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Altered Frequencies of $CD4^+$ $CD25^+$ $Foxp3^+$ and $CD8^+$ $CD25^+$ $Foxp3^+$ Regulatory T Cells in Pre-eclampsia

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ABSTRACT

Regulatory T cells are of utmost importance for tolerating the fetus. In some pregnancy complications such as pre-eclampsia, the frequency of $CD4^+CD25^+Foxp3^+$ regulatory T cells is altered, but there is no consistency regarding the results. Besides, little is known about the frequency of $CD8^+CD25^+Foxp3^+$ Treg cells in pregnancy complications. Therefore, we aimed to investigate the frequency of both $CD4^+$ and $CD8^+$ regulatory T cells in the peripheral blood of women afflicted by preeclampsia.

Ten non-pregnant, ten healthy pregnant, and ten preeclamptic women participated in this study. Four colors flow cytometry method was used to identify the frequency of the $CD4^+$ and $CD8^+$ regulatory T cells in the peripheral blood.

Results indicated that the frequencies of $CD4^+CD25^+Foxp3^+$ and $CD8^+CD25^+Foxp3^+$ cells were significantly lower in preeclamptic women compared to healthy pregnant and non-pregnant ones ($p<0.05$). A positive correlation was also observed between $CD4^+$ and $CD8^+$ regulatory T cells ($R=0.532$, $p=0.002$). Moreover, $CD4^+$ regulatory T cells negatively correlated with systolic and diastolic blood pressures ($R=-0.760$ and -0.753, respectively; $p<0.001$). $CD8^+$ regulatory T cells also had a negative correlation with systolic ($R=-0.503$, $p=0.001$) and diastolic ($R=-0.590$, $p=0.005$) blood pressures.

In conclusion, a reduction in the frequencies of both $CD4^+$ $CD25^+$ $Foxp3^+$ and $CD8^+CD25^+Foxp3^+$ regulatory T cells might be important in the pathogenesis of pre-eclampsia.

Keywords: CD4; CD8; CD25; Foxp3; Pre-eclampsia; Pregnancy; Regulatory T cells

INTRODUCTION

During a normal pregnancy, a semi allogeneic fetus is engrafted to the maternal decidua without any symptoms of immune rejection. There are several local and systemic regulatory mechanisms which protect the fetus against maternal immune system such as uterine entrapment of antigen presenting cells, release of inhibitory exosomes via trophoblast cells into maternal circulation, and presence of regulatory molecules such as membrane bound and soluble forms of HLA-G inhibitory molecule.[1,2] When the maternal immune system does not tolerate the fetus well,

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pregnancy related disorders may occur.[3] Pre-eclampsia (PE) is one of the important pregnancy complications which occurs in the third trimester and is characterized by high blood pressure (BP) and proteinuria. Several factors including angiogenic factors, endothelial and vascular dysfunction, and immune system play roles in the pathophysiology of pre-eclampsia.[4] There are evidences that show both innate and adaptive immune systems are involved in the pathogenesis of PE. One of the most important cell populations of the adaptive immune system which may play a role in PE are T lymphocytes and their regulatory subpopulations.[5,6] Both $CD4^+$ and $CD8^+$ regulatory T cells are important modulator of the immune responses and play role in pregnancy.[7,8] Several studies have evaluated the probable association between the frequency of these cells and pre-eclampsia. Although changes in the frequency and function of $CD4^+$ regulatory T cells are reported to be associated with different pregnancy complications such as recurrent spontaneous abortion and preterm labor,[9-12] there is not a constancy regarding the frequency of $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T cells in the peripheral blood of preeclamptic patients. To explain the issue more clearly, it should be noted that several investigators reported a decrease in the frequency of $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T cells in preeclamptic women compared to healthy pregnant women; while others did not observe any significant changes.[13-15] In addition to the well-known $CD4^+$ $CD25^+$ $Foxp3^+$ Treg cells, there is also a regulatory population within $CD8^+$ T cells which express CD25 and Foxp3 molecules.[16] This subset is studied in some immunological states such as tumor and organ transplantation,[17-19] but little is known about these regulatory T cells in normal and complicated pregnancies.

