ORIGINAL RESEARCH

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Proportional changes of CD4+CD25+Foxp3+ Regulatory T cells in maternal peripheral blood during pregnancy and labor at term and preterm

Abstract

Purpose: To evaluate the proportional changes of CD4+CD25+Foxp3+ regulatory T cells (Tregs) in maternal peripheral blood during pregnancy and labor at term and preterm.

Methods: Peripheral blood was collected from 20 non-pregnant controls and 139 pregnant women (60 at different gestational ages, 48 at term with or without labor, and 31 in threatened or actual preterm labor). CD4+CD25+Foxp3+ Tregs in peripheral blood samples were analyzed in peripheral blood samples by flow cytometry. Placentas from preterm women were examined for the presence of histological chorioamnionitis (HC).

Results: The percentage of circulating CD4+CD25+Foxp3+ Tregs was significantly increased in women during the first trimester compared with non-pregnant controls ($P<0.0001$), peaking during the second trimester and then declining slightly in the third trimester. There was a significantly lower level of CD4+CD25+Foxp3+ Tregs in women with term labor than in those at term without labor ($P<0.0001$). Women admitted in preterm labor had a lower proportion of CD4+CD25+Foxp3+ cells than those admitted with threatening preterm labor ($P<0.0001$). Preterm women with evidence of HC had decreased proportion of CD4+CD25+Foxp3+ cells than those without HC in preterm deliveries ($P=0.008$). Moreover, the percentages of CD4+CD25+Foxp3+ cells in preterm subjects, irrespective of the HC status, were significantly diminished compared with women with normal pregnancy at the same gestational age ($P<0.0001$).

Conclusion: Our data reveal a marked elevation of peripheral blood CD4+CD25+Foxp3+ Tregs during early pregnancy, but a dramatic decline during labor, either at term or preterm, suggesting their involvement in the maintenance of pregnancy and the initiation of labor.

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Pregnancy raises a major challenge to the maternal immune system, as the immune system has to tolerate fetal alloantigens encoded by paternal genes. CD4⁺ CD25⁺ regulatory T cells (Tregs) are well recognized to play an important role in the maintenance of maternal tolerance to the fetus [1-5]. They can be detected in the human decidua and peripheral blood throughout pregnancy [5]. Severely reduced numbers of Tregs have been found in women either following spontaneous abortion or with pregnancy complications (e.g., premature rupture of membranes and pre-eclampsia) in comparison with those with uncomplicated pregnancy, suggesting the association between Treg levels and pregnancy [6-9]. Accumulating evidence indicates that the transcription factor, forkhead box P3 (Foxp3), is involved in Treg lineage commitment [10-14]. Foxp3 deficiency impairs Treg development and results in severe autoimmune/inflammatory disease in humans and rodents [11,12], whereas ectopic expression of Foxp3 in CD25⁺ CD4⁺ naive T cells can convert them to cells functionally and phenotypically similar to Tregs [13,14], indicating the key role of Foxp3 in the development and function of CD4⁺ CD25⁺ Tregs.

Tregs can selectively express multiple toll-like receptors (TLRs) [15] that play an essential role in innate immunity by recognizing conserved pathogen-associated molecular patterns [16]. Interestingly, the suppressive effect of CD4⁺ CD25⁺ Tregs can be blocked by microbial induction of the Toll pathway on dendritic cells (DCs), thereby facilitating the activation of pathogen-specific adaptive immune responses [17]. Preterm labor has been suggested to be closely linked to microbial infection [18,19]. Molecular studies demonstrated the involvement of TLR4 in lipopolysaccharide-induced preterm birth [20,21]. Our pilot study also found a marked elevation in TLR4 expression in the peripheral blood and placenta of patients with preterm labor (data not shown). In light of these findings, we hypothesized that there was an abnormal alteration in the proportion of Tregs associated with preterm labor that might impair the maternal immune homeostasis and thus contribute to preterm birth. To test this hypothesis, in this study we measured the proportional changes of CD4⁺CD25⁺ Foxp3⁺ Tregs in peripheral blood during pregnancy and labor at term and preterm, and analyzed the associations between the frequency of circulating CD4⁺CD25⁺Foxp3⁺ Tregs and pregnancy and preterm labor.

