Reproductive Sciences
2017, Vol. 24(7) 1025-1032

DOI: 10.1177/1933719116676395
journals.sagepub.com/home/rsx
SAGE

Regulatory T Cells Show Dynamic Behavior During Late Pregnancy, Delivery, and the Postpartum Period

Jorge Lima, MD[1,2], Catarina Martins, PhD[2], Glória Nunes, BSc[2], Maria-José Sousa, MD, PhD[3,4], Jorge C. Branco, MD, PhD[5], and Luís-Miguel Borrego, MD, PhD[2,6]

Abstract

Regulatory T cells (Tregs) are critical immunomodulators during early pregnancy by preventing maternal T-cell activation against fetal cells. However, how populations of maternal Tregs vary during and after pregnancy in humans is still unclear. Therefore, we investigated Treg subsets in the peripheral blood of pregnant women from late pregnancy through the postpartum period. To accomplish this, the following circulating Treg subsets were analyzed in 43 healthy pregnant women and 35 nonpregnant women by flow cytometry during the third trimester, on the day of delivery, and postpartum: $CD4^{Dim}CD25^{Hi}$, $CD4^{+}CD25^{Hi}Foxp3^{+}$, and $CD4^{+}CD25^{Hi}CD127^{-/dim}$. Additionally, the expression levels of the transcription factor Foxp3 in $CD4^{Dim}CD25^{Hi}$ Treg were analyzed. We have found that $CD4^{Dim}CD25^{Hi}$ Treg subset significantly decreased in the pregnant women on the day of delivery relative to the third trimester ($P < .05$), and that all Treg subsets significantly increased postpartum compared to the third trimester and the day of delivery ($P < .05$). Moreover, the Foxp3 expression ratios within the $CD4^{Dim}CD25^{Hi}$ Treg subset decreased during pregnancy and until delivery compared to those measured in the nonpregnant women and significantly increased postpartum compared to the third trimester and the day of delivery ($P < .05$). Thus, despite their established role in offering immunoprotection to the fetus in early pregnancy, the number of circulating Tregs also varies from late pregnancy to the postpartum period. Our results offer an explanation for the possible effects of pregnancy on the clinical outcomes of some autoimmune diseases during the postpartum period.

Keywords

regulatory T cells, immunology, Foxp3, immunomodulation, pregnancy

Background

The survival of a semiallogeneic fetus is a unique immunological challenge during pregnancy. Regulatory T cells (Tregs) are a specialized T-cell subpopulation involved in preventing autoimmunity and graft rejection,[1] as they are potent suppressors of inflammatory immune responses. Additionally, Tregs prevent maternal T-cell activation against fetal cells, and this protection of the fetus from the maternal immune system has been widely reported in both mice and humans.[2-4] A reduction in maternal Treg populations could cause failure of immunological tolerance to the fetus and has been associated with obstetrical complications, such as miscarriage, preeclampsia, and preterm labor.[5-10]

Although numerous Treg-specific markers have been proposed for mice and humans, the transcription factor Foxp3 is still the most consistent marker, and this protein seems to be essential for the function of these immunosuppressive cells.[11,12] In humans, the detection of $CD4^{Dim}CD25^{Hi}$, $CD4^{+}CD25^{Hi}Foxp3^{+}$, and $CD4^{+}CD25^{Hi}CD127^{-/dim}$ T-cell populations remains one of the most common analytical strategies used to identify Treg subsets.[3,13-15]

Treg expansion in the decidua of normal pregnant women is most robust during early pregnancy and decreases as term approaches, suggesting that the immune regulatory processes that occur at the maternal–fetal interface are dynamic and

[1] Department of Obstetrics and Gynecology, CUF Descobertas Hospital, Lisbon, Portugal
[2] Department of Immunology, Chronic Diseases Research Center (CEDOC), Faculty of Medical Sciences, NOVA Medical School, Lisbon, Portugal
[3] Centro de Medicina Laboratorial Germano Sousa, Lisbon, Portugal
[4] Department of Clinical Pathology, Hospital Prof. Fernando Fonseca, E.P.E., Amadora, Portugal
[5] Obstetrics and Gynecology, Private Medical Clinic, Lisbon, Portugal
[6] Department of Immunoallergy, CUF Descobertas Hospital, Lisbon, Portugal

Corresponding Author:
Jorge Lima, Department of Obstetrics and Gynecology, CUF Descobertas Hospital, Parque das Nações, Rua Mário Botas, 1998-018 Lisbon, Portugal.
Email: jorgeramoslima@sapo.pt

provide immune protection for the fetus.[4,16] Variations in Treg numbers during human pregnancy occur not only at the decidua but also in the maternal peripheral blood.[6,17]

Treg subsets in peripheral blood have been explored in normal human pregnancy and during the postpartum period in several studies; however, the results have been highly variable, with some reports showing a decrease[18] or an increase[3,10,19-22] in the first trimester, a decrease[18,23] or an increase[3,10,19,20,24] in the second trimester, or a decrease[3,4,10,18-20,22] or an increase[24] in the third trimester, and others showing either no changes[22] or a decline[10,24] during labor and a decrease[3,10,19] or an increase[25] in the postpartum period. These discrepancies can be attributed to differences in study design, as some studies do not include the postpartum period,[4,18,20,22-24] and in others, not all women contribute samples at all time points.[3,10,18-20,22,25] Contrasting reports of Treg patterns may also be attributed to differences in sample size or the use of different and sometimes nonspecific markers for Treg subsets. Therefore, the currently available data on variations in maternal Treg subsets during pregnancy need reevaluation. Consequently, the aim of this study was to characterize the evolution of Treg subsets (defined as $CD4^{Dim}CD25^{Hi}$, $CD4^{+}CD25^{Hi}Foxp3^{+}$, and $CD4^{+}CD25^{Hi}CD127^{-/dim}$ T cells) and the expression of Foxp3 within $CD4^{Dim}CD25^{Hi}$ Treg subset in the peripheral blood of normal pregnant women from late pregnancy through the postpartum period and to compare these measurements with those taken from nonpregnant women.