Considering the important role of regulatory T cells in a normal pregnancy, the present study was carried out to investigate the frequency of $CD25^+$ $Foxp3^+$ regulatory cells within both $CD4^+$ and $CD8^+$ T cells in the peripheral blood of preeclamptic women and compare them with healthy pregnant and non-pregnant women.

MATERIALS AND METHODS

Subjects and Samples

Ten pregnant women with de-novo hypertension and proteinuria were included in the preeclamptic patient group. Inclusion criteria for the patients were: a) systolic blood pressure≥140 mmHg or diastolic blood pressure≥90 mmHg, b) proteinuria (at least 1^+ protein in the urine test strip or more than 0.3 gr protein in 24-hour urine).[20] Ten women with healthy pregnancy (HP) were also included in the study (age- and gestational month-matched to the PE group). These subjects had at least one previous successful pregnancy without any complication. Subjects who had chronic hypertension, gestational diabetes, HELLP (hemolysis, elevated liver enzymes, and low platelet count) syndrome, cancer, active infection or autoimmune disorders were excluded from the studied groups. Ten healthy non-pregnant (NP) women without any history of pregnancy at the secretory phase of the menstrual cycle were also included as control. These subjects were age-matched with the two previous groups. Ten mL of heparinized blood was collected from all participants after obtaining written informed consents approved by local ethics committee ofShiraz University of Medical Sciences, Shiraz, Iran (N.: 1395-S144). Healthy pregnant and preeclamptic subjects were selected from women who referred to Zeynabiye and Hafez hospitals, Shiraz University of Medical Sciences, Shiraz, Iran. Clinical features of patients and controls are indicated in Table 1.

Peripheral blood mononuclear cell (PBMC) Isolation and Cryopreservation

PBMCs were isolated from ten ml of peripheral blood by density centrifugation using Lymphoprep (Axis-Shield, Oslo, Norway). Mononuclear cells were washed using RPMI 1640 media (Gibco by Thermo Fisher Scientific, New York, USA) and counted, then transferred to cryovials containing freezing media and preserved in liquid nitrogen tank until the time of experiments. The preservation period was less than one month for each sample.

Flow Cytometry

At the time of the experiments, cryotubes were thawed and the cells were washed two times using RPMI 1640 media. Viability of the cells was determined by trypan blue exclusion test and more than 95% of the cells were viable. Human serum was added to the cell suspension (10% v/v) in order to block Fc receptors (10 min at 4ºC).

100 µL of the suspension containing $1*10^6$ cells was transferred to the flow cytometry tube and

Table 1. Clinical and demographic characteristics of preeclamptic, healthy pregnant, and non-pregnant women

	Non-pregnant (n=10)	Healthy pregnant (n=10)	Preeclamptic (n=10)	*p* value
Age (Years±SD)	28.4±2.6	28.6±2.0	29.7±1.9	0.393
Pregnancy month (Mean±SD)	-	8.4±0.5	8.6±0.5	0.398
Gravidity (Mean±SD)	-	2.3±0.4	1.8±0.7	0.105
Systolic BP (Mean±SD)	107±6.7	104±5.1	134±6.9	<0.001*
Diastolic BP (Mean±SD)	73±4.2	73±4.8	93±4.8	<0.001*
Urine dipstick protein test	0	0	1^+ or 2^+ **	-

One-way ANOVA and independent samples T test were used for data analysis. *p* values less than 0.05 were considered to be statistically significant. *The difference was between the preeclamptic group and the two other groups. ** Four patients had 1^+ and six had 2^+ protein in the urine dipstick. BP: blood pressure

incubated with specific antibodies against CD3-PerCP, CD4-APC, CD8-APC, and CD25-FITC. Following 20 minutes incubation at 4ºC, the cells were washed with 2% fetal bovine serum/phosphate buffered saline (FBS/PBS). Intracellular staining was performed using anti Foxp3 antibody (PE) and human Foxp3 buffer set. All antibodies were purchased from Biolegend (Biolegend, San Diego, California, USA). After staining, the cells were fixed using 1% paraformaldehyde and $1*10^5$ cells were analyzed using 4 color FACSCalibur flow cytometer (BD Biosciences, San Jose, California, USA). Frequencies of the $CD4^+$ $CD25^+$ $Foxp3^+$ and $CD8^+$ $CD25^+$ $Foxp3^+$ regulatory T cells were determined using FlowJo Software (Version 7.6.1).