Materials and Methods

Subjects

The study, conducted between August 2009 and June 2010 was approved by the Ethical Committee of Central South University, Changsha, China. Informed consent was obtained from each participant. A total of 139 pregnant women were enrolled, of which 20 were at 7-12 weeks of gestation (within the first trimester), 20 were at 24-28 weeks of gestation (within the second trimester), 48 were at 37-42 weeks of gestation who were admitted for delivery (28 having cesarean section with and 20 without labor), and 51 were at 28-36 weeks of gestation (16 with threatening and 15 actual preterm labor and 20 with normal pregnancy as control group). Of the 16 subjects with threatening preterm labor, 6 delivered within 5 days after admission, and the remaining 10 did not deliver after tocolysis. All women with actual preterm labor delivered within 24 h after admission. An additional 20 non-pregnant healthy women at reproductive age were enrolled for comparison. Gestational age was assessed by date of last menstrual period or by ultrasound performed during the first trimester or early second trimester. Term labor was considered as delivery between 37 and 42 weeks of gestation, presenting with painful uterine contractions at least once every 5 minutes for more than 1 hour and with cervical effacement or dilatation. Threatening preterm labor was defined as the presence of at least one regular uterine contraction in 10 min and effacement of cervix, while the preterm labor was defined as the presence of ≥ 4 regular uterine contractions in 20 min with each contraction duration lasting ≥ 30 s, effacement of more than 75% of the cervical canal, or cervical dilation more than 2 cm). Placental biopsies were taken from women who delivered at preterm and examined for the presence of histological chorioamnionitis according to the criteria of Blanc [22].

Women were excluded from the study who had any of the following: a preexisting condition, including premature rupture of fetal membranes, hypertensive disorder, diabetes mellitus, heart disease, hepatitis, nephritis, autoimmune diseases, or endocrine diseases; a history of recurrent spontaneous abortion, birth defects, stillbirths or other adverse pregnancy outcomes; usage of hormone drugs within 3 months before enrollment; clinical signs of intrauterine infection, including maternal temperature $\geq 37.8^{\circ}\text{C}$, the presence of uterine tenderness, fetal tachycardia >160 beats/min, or WBC >15000 cells/ mm^3 in maternal blood; and more than one fetus.

Peripheral Blood Collection and Cell Isolation

Fresh peripheral venous blood samples were obtained from all the 159 subjects. Peripheral blood mononuclear cells (PBMCs) were isolated within 3 h by standard Ficoll-Hypaque density gradient centrifugation.

Flow cytometry

The isolated PBMCs were analyzed for CD4⁺CD25⁺Foxp3⁺ Tregs using the Human Treg Staining Kit (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions. Briefly, PBMCs were incubated with fluorescein isothiocyanate (FITC)- and allophycocyanin (APC)-conjugated CD4/CD25 cocktail monoclonal antibodies for 30 min at 4 °C, followed by the addition of freshly prepared Fixation/Permeabilization Buffer, and incubation for 45 min at 4 °C. Cells were washed twice and then incubated with phycoerythrin (PE)-labeled anti-human Foxp3 monoclonal antibody for 45 min at 4 °C. As negative controls, PE-labeled rat IgG2a isotype antibodies were used. The stained cells were analyzed using FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

Statistical analysis

All data were presented as median and range. Because the data were not distributed normally, the Mann-Whitney U test was applied to analyze differences in the percentage of CD4⁺CD25⁺Foxp3⁺ Tregs between groups, and the Kruskal-Wallis test was used for analysis of the differences in clinical characteristics. $P < 0.05$ was considered a statistically significant difference.