Materials and Methods

Study Population

This was a prospective observational study that followed healthy pregnant women overtime to characterize (through cell quantification and phenotype identification) peripheral blood Tregs ($CD4^{Dim}CD25^{Hi}$, $CD4^{+}CD25^{Hi}Foxp3^{+}$, and $CD4^{+}CD25^{Hi}CD127^{-/dim}$ T cells) and Foxp3 expression in $CD4^{Dim}CD25^{Hi}$ Tregs from late pregnancy through the postpartum period. Furthermore, changes in the above parameters were compared between the pregnant women and nonpregnant women to identify associations with pregnancy.

We recruited sequential nonlaboring pregnant women with singleton pregnancies who were attending routine obstetrical care while in the third trimester of pregnancy. Furthermore, we also recruited sequential nonpregnant women who were attending routine annual well-woman examinations.

Pregnant women were recruited if their fetuses exhibited appropriate growth (as measured by uterine fundal height and by an ultrasound performed after 28 weeks of gestation) and if they had no pregnancy complications (either prior or after study inclusion). The following exclusion criteria were applied to all of the women: diabetes, hypertension, autoimmune or vascular disease, infection (HIV, syphilis, or hepatitis B or C), or smoking during the 6 months prior to study inclusion. Furthermore, pregnant women were excluded if they had a preterm delivery (<37 weeks of gestation), signs of infection, if they required labor induction, or if they required administration of prenatal medication (other than vitamins and iron supplements). We also excluded nonpregnant women who were taking oral contraceptives, as these drugs may influence peripheral blood lymphocytes.[26]

All of the women were recruited between July 2013 and March 2014 from *CUF Descobertas Hospital* in Lisbon (Portugal). The ethics committee of the hospital approved our study protocol, and all the recruited women provided written informed consent before study inclusion.

Study Procedures

To characterize peripheral blood Tregs and Foxp3 expression from late pregnancy through the postpartum period, 3 blood samples were collected: the first was collected during the third trimester of pregnancy, the second was collected on the day of delivery (within 15 minutes after placental expulsion and oxytocin administration), and the last was collected during the postpartum period (at least 6 weeks after delivery). A single peripheral blood sample was taken from the nonpregnant women at a planned visit, which occurred during the follicular phase of the menstrual cycle.

Demographics, anthropometrics (body mass index [BMI]), obstetric history, and systolic and diastolic blood pressures were collected as baseline data for all the women. Gestational age (at baseline and at delivery), type of analgesia and/or anesthesia, and mode of delivery were collected only for the pregnant women. Gender, birth weight, and 1-minute and 5-minute Apgar scores were collected for the newborns.

Flow Cytometry Analysis and Laboratory Measurements

Blood samples (anticoagulated with EDTA) were evaluated by multicolor flow cytometry in a BD FACS Calibur (BD Biosciences, San Jose, California) equipped with 2 lasers (488 nm air-cooled argon–ion laser and 635 nm red diode laser). All samples were processed within 24 hours of collection.

A BD Multitest IMK kit (BD Biosciences) was used to obtain absolute counts of lymphocyte subsets and to characterize these subsets according to the manufacturer's instructions.

For Treg phenotyping, a panel of monoclonal antibodies (mAbs) including anti-CD3-FITC (clone SK7; BD Biosciences), anti-CD25 PE (clone BC96; Biolegend, San Diego, California), anti-CD4 PerCP Cy5.5 (clone SK3; Biolegend), and anti-CD127 Alexa Fluor 647 (clone A019D5; Biolegend) antibodies was employed with a lyse–wash protocol using BD FACS lysing solution (BD Biosciences). The Human FoxP3 Buffer Set (BD Pharmingen, San Jose, California) was also used to characterize Foxp3 expression. In brief, cells were lysed, washed, and then incubated with the surface mAbs anti-CD4 and anti-CD25 (referred to the above). Staining with Anti-Foxp3 Alexa Fluor 488 (clone 259D/C7; BD Pharmingen) was performed after fixing and permeabilizing the cells with the reagents supplied in the Human FoxP3 Buffer Set kit. A minimum of 10 000 CD4 T cells were acquired per tube, and