Statistical Analysis

Statistical analysis was performed using SPSS Statistics for Windows, version 18.0 (SPSS Inc., Chicago, Ill., USA). One Sample Kolmogorov-Smirnov and Levene´s test were used in order to check normal distribution of the variables and homogeneity of variances, respectively. Comparisons between two groups and more than two groups were performed using independent samples T test and one-way ANOVA followed by Tukey´s post hoc test, respectively. Spearman's rank correlation coefficient was used to analyze the relationship between frequency of Treg cell subsets and quantitative continuous variables. *p* values less than 0.05 were considered statistically significant. Graphpad prism software version 5 was used for plotting graphs.

RESULTS

Frequency of $CD4^+CD25^+Foxp3^+$ Treg cells in the peripheral blood

The percentage of $CD4^+CD25^+Foxp3^+$ regulatory T cells in total $CD3^+CD4^+$ cells was determined in non-pregnant, healthy pregnant, and preeclamptic women. Results revealed statistical differences between groups regarding the frequency of these cells (*p* value<0.001). Healthy pregnant women had the highest level of regulatory T cells (1.72±0.33 %), followed by NP (1.21±0.19 %) and PE women (0.86±0.09 %). Figure 1-A shows the differences between studied groups regarding the frequency of $CD4^+CD25^+Foxp3^+$ regulatory cells in total $CD4^+$ T cells pool. Figure 2 depicts the flow cytometric analysis and the gating strategy for detection of $CD4^+$ and $CD8^+$ regulatory T cells.

Frequency of $CD8^+CD25^+Foxp3^+$ Treg cells in the Peripheral Blood

Percentage of $CD8^+$ regulatory T cells was determined in the peripheral blood as depicted in Figure 2. Mean±SD frequencies of $CD8^+CD25^+Foxp3^+$ cells in the peripheral blood of participants were as follows: 0.92±0.17% in HP women, 0.73±0.14% in NP women, and 0.52±0.18% in preeclamptic ones (*p* value<0.001). Healthy pregnant and non-pregnant groups had significant differences regarding the frequency of $CD8^+$ regulatory T cells with preeclamptic group (Figure 1-B).

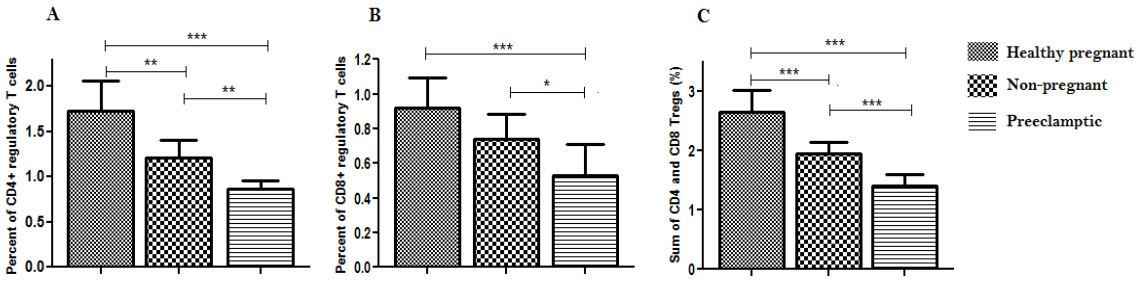

Figure 1. Frequencies of $CD4^+CD25^+Foxp3^+$ and $CD8^+CD25^+Foxp3^+$ regulatory T cells in the peripheral blood of preeclamptic, healthy pregnant, and non-pregnant women . A) There were significant differences regarding the frequency of $CD4^+$ regulatory T cells between 3 studied groups. Healthy pregnant women had the highest and preeclamptic patients had the lowest frequency of level of $CD4^+$ regulatory T cells. B) PE patients had significant differences regarding $CD8^+$ regulatory T cells with two other groups. C) Sum of $CD4^+$ and $CD8^+$ regulatory T cells pools was significantly different between studied groups, and PE patients had the lowest frequency of total regulatory cells. One-way ANOVA followed by Tukey´s post hoc test was used for data analysis. *p* value < 0.05 was considered statistically significant. *<0.05, **<0.01, *<0.001.**
PE: preeclamptic; Treg: regulatory T cells