Results

Clinical characteristics

The participants in this study were assigned to the following 8 groups: Group I, non-pregnant women; Group II, normal pregnancy at 7-12 weeks; Group III, normal pregnancy at 24-28 weeks; Group IV, delivery by cesarean section at 37-42 weeks of gestation without labor; Group V, delivery by cesarean section with term labor at 37-42 weeks; Group VI, threatening preterm labor at 28-36 weeks of gestation; Group VII, preterm labor at 28-36 weeks; and Group VIII, normal pregnancy at 28-36 weeks.

TABLE 1. Clinical characteristics of subjects in different groups

Variables	Group I (n=20)	Group II (n=20)	Group III (n=20)	Group IV (n=20)	Group V (n=28)	Group VI (n=16)	Group VII (n=15)	Group VIII (n=20)	P value
Maternal age (years)	26±4	26±4	28±2	27±2	27±2	26±3	26±2	28±2	0.06
Gestational age (weeks)	NA	9±1	26±1	39±1	39±1	32±2	31±2	32±2	0.823 ^b 0.163 ^c
Newborn mass (g)	NA	NA	NA	3587±281	3450±385	NA	1781±632	NA	0.198 ^a
Apgar score(1 min)	NA	NA	NA	9.0±0.6	8.9±0.6	NA	7.3±1.2	NA	0.469 ^a

Group I, non-pregnant women; Group II, normal pregnancy at 7-12 weeks; Group III, normal pregnancy at 24-28 weeks; Group IV, delivery by cesarean section at 37-42 weeks of gestation without labor; Group V, delivery by cesarean section with term labor at 37-42 weeks; Group VI, threatening preterm labor at 28-36 weeks of gestation; Group VII, preterm labor at 28-36 weeks; Group VIII, normal pregnancy at 28-36 weeks; and NA, not applicable.

All data are presented as mean ± standard deviation.

^aP value for groups IV versus V; ^bP value for groups VI versus VII; and ^cP value for group III versus groups IV and V (Kruskal-Wallis test).

TABLE 2. The absolute number and percentage of CD4⁺CD25⁺Foxp3⁺ Tregs in peripheral blood for each group

	Group I (n=20)	Group II (n=20)	Group III (n=20)	Group IV (n=20)	Group V (n=28)	Group VI (n=16)	Group VII (n=15)	Group VIII (n=20)
Tregs/μl blood	54 (46-68)	83 (71-105)	90 (66-109)	83 (75-101)	44 (37-53)	64 (41-90)	18 (13-34)	87 (81-104)
% Tregs	3.1 (2.7-3.3)	6.4 (6.0-8.3)	7.9 (6.2-9.3)	7.5 (6.6-7.9)	3.5 (3.0-4.2)	5.3 (3.8-7.4)	1.6 (1.1-2.7)	7.4 (6.9-8.8)

Group I, non-pregnant women; Group II, normal pregnancy at 7-12 weeks; Group III, normal pregnancy at 24-28 weeks; Group IV, delivery by cesarean section at 37-42 weeks of gestation without labor; Group V, delivery by cesarean section with term labor at 37-42 weeks; Group VI, threatening preterm labor at 28-36 weeks of gestation; Group VII, preterm labor at 28-36 weeks; and Group VIII, normal pregnancy at 28-36 weeks.

All data are presented as median and interquartile range.

FIGURE 1. Flow cytometric gating strategy for analysis of CD4⁺CD25⁺Foxp3⁺ Tregs in peripheral blood. Peripheral blood lymphocytes were stained with FITC-labeled anti-CD4, APC-labeled anti-CD25, and PE-labeled anti-human Foxp3 monoclonal antibodies or with APC-labeled IgG2a isotype control (d). (a, R2), lymphocytes; (b, R1), CD4⁺ population; (c, R3), CD4⁺CD25⁺ population; and (e, R4), CD4⁺CD25⁺Foxp3⁺ population.

nancy at 28-36 weeks. The clinical data of all the groups are summarized in Table 1. There was no statistically significant difference among the studied groups in maternal age, gestational age, newborn mass, and 1 min Apgar score.