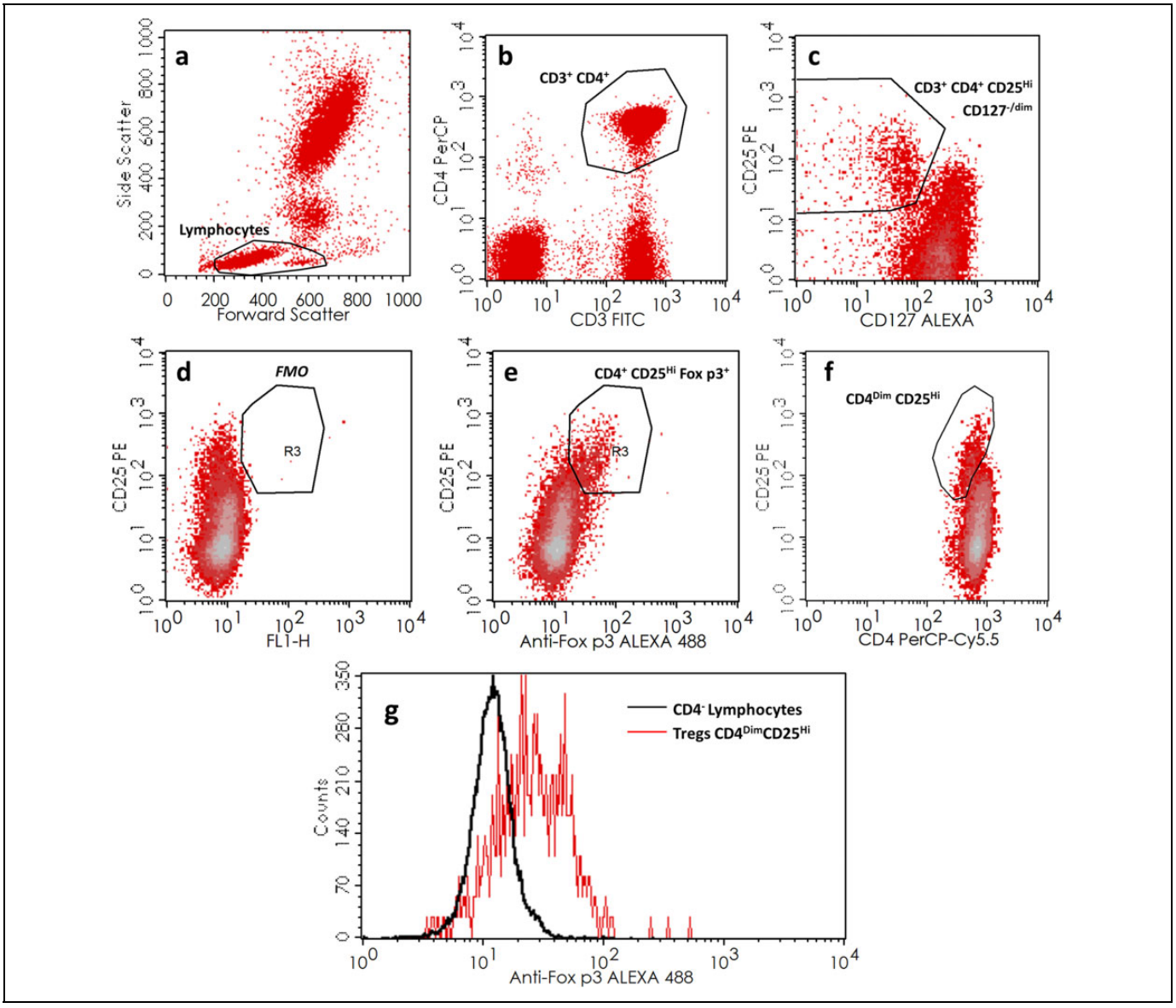

Figure 1. Gating strategy for the identification of Tregs. A, Lymphocyte gate on FSC/SSC. B, Identification of CD4 T cells within lymphocyte populations ($CD3^+CD4^+$ T cells). C, Identification of $CD4^+CD25^{Hi}CD127^{-/dim}$ Tregs. D and E, Identification of $CD4^+CD25^{Hi}Foxp3^+$ Tregs with dot plots of fluorescence minus one (FMO) (D) and Foxp3 (E) tubes. F, CD4 versus CD25 dot plots showing the identification of $CD4^{Dim}CD25^{Hi}$ Tregs. G, Histogram for the evaluation of Foxp3 expression in $CD4^{Dim}CD25^{Hi}$ Tregs (red line), overlaid on that for the expression of Foxp3 within $CD4^-$ lymphocytes (black line). Tregs indicates regulatory T cells; FSC/SSC, Lymphocyte gate on forward scatter/side scatter.

data analysis was performed using CellQuest software (BD Biosciences).

The gating strategies used are described in Figure 1. In brief, CD4 T cells were gated within the lymphocyte cluster using a combined Boolean gating strategy. Bivariate dot plots of CD25 versus CD127, CD25 versus Foxp3, and CD25 versus CD4 were further used to identify $CD4^{Dim}CD25^{Hi}$, $CD4^+CD25^{Hi}Foxp3^+$, and $CD4^+CD25^{Hi}CD127^{-/dim}$ regulatory subsets, which were quantified as percentages of total CD4 T cells. Fluorescence minus one tubes and internal cell populations were used as negative controls to assess CD25, CD127, and Foxp3 positivity.[27,28]

The expression of the transcription factor Foxp3 was assessed within the $CD4^{Dim}CD25^{Hi}$ Treg subset using the geometric mean values of mean fluorescence intensity (MFI) units, which were converted into a ratio (MFI of Foxp3 in $CD4^{Dim}CD25^{Hi}$ Treg/MFI of Foxp3 in $CD4^-$ lymphocytes) as previously described to reduce the impact of day-to-day variation.[29]

Statistical Analysis

Data normality was assessed using Shapiro-Wilk test. Baseline data were presented as means ($\pm$standard deviations) if normally distributed or as medians and ranges if not normally

Table 1. Demographic and Clinical Characteristics of Nonpregnant Women and Pregnant Women and Their Newborns.