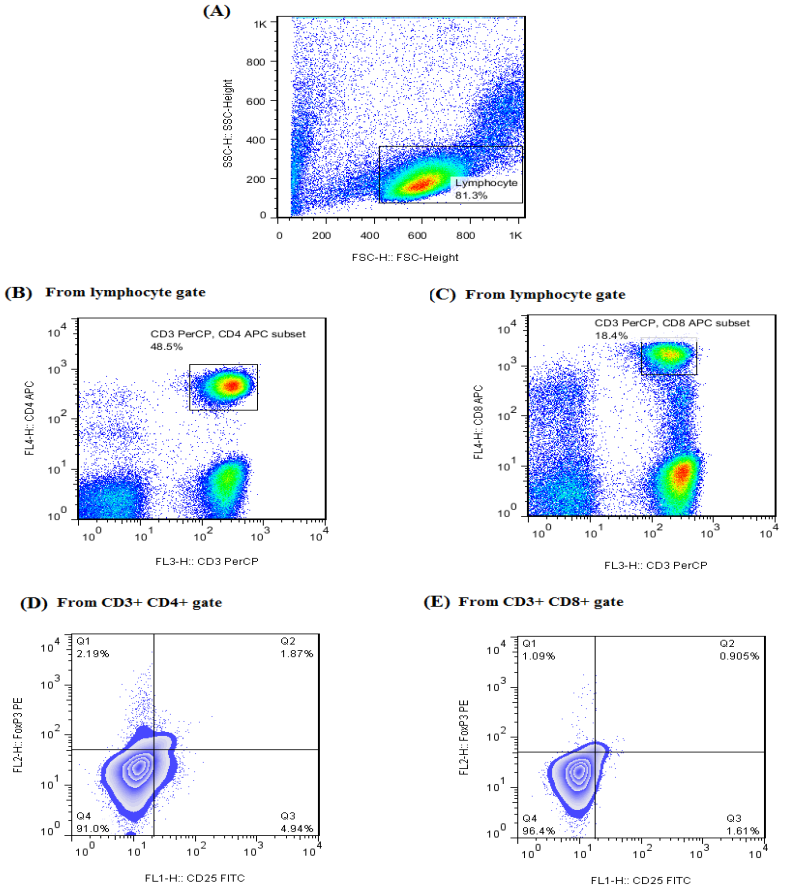

Figure 2. Gating strategy for the detection of $CD4^+$ and $CD8^+$ regulatory T cells in the peripheral blood of preeclamptic, healthy pregnant, and non-pregnant women. For gating, A) lymphocytes were gated based on the forward & side scatter parameters, B) then $CD3^+CD4^+$ or C) $CD3^+CD8^+$ T cells were selected of the lymphocyte gate. CD25 and Foxp3 markers were plotted against each other and the frequency of the D) $CD3^+CD4^+CD25^+Foxp3^+$ and E) $CD3^+CD8^+CD25^+Foxp3^+$ regulatory cells was determined in the pool of $CD4^+$ or $CD8^+$ T cells. FSC: forward scatter; SSC: side scatter