Analysis of the percentage of CD4⁺CD25⁺Foxp3⁺ Tregs in peripheral blood

Figure 1 shows flow cytometric gating strategy for the CD4⁺CD25⁺Foxp3⁺ population in peripheral blood. The absolute number and percentage of CD4⁺CD25⁺Foxp3⁺ Tregs in all the studied groups are given in Table 2. The changes in the percentage of CD4⁺CD25⁺Foxp3⁺ Tregs were consistent with the variations of the absolute Treg count in blood, indicating that the CD4⁺CD25⁺Foxp3⁺ population was strongly regulated during pregnancy. Notably, the percentage of circulating CD4⁺CD25⁺Foxp3⁺ Tregs was significantly increased in women during the first trimester compared with non-pregnant controls ($P<0.0001$), peaking during the second trimester and then slightly declining in the third trimester (Figure 2). No significant differences in the proportion of the cell population (% Tregs) were detected among the pregnant women at different gestational ages ($P=0.855$). The proportion of CD4⁺CD25⁺Foxp3⁺ Tregs was significantly lower in women at

term in labor relative to those at term without labor (3.5% [3.0-4.2%] versus 7.5% [6.6-7.9%], $P<0.0001$) (Figure 3). Women admitted in preterm labor had lower proportion of CD4⁺CD25⁺Foxp3⁺ Tregs than did those with threatening preterm labor in admission (1.6% [1.1-2.7%] versus 5.3% [3.8-7.4%], $P<0.0001$) (Figure 4).

Sixteen (76%) of the 21 subjects who delivered at preterm were diagnosed with chorioamnionitis. Interestingly, preterm women with evidence of chorioamnionitis had a significantly lower proportion of CD4⁺CD25⁺Foxp3⁺ Tregs than those without chorioamnionitis in preterm deliveries (1.7% [1.2-2.7%] versus 3.3% [2.7-5.1%], $P=0.008$) (Figure 5). There was a similar proportion of CD4⁺CD25⁺Foxp3⁺ Tregs between women without HC during preterm labor and those with term labor. Moreover, the preterm women without chorioamnionitis had a significantly lower frequency of circulating CD4⁺CD25⁺Foxp3⁺ Tregs than those with normal pregnancy at the same gestational age ($P<0.0001$).

Discussion

Here we demonstrated a dramatic increase of circulating CD4⁺CD25⁺Foxp3⁺ Tregs in pregnant women at the first trimester of gestation in comparison with non-pregnant controls,

FIGURE 2. Analysis of the proportional changes of CD4+CD25+Foxp3+ Tregs in peripheral blood. The percentage of circulating CD4+CD25+Foxp3+ Tregs is significantly increased with advancing gestational ages compared to non-pregnant controls, but there is no statistical significance in the proportion of circulating Tregs between women at the early third trimester and at term without labor. Group I, non-pregnant women; Group II, 7-12 weeks of gestation; Group III, 24-28 weeks of gestation; Group IV, delivery by cesarean section at 37-42 weeks of gestation without labor; and Group VIII, 28-36 weeks of gestation. *** $P < 0.0001$ versus non-pregnant controls.

FIGURE 3. Comparisons of the percentages of circulating CD4+CD25+Foxp3+ Tregs in women having cesarean section (CS) at term with labor ($n=28$) and without labor ($n=20$). *** $P < 0.0001$.

FIGURE 4. Comparison of circulating CD4+CD25+Foxp3+ Tregs between women admitted at threatening preterm labor (TPL; $n=16$) and actual preterm labor (PL; $n=15$). The short horizontal lines represent median values for each group.

FIGURE 5. Analysis of the association of the percentage of circulating CD4+CD25+Foxp3+ Tregs with the status of histological chorioamnionitis (HC) in preterm deliveries. Preterm women with evidence of HC have a significantly lower proportion of CD4+CD25+Foxp3+ Tregs than those without HC in preterm deliveries. There were similar frequencies of the Tregs between preterm subjects without HC and term subjects. The short horizontal lines represent median values for each group. -HC, absence of HC; +HC, presence of HC.