Characteristic	Nonpregnant Woman (n = 35)	Pregnant Women (n = 43)
Age, median (range), years	35.0 (20-40)	32.0 (25-41)[a]
Parity: nulliparous, n (%)	5 (14.3)	24 (55.8)[a]
Gestational age, median (range), weeks		
Third trimester		33.0 (31-35)
Day of delivery		39.0 (37-41)
Postpartum evaluation, median (range), days		45 (41-58)
Mode of delivery, n (%)		
Vaginal		18 (41.8)
Cesarean		25 (58.2)
Newborns		
Birth weight in grams, mean (± standard deviation)		3265.0 (±393.5)
Gender: male, n (%)		22 (51)
Apgar score, median (range)		
1-minute Apgar score		9 (6-10)
5-minute Apgar score		10 (9-10)

[a]Statistically significant differences ($P < .05$) identified between the healthy pregnant women and the healthy nonpregnant women.

distributed. Categorical variables were described using absolute and relative frequencies. Treg counts and percentages were presented as medians and ranges. If normally distributed, 2 independent groups were compared using Student *t* tests; otherwise, Mann-Whitney *U* tests were used. If normally distributed, paired data were compared using paired Student *t* tests; otherwise, Wilcoxon signed-rank tests were used. For normally distributed data, comparisons between more than 2 groups were performed using analysis of variance; otherwise, Kruskal-Wallis tests were used. Statistical significance was defined by a P value <.05. The P values for the comparisons of Treg subsets and Foxp3 expression levels at different time points were adjusted for multiplicity using the Benjamini and Yekutieli method.[30] All of the data were analyzed using R software, version 3.12 for Windows.

Results

Baseline Characteristics

Our study included a total of 78 healthy women (43 pregnant and 35 nonpregnant). The demographic and clinical data collected for all the participants and their newborns are presented in Table 1. The mean BMI was 21.5 (±2.8) kg/m^2 for the nonpregnant women and 26.2 (±2.8) kg/m^2 for the pregnant women. All the women had normal blood pressures (mean systolic blood pressure: 119.8 [±10.5] mm Hg for the nonpregnant women and 115.7 [±9.3] mm Hg for the pregnant women; mean diastolic blood pressure: 74.7 [±7.4] mm Hg for the nonpregnant women and 67.4 [±7.4] mm Hg for the pregnant women). The median time since the last pregnancy (regardless of whether the pregnancies were interrupted or resulted in a live birth) for the nonpregnant women was 169 (23-449) weeks. For the pregnant women, the median gestational age at the third trimester of pregnancy was 33.0 (31-35) weeks, and on day of delivery, it was 39.0 (37-41) weeks. The group of pregnant women was significantly younger ($P < .05$) and included significantly more nulliparous women ($P < .05$) than the group of nonpregnant women.

All vaginal deliveries were spontaneous and received epidural analgesia during labor. All cesarean sections were performed with regional anesthesia. All women were discharged from the hospital 2 days after a vaginal delivery or 3 days after a cesarean section. Postpartum evaluations were undertaken at a median of 45 (41-58) days after delivery.

Characterization of Tregs

The Treg characterization results for all of the enrolled women are presented in Figure 2. Relative to during the third trimester, on the day of delivery, a significant decrease in the percentage of $CD4^{Dim}CD25^{Hi}$ Tregs ($P < .05$) was found, but no other significant differences were identified for percentages of Tregs between any of the other study visits. However, postpartum, there was a significant increase ($P < .05$) in the absolute counts of Tregs ($CD4^{Dim}CD25^{Hi}$, $CD4^{+}CD25^{Hi}Foxp3^{+}$, and $CD4^{+}CD25^{Hi}CD127^{-/dim}$) compared with the values obtained during the third trimester and on the day of delivery. The absolute counts and percentages of $CD4^{Dim}CD25^{Hi}$ Tregs, $CD4^{+}CD25^{Hi}Foxp3^{+}$ Tregs, and $CD4^{+}CD25^{Hi}CD127^{-/dim}$ Tregs did not significantly differ ($P \geq .05$) between the nonpregnant and pregnant women at any of the study visits.

Foxp3 Expression in $CD4^{Dim}CD25^{Hi}$ Tregs

The ratios of Foxp3 expression in $CD4^{Dim}CD25^{Hi}$ Tregs for all of the enrolled women are presented in Figure 3. This ratio was significantly lower ($P < .001$) during the third trimester and on the day of delivery in the pregnant women compared to the nonpregnant women. Postpartum, the ratios of Foxp3 expression in $CD4^{Dim}CD25^{Hi}$ Tregs significantly increased ($P < .001$) compared to the values measured during the third trimester and on the day of delivery, reaching expression levels similar to those observed in the control group of nonpregnant women. No statistically significant differences ($P \geq .05$) were identified for this ratio between the third trimester and the day of delivery.

Discussion

In the present study, we investigated whether Treg subsets varied in the peripheral blood of normal women between late pregnancy and the postpartum period. We found that percentages of $CD4^{Dim}CD25^{Hi}$ Tregs in pregnant women decreased significantly on the day of delivery compared to those measured during the third trimester ($P < .05$). This variation was not observed in the other tested subsets of Tregs ($CD4^{+}CD25^{Hi}Foxp3^{+}$ and $CD4^{+}CD25^{Hi}CD127^{-/dim}$ T cells), which can be explained by the heterogeneity of Treg