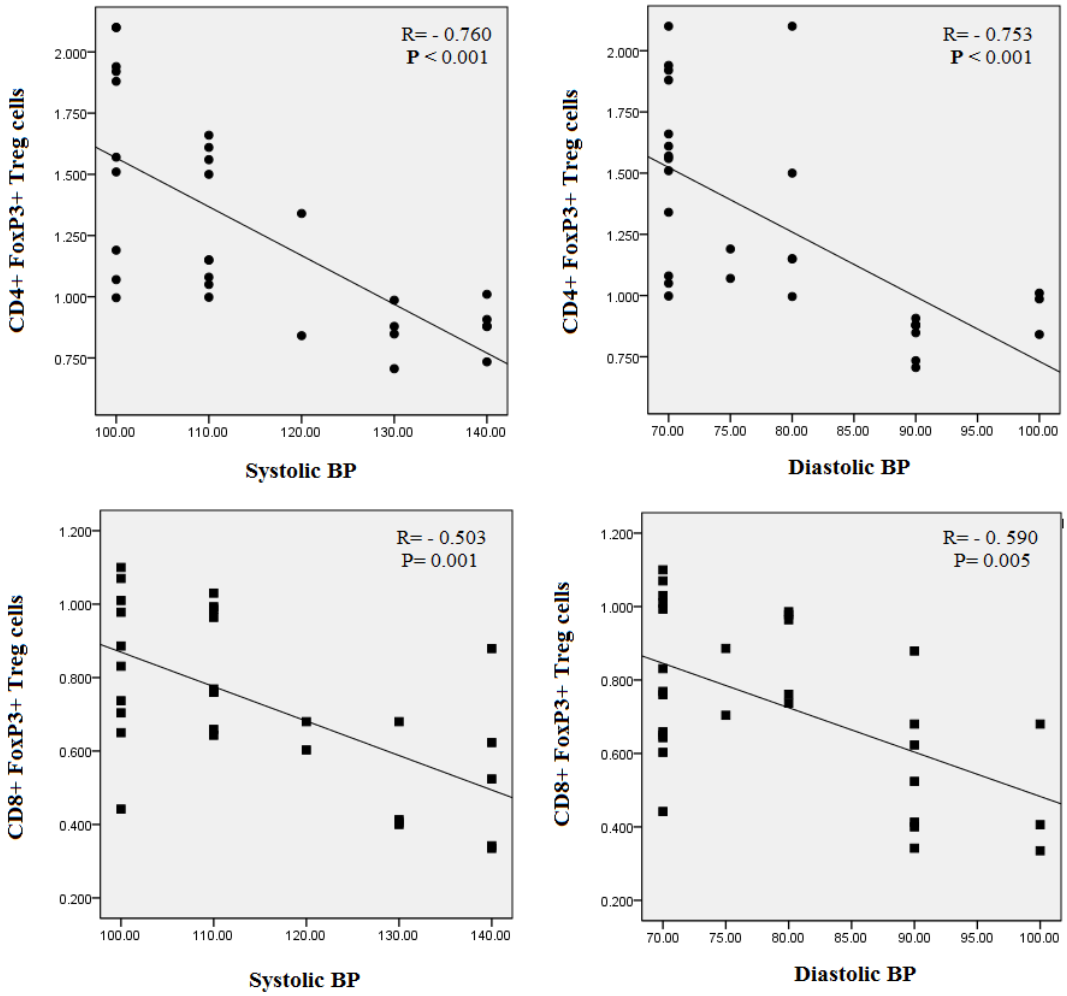

Figure 3. Correlation between percentage of regulatory T cells subsets and blood pressure in all preeclamptic, healthy pregnant, and non-pregnant women. Both $CD4^+$ and $CD8^+$ regulatory T cells had negative correlation with systolic and diastolic blood pressures. Spearman's rank correlation coefficient was used for data analysis. *p* value < 0.05 was considered statistically significant.

BP: blood pressure; P: *p* value; R: correlation coefficient; Treg: regulatory T cells

Correlation between $CD4^+$ and $CD8^+$ regulatory T cells frequencies

Interestingly, a positive correlation was also detected between $CD4^+$ and $CD8^+$ regulatory T cells in the peripheral blood of all participants (R=0.532, p=0.002). As different regulatory mechanisms work in concert to maintain immune tolerance in the body, the sum of $CD4^+$ and $CD8^+$ regulatory T cells was also determined in different groups. A significant difference was detected between groups regarding total $CD4^+$ and $CD8^+$ regulatory T cell pools (p<0.001). In line with our previous results, preeclamptic patients had the lowest frequency of total regulatory T cells in the peripheral blood (1.39±0.19%). The mean±SD frequency of total Treg cells in HP and NP subjects was 2.64±0.35 and 1.94±0.19 %, respectively (Figure 1-C).

Spearman's rank correlation coefficient was used to analyze the relationship between frequency of $CD4^+$ and $CD8^+$ regulatory T cell subsets and variables such as age, systolic BP, and diastolic BP. There was no correlation between age and subsets of regulatory T cells. Both $CD4^+$ and $CD8^+$ regulatory T cells had significant negative correlations with both systolic and diastolic BP (Figure 3).