peaking during the second trimester and then slightly declining during the third trimester. This finding is consistent with an earlier report [23]. The elevation of CD4⁺CD25⁺Foxp3⁺ Tregs during pregnancy suggests a protective role against maternal immune reactions that could compromise survival and normal development of the placenta and embryo/fetus. Consistent with this view, patients with recurrent spontaneous abortion were found to have a significantly lower proportion of CD4⁺CD25⁺ Tregs in comparison with women with normal early pregnancy [2,9]. Hyun-Joo *et al.*, reported that, during uncomplicated pregnancy, the proportion of CD4⁺ CD25^{bright} Tregs decreased with advancing gestational age; the highest levels were seen during the first trimester and lower levels in the second and third trimesters [7]. This difference may be explained by the relative small sample size in the previous study, where only two pregnancies during the first trimester were included, or the use of different markers for Tregs.

This is the first investigation of the alteration of circulating CD4⁺CD25⁺Foxp3⁺ Tregs in preterm labor. Intrauterine infection has been implicated as a major etiologic factor resulting in the pathogenesis of preterm labor [18,24-26]. Consistent with this, 16 of our studied preterm subjects (76%) presented with chorioamnionitis. Notably, the presence of chorioamnionitis during preterm labor is linked to a significantly lower percentage of circulating CD4⁺CD25⁺Foxp3⁺ Tregs, suggesting an involvement of Tregs in the infection-associated preterm labor. This finding is consistent with a previous study [7], where the CD4⁺CD25^{bright} Treg population decreased in pregnancies complicated by premature rupture of membranes; however, the women without chorioamnionitis in preterm labor had similar levels of circulating CD4⁺CD25⁺Foxp3⁺ Tregs to those in term labor. These data highlight a potentially important role for CD4⁺CD25⁺Foxp3⁺ Tregs in pregnancy where decreased levels of CD4⁺CD25⁺Foxp3⁺ Tregs might be the consequence of microbial infection that results in the breakdown of the maternal immune homeostasis and thus contributes to the onset of preterm labor.

It is well accepted that the decline of Tregs in the postpartum period is associated with the withdrawal of the immunological stimulus of the allograft [23]. Interestingly, the percentage of the circulating CD4⁺CD25⁺Foxp3⁺ Tregs was found to be significantly diminished during term or preterm labor. Consistent with our findings, Sindram-Trujillo and colleagues have reported that there is a reduction of the CD4⁺CD25⁺ T cell subpopulation in decidua basalis and parietalis from women with spontaneous vaginal delivery in comparison with those with elective cesarean section without labor [27]. Recently, Galazka *et al.* demonstrated that the CD4⁺CD25⁺Foxp3⁺

Tregs in the deciduas of pregnant women decreased with the successive stages of labor [28]. In contrast, levels of many inflammatory cytokines such as interleukin (IL)-1, 6, 8 and tumor necrosis factor- α (TNF- α) increase at the beginning of labor [29,30]. IL-17-producing Th17 cells are well established to be reciprocally related to Foxp3⁺ Tregs and can rapidly initiate an inflammatory response [31]. A recent study documented that Th17 cell number increased in the chorioamniotic membrane of preterm delivery cases with chorioamnionitis [32]. Based on these findings, we propose that the decline in the levels of CD4⁺CD25⁺Foxp3⁺ Tregs was coupled with the activation of inflammatory cascades, which subsequently contributed to labor progression. The exact mechanism of the Treg decline during labor remains unclear. It may be partially due to the elevation of inflammatory cytokines, since some of them such as IL-6 can block the suppressive effect of CD4⁺CD25⁺ Tregs through the Toll pathway on DCs and inhibit transforming growth factor- β (TGF- β)-induced Treg differentiation [17,33].

In conclusion, our data demonstrate that the proportion of circulating CD4⁺CD25⁺Foxp3⁺ Tregs is increased with advancing gestation age during early pregnancy but decreased during labor at term or preterm, and that women in preterm labor with evidence of intrauterine infection have a reduced proportion Tregs. These findings suggest an important role for circulating CD4⁺CD25⁺Foxp3⁺ Tregs in the maintenance of pregnancy and the initiation of labor.

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