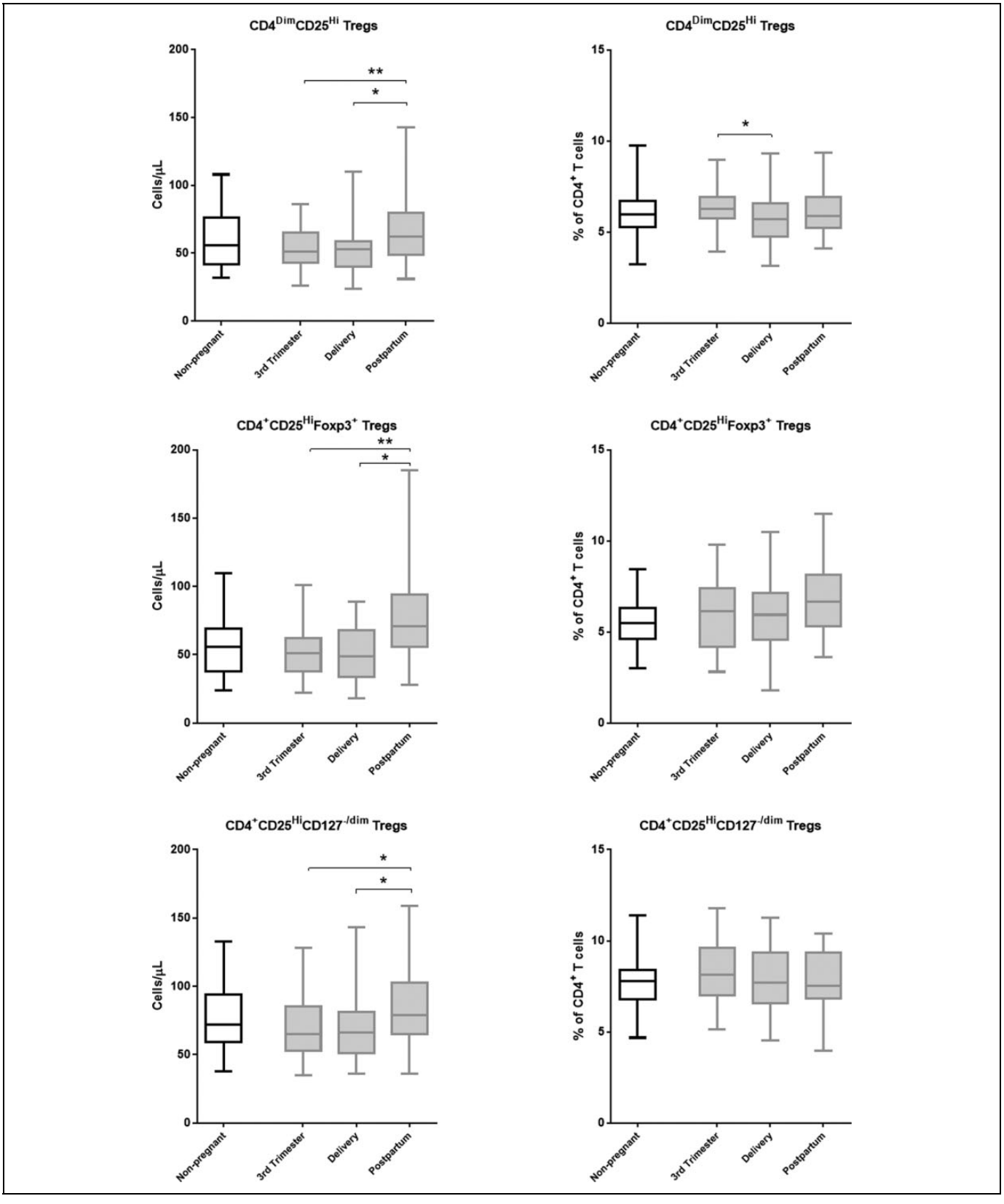

Figure 2. Absolute counts and percentages of regulatory T cells (Tregs) in peripheral blood samples. Delivery, within 15 minutes after placental expulsion; postpartum, at least 6 weeks after delivery. The bottom of the box represents the 25th percentile, the top of the box represents the 75th percentile, the horizontal line inside the box represents the median, the bottom whiskers represent the minimum value, and the top whiskers represent the maximum value. *$P < .05$; **$P < .001$.

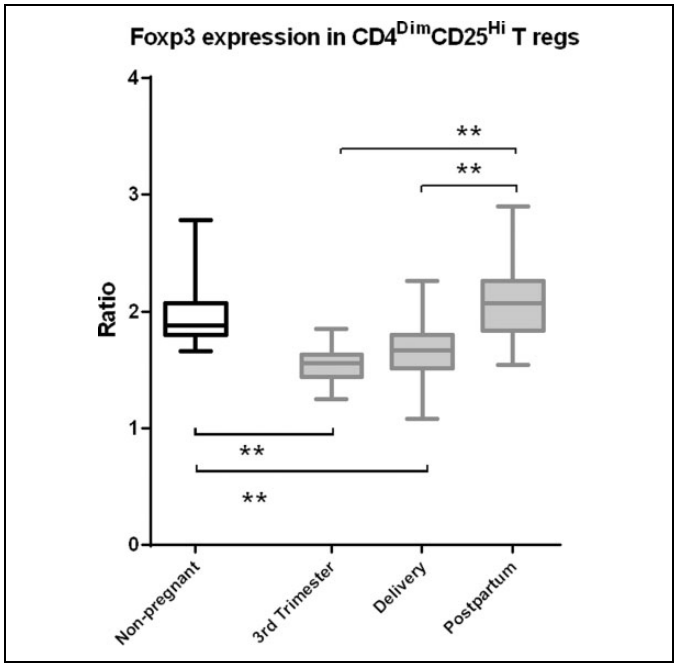

Figure 3. Foxp3 expression in $CD4^{Dim}CD25^{Hi}$ regulatory T cells (Tregs). Delivery, within 15 minutes after placental expulsion and postpartum, at least 6 weeks after delivery. The bottom of the box represents the 25th percentile, the top of the box represents the 75th percentile, the horizontal line inside the box represents the median, the bottom whiskers represent the minimum value, and the top whiskers represent the maximum value. *$P < .05$; **$P < .001$.

populations. For example, Loewendorf et al[31] demonstrated that defining Tregs as $CD4^{+}CD25^{Hi}CD127^{-/dim}$ underestimates the number of CD4 T cells that express Foxp3.

