how Tregs, a preventive arm of adaptive immunity, are affected by the two most well-characterized risk factors for NEC, growth restriction and prematurity, immediately at birth prior to environmental influences [8,9,13]. Tregs have been shown to be critical for protection against intestinal ischemic and inflammatory injury [17,18]. IUGR is associated with placental insufficiency and fetal hypoxia [19,20], which likely leads to hypoxic conditions shown to affect the development of Treg populations [21–27]. Given that epithelial ischemia is part of the cascade that ultimately leads to NEC, a decrease in numbers or function of Tregs not only locally but systemically may be a contributing factor.

Previous studies have demonstrated a decreased ratio of Tregs to effector CD4+ cells locally in the intestinal tissue of premature newborns with NEC [5]. Systemically, it has been suggested that Foxp3 expression decreases with decreasing birth weight and increasing gestational age [2,6]. Our study further qualified previous findings by definition of the Treg suppressive index. Using this measurement, we showed that despite decreased percentages of circulating Tregs in the cord blood, growth-restricted babies actually had intact individual Foxp3 expression. This suggests that an intrauterine insult that results in growth restriction may also result in an immune phenotype skewed towards a proinflammatory state; Treg therefore remain functional but are fewer in number. In contrast, premature babies showed an increased percentage of Tregs in their CD4+ T-cell population, resulting in the appearance of intact Foxp3 expression. However, suppressive indices were lower in younger infants and actually increased through gestation, suggesting a physiologic clonal deletion of less functional Tregs through gestation, preparing the infant for interaction with the external environment. Through these different mechanisms, Treg numbers or function may play a role in the multifactorial etiology of NEC.

Establishment of normal baseline values of Tregs in human babies and how they are quantitatively and qualitatively affected by prematurity and growth restriction lays the groundwork for future research in diseases of premature and IUGR infants, especially necrotizing enterocolitis. Linking the findings of Treg populations and dynamics in peripheral blood and how they change in the subset of babies who go on to develop NEC has exciting implications for risk stratification, diagnosis and even therapy. Regulatory T cells remain an exciting branch of immunology research, but much remains to be discovered with respect to their role in diseases of prematurity and how they are influenced by the combination of baby, mother and environment.

Acknowledgments

Scott & White Research Grant Program, Scott & White Blood Bank, and Brett Mitchell, PhD for expert advice.
Fig. 2. Treg characteristics through gestation. A. Representative samples of flow cytometric analysis of term appropriate for gestational age infant showing all cells gated in first panel, CD3+/CD4+ cells gated in second panel representing CD4+ T cells and FoxP3high/CD25high cells gated in third panel representing Tregs. B. Flow cytometry for late preterm appropriate for gestational age infant (33–36 weeks). C. Flow cytometry for early preterm appropriate for gestational age (23–32 weeks). D. Linear regression shows a gradual decrease in Treg proportions from 23 weeks gestational age until term (r = −0.222). E. Concurrently, there is an increase in the Treg suppressive index (r = 0.119).
References