Our results are consistent with those of previous human studies showing that Treg subsets in peripheral blood decrease on the day of delivery. These studies[10,24] suggest that Tregs play a role in the immunological changes that occur before labor, and that human labor may be initiated as an effect of the decrease in Tregs. In addition, similar to the results published by Galazka et al[32] that demonstrated a decrease in Tregs in the decidua during labor, the decrease in Tregs in peripheral blood might also reflect an activation of the inflammatory immune system on the day of delivery. Furthermore, hormone changes owing to pregnancy appear to impact Treg populations in humans. Xiong et al[3] showed that Treg subsets in peripheral blood are positively correlated with estrogen levels, a relationship that might play an important immunomodulatory role during pregnancy. Additionally, another study recently demonstrated that membrane progesterone receptor α is present on Tregs, suggesting an association between the immune system and the initiation of human labor; notably, in the referenced study, the highest Treg values were measured during the third trimester, and these values decreased on the day of delivery.[33] However, the decrease in Tregs on the day of delivery found in our study is small compared to those reported in preceding studies.[10,24] This difference in Tregs' variation might be explained by the use of different approaches to characterize Treg subsets and by different sample sizes. Therefore, the exact mechanism of Tregs' decline in peripheral blood on the day of delivery remains unclear.

In the present study, we used 3 analytical strategies to characterize Treg subsets ($CD4^{Dim}CD25^{Hi}$, $CD4^{+}CD25^{Hi}Foxp3^{+}$, and $CD4^{+}CD25^{Hi}CD127^{-/dim}$ T cells) and found similar patterns overall, namely, that significantly higher counts of Tregs were present in circulating blood in the postpartum period compared to the third trimester and the day of delivery ($P < .05$). These results are consistent with the conclusions of Wegienka et al[25] but are inconsistent with earlier studies.[3,10,19] The increase in absolute Treg counts suggests that an immunosuppressed environment, which allows maternal–fetal tolerance in pregnancy, is also present postpartum. According to our findings, we hypothesize that Treg expansion is a potential physiological mechanism for downregulating the activation of maternal immunological events (ie, causing less susceptibility to systemic inflammatory responses) that occur during the postpartum period. Nevertheless, an explanation for the Tregs' increase during this period is still under investigation.

We also studied Foxp3 expression levels in $CD4^{Dim}CD25^{Hi}$ Tregs. We found that during pregnancy and until delivery, Foxp3 expression was lower within the $CD4^{Dim}CD25^{Hi}$ Tregs in the pregnant women compared to the nonpregnant women. It has been reported that progesterone and 17β-estradiol reduce Foxp3 expression in Tregs in midpregnancy.[23] Our data are consistent with these findings, which probably extend through the third trimester and delivery. Postpartum, Foxp3 expression increased significantly compared to the third trimester and the day of delivery, reaching levels similar to those measured in the control group of nonpregnant women. This confirms that pregnancy causes modulation in Foxp3 expression.

Furthermore, postpartum, we observed fluctuations in Foxp3 expression that showed a similar pattern to that previously described for $CD24^{Hi}CD38^{Hi}$ regulatory B cells in healthy human pregnancies, namely, absolute counts and percentages increased significantly compared to those measured during the third trimester of pregnancy and on the day of delivery.[34] These results are concordant with recent studies in human cell lines, demonstrating that regulatory B cells play an important role in Treg differentiation by increasing Foxp3 expression in these cells.[35,36]

Our study included only women with a normal singleton pregnancy between 37 and 41 weeks of gestation to obtain a relatively homogenous sample and reduce potential bias. In addition, all the enrolled pregnant women contributed samples at all of the study's time points (third trimester, delivery day, and postpartum). Preferably, samples from additional time points, such as during the first and second trimesters of pregnancy, would be included, as well as the monitoring of women from before becoming pregnant through the postpartum period. However, these additional time points were beyond the scope of this study.

We also accounted for the phase of the menstrual cycle during which the samples were collected from the control group. Peripheral blood samples were collected from the

nonpregnant women during the follicular phase of the menstrual cycle, as hormone status during the luteal phase is comparable to that during pregnancy.[37] However, a previous study[38] reported that Treg subsets expand during the follicular phase, reaching a peak immediately before ovulation (to induce immune tolerance to facilitate implantation) and then decline in the subsequent luteal phase. This may have contributed to the absence of significant differences in Treg numbers between the nonpregnant and pregnant groups. Additionally, because crucial immunological events are more likely to occur at the fetal–maternal interface, it is important that future studies compare Tregs in both the peripheral blood and the decidua from the same healthy pregnant women.

In this study, we performed complementary measurements of both percentages and absolute counts of different Treg subsets. The use of percentages allows for the interpretation of the relative fluctuations in Treg cell subsets from late pregnancy through the postpartum period. Although absolute counts were also measured, pregnancy is characterized by variable degrees of hemodilution, during which changes in total numbers of circulating Tregs may not necessarily reflect variations in these subsets. Further functional and epigenetic experiments are required to identify T cells, expressing Foxp3 as Tregs because activated T cells without regulatory function can also present a Foxp3-positive phenotype.[39]

In conclusion, we found that $CD4^{+}CD25^{Hi}$ Treg subset decreases significantly on the day of delivery compared to those measured during the third trimester in healthy pregnant women. We also showed significant increases in circulating Treg subsets and in Foxp3 expression in $CD4^{Dim}CD25^{Hi}$ Treg subsets in the postpartum period compared to during the third trimester and on the day of delivery. Our results support that Tregs, despite their established immunoprotective role for the fetus in early pregnancy, present dynamic behavior in the postpartum period. Although enhancement of Treg function may contribute to systemic changes that occur in the maternal immune system, modifying for instance the clinical course of some autoimmune diseases,[40,41] this does not necessarily increase maternal susceptibility to infections. We suggest that variations in Treg numbers from late pregnancy through the postpartum period are reflective to pregnancy's effect on the maternal immune system and may permanently affect the immune status of a woman and have implications for future pregnancies.

Authors' Note

Jorge Lima conceived of the original research idea, while all of the authors designed the study and created the study protocol. Jorge Lima recruited the patients and collected the data. Catarina Martins and Glória Nunes analyzed the blood samples using flow cytometry. Luís-Miguel Borrego supervised all the work and the research protocol. All of the authors contributed to data analysis and interpretation. Jorge Lima drafted the manuscript, and all of the authors revised it and contributed to it intellectually. All of the authors have approved the final version of the manuscript. All procedures performed in studies involving human participants were conducted in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This article does not contain any studies with animals performed by any of the authors. Informed consent was obtained from all individual participants included in the study.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was partially funded by José de Mello Saúde.

References

1. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol*. 1995;155(3):1151-1164.
2. Aluvihare VR, Kallikourdis M, Betz AG. Regulatory T cells mediate maternal tolerance to the fetus. *Nat Immunol*. 2004;5(3): 266-271.
3. Xiong YH, Yuan Z, He L. Effects of estrogen on CD4(+) CD25(+) regulatory T cell in peripheral blood during pregnancy. *Asian Pac J Trop Med*. 2013;6(9):748-752.
4. Heikkinen J, Mottonen M, Alanen A, Lassila O. Phenotypic characterization of regulatory T cells in the human decidua. *Clin Exp Immunol*. 2004;136(2):373-378.
5. Zenclussen AC, Gerlof K, Zenclussen ML, et al. Abnormal T-cell reactivity against paternal antigens in spontaneous abortion: adoptive transfer of pregnancy-induced CD4+CD25+ T regulatory cells prevents fetal rejection in a murine abortion model. *Am J Pathol*. 2005;166(3):811-822.
6. Sasaki Y, Darmochwal-Kolarz D, Suzuki D, et al. Proportion of peripheral blood and decidual CD4(+) CD25(bright) regulatory T cells in pre-eclampsia. *Clin Exp Immunol*. 2007;149(1):139-145.
7. Darmochwal-Kolarz D, Saito S, Rolinski J, et al. Activated T lymphocytes in pre-eclampsia. *Am J Reprod Immunol*. 2007; 58(1):39-45.
8. Nakashima A, Shima T, Inada K, Ito M, Saito S. The balance of the immune system between T cells and NK cells in miscarriage. *Am J Reprod Immunol*. 2012;67(4):304-310.
9. Koucky M, Malickova K, Cindrova-Davies T, et al. Low levels of circulating T-regulatory lymphocytes and short cervical length are associated with preterm labor. *J Reprod Immunol*. 2014;106: 110-117.
10. Xiong H, Zhou C, Qi G. Proportional changes of CD4+ CD25+Foxp3+ regulatory T cells in maternal peripheral blood during pregnancy and labor at term and preterm. *Clin Invest Med*. 2010;33(6):E422.
11. Roncador G, Brown PJ, Maestre L, et al. Analysis of FOXP3 protein expression in human CD4+CD25+ regulatory T cells at the single-cell level. *Eur J Immunol*. 2005;35(6):1681-1691.

12. Finak G, Langweiler M, Jaimes M, et al. Standardizing flow cytometry immunophenotyping analysis from the Human ImmunoPhenotyping Consortium. *Sci Rep*. 2016;6:20686.
13. Liu W, Putnam AL, Xu-Yu Z, et al. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. *J Exp Med*. 2006;203(7):1701-1711.
14. Seddiki N, Santner-Nanan B, Martinson J, et al. Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. *J Exp Med*. 2006; 203(7):1693-1700.
15. Santner-Nanan B, Peek MJ, Khanam R, et al. Systemic increase in the ratio between Foxp3+ and IL-17-producing CD4+ T cells in healthy pregnancy but not in preeclampsia. *J Immunol*. 2009; 183(11):7023-7030.
16. Saito S, Nakashima A, Shima T, Ito M. Th1/Th2/Th17 and regulatory T-cell paradigm in pregnancy. *Am J Reprod Immunol*. 2010;63(6):601-610.
17. Tilburgs T, Roelen DL, van der Mast BJ, et al. Differential distribution of CD4(+)CD25(bright) and CD8(+)CD28(-) T-cells in decidua and maternal blood during human pregnancy. *Placenta*. 2006;27(suppl A):S47-S53.
18. Kisielewicz A, Schaier M, Schmitt E, et al. A distinct subset of HLA-DR+-regulatory T cells is involved in the induction of preterm labor during pregnancy and in the induction of organ rejection after transplantation. *Clin Immunol*. 2010;137(2):209-220.
19. Somerset DA, Zheng Y, Kilby MD, Sansom DM, Drayson MT. Normal human pregnancy is associated with an elevation in the immune suppressive CD25+ CD4+ regulatory T-cell subset. *Immunology*. 2004;112(1):38-43.
20. Steinborn A, Haensch GM, Mahnke K, et al. Distinct subsets of regulatory T cells during pregnancy: is the imbalance of these subsets involved in the pathogenesis of preeclampsia? *Clin Immunol*. 2008;129(3):401-412.
21. Sasaki Y, Sakai M, Miyazaki S, Higuma S, Shiozaki A, Saito S. Decidual and peripheral blood CD4+CD25+ regulatory T cells in early pregnancy subjects and spontaneous abortion cases. *Mol Hum Reprod*. 2004;10(5):347-353.
22. Seol HJ, Oh MJ, Lim JE, Jung NH, Yoon SY, Kim HJ. The role of CD4+CD25bright regulatory T cells in the maintenance of pregnancy, premature rupture of membranes, and labor. *Yonsei Med J*. 2008;49(3):366-371.
23. Mjosberg J, Svensson J, Johansson E, et al. Systemic reduction of functionally suppressive CD4dimCD25highFoxp3+ Tregs in human second trimester pregnancy is induced by progesterone and 17beta-estradiol. *J Immunol*. 2009;183(1):759-769.
24. Areia A, Vale-Pereira S, Alves V, et al. Can membrane progesterone receptor alpha on T regulatory cells explain the ensuing human labour? *J Reprod Immunol*. 2015;113:22-26.
25. Wegienka G, Havstad S, Bobbitt KR, et al. Within-woman change in regulatory T cells from pregnancy to the postpartum period. *J Reprod Immunol*. 2011;88(1):58-65.
26. Auerbach L, Hafner T, Huber JC, Panzer S. Influence of low-dose oral contraception on peripheral blood lymphocyte subsets at particular phases of the hormonal cycle. *Fertil Steril*. 2002;78(1):83-89.
27. Hulspas R, O'Gorman MR, Wood BL, Gratama JW, Sutherland DR. Considerations for the control of background fluorescence in clinical flow cytometry. *Cytometry B Clin Cytom*. 2009;76(6): 355-364.
28. Keeney M, Gratama JW, Chin-Yee IH, Sutherland DR. Isotype controls in the analysis of lymphocytes and CD34+ stem and progenitor cells by flow cytometry–time to let go! *Cytometry*. 1998;34(6):280-283.
29. Dendrou CA, Fung E, Esposito L, Todd JA, Wicker LS, Plagnol V. Fluorescence intensity normalisation: correcting for time effects in large-scale flow cytometric analysis. *Adv Bioinformatics*. 2009;2009:476106.
30. Benjamini Y, Yekutieli D. The control of the false discovery rate in multiple testing under dependency. *Ann Stat*. 2001;29(4): 1165-1188.
31. Loewendorf AI, Nguyen TA, Yesayan MN, Kahn DA. Normal human pregnancy results in maternal immune activation in the periphery and at the uteroplacental interface. *PLoS One*. 2014; 9(5):e96723.
32. Galazka K, Wicherek L, Pitynski K, et al. Changes in the subpopulation of CD25+ CD4+ and FOXP3+ regulatory T cells in decidua with respect to the progression of labor at term and the lack of analogical changes in the subpopulation of suppressive B7-H4 macrophages—a preliminary report. *Am J Reprod Immunol*. 2009;61(2):136-146.
33. Areia A, Vale-Pereira S, Alves V, Rodrigues-Santos P, Moura P, Mota-Pinto A. Membrane progesterone receptors in human regulatory T cells: a reality in pregnancy. *BJOG*. 2015;122(11): 1544-1550.
34. Lima J, Martins C, Leandro MJ, et al. Characterization of B cells in healthy pregnant women from late pregnancy to post-partum: a prospective observational study. *BMC Pregnancy Childbirth*. 2016;16(1):1-13.
35. Flores-Borja F, Bosma A, Ng D, et al. CD19+CD24hiCD38hi B cells maintain regulatory T cells while limiting TH1 and TH17 differentiation. *Sci Transl Med*. 2013;5(173):173ra23.
36. Kessel A, Haj T, Peri R, et al. Human CD19(+)CD25(high) B regulatory cells suppress proliferation of CD4(+) T cells and enhance Foxp3 and CTLA-4 expression in T-regulatory cells. *Autoimmun Rev*. 2012;11(9):670-677.
37. Shinoda R, Watanabe M, Nakamura Y, Maruoka H, Kimura Y, Iwatani Y. Physiological changes of Fas expression in peripheral lymphocyte subsets during the menstrual cycle. *J Reprod Immunol*. 2003;60(2):159-168.
38. Arruvito L, Sanz M, Banham AH, Fainboim L. Expansion of CD4+CD25+and FOXP3+ regulatory T cells during the follicular phase of the menstrual cycle: implications for human reproduction. *J Immunol*. 2007;178(4):2572-2578.
39. Morgan ME, van Bilsen JH, Bakker AM, et al. Expression of FOXP3 mRNA is not confined to CD4+CD25+ T regulatory cells in humans. *Hum Immunol*. 2005;66(1):13-20.
40. Habibagahi M, Habibagahi Z, Jaberipour M, Aghdashi A. Quantification of regulatory T cells in peripheral blood of patients with systemic lupus erythematosus. *Rheumatol Int*. 2011;31(9): 1219-1225.
41. Perricone C, de Carolis C, Perricone R. Pregnancy and autoimmunity: a common problem. *Best Pract Res Clin Rheumatol*. 2012;26(1):47-60.