DISCUSSION

In the present study, we found that the conventional $CD4^+CD25^+Foxp3^+$ regulatory T cells were significantly lower in preeclamptic subjects compared to healthy pregnant and non-pregnant women. Several experiments have investigated the alteration of the peripheral blood $CD4^+$ regulatory T cells frequencies during normal pregnancy and pre-eclampsia, but there is no constancy regarding data. For example, Toldi et al.[21,22] and Prins et al.[13] have found that the number of $CD4^+$ Treg cells decrease in pre-eclampsia compared to normal pregnancy which is in line with results of the present study. We detected -0.50 and -0.28 fold change decreases in total regulatory T cells pools in PE patients compared to healthy pregnant and non-pregnant women, respectively. On the other hand, Hu et al.,[23] Nagayama et al.,[24] and Paeschke et al.[15] have reported that there were not any significant differences regarding the percentage of regulatory T cells among non-pregnant, normal pregnant and preeclamptic groups. Steinborn and his colleagues also have reported a significant decrease in the frequency of peripheral blood $CD4^+CD25^+Foxp3^+$ Treg cells in third trimester of pregnancy compared to non-pregnant women,[25] while our results indicated that healthy pregnant women had higher $CD4^+$ regulatory T cells compared to non-pregnant subjects. We detected a 0.41 fold change increase in $CD4^+$ regulatory T cells in normal pregnant women compared to non-pregnant controls. This increase in the frequency of $CD3^+CD4^+CD25^+Foxp3^+$ Treg cells in a normal pregnancy might be regulated through hormones such as estrogen and androgens,[26-28] as these hormones modulate $CD4^+$ T cells polarization[26] and also augment Foxp3 expression in $CD4^+CD25^+Foxp3^+$ regulatory T cells.[27-29]

Regarding $CD8^+$ regulatory T cells, many studies have highlighted the importance of these cells in different clinical conditions such as organ transplantation and tumors.[16-19] In case of pregnancy, only one article has recently been published which investigated the frequency of these cells in normal pregnancy and pre-eclampsia. Wang and his colleagues reported that the peripheral blood frequency of $CD8^+CD25^+Foxp3^+$ regulatory T cells was significantly lower in preeclamptic patients compared to healthy pregnancy.[30] We also had similar results regarding this cell subset, as preeclamptic patients had the lowest frequency of $CD8^+CD25^+Foxp3^+$ regulatory T cells in the peripheral blood. The fold change decreases of this population in PE patients were also similar to $CD4^+$ regulatory T cells. The positive correlation between $CD4^+$ and $CD8^+$ regulatory T cells may suggest that these cells work in concert to establish immune tolerance, as the differences in pairwise comparisons between groups are more significant when the sum of $CD4^+$ and $CD8^+$ regulatory T cells is considered, compared to each regulatory cell population alone. So, the decrease in both $CD4^+$ and $CD8^+$ regulatory T cells in PE patients might play a role in the pathogenesis of pre-eclampsia. Simultaneous investigation of these regulatory T cells may provide a more valuable insight regarding immune tolerance mechanisms in different pathological contexts such as pre-eclampsia.

We also observed that $CD4^+$ and $CD8^+$ regulatory T cells negatively correlated to systolic and diastolic blood pressures. Considering the importance of $Foxp3^+$ regulatory T cells in other hypertension related diseases such as coronary artery disease or pulmonary hypertension,[31,32] we propose that the decrease in the $Foxp3^+$ regulatory T cells might play a role in the pathogenesis of pregnancy hypertension, as preeclamptic women had the lowest frequency of these cells in the peripheral blood.

The main limitation of our study was the sample size. Also, we were not able to assess the function of Treg cells in the context of pregnancy and pre-eclampsia. Therefore, additional experiments that simultaneously investigate both the frequency and function of different regulatory T cell subsets, with increased sample size, are needed to provide a more accurate insight regarding the maternal immune tolerance and pre-eclampsia.

In summary, our results lead to the following conclusions: a) the frequencies of both $CD3^+CD4^+CD25^+Foxp3^+$ and $CD3^+CD8^+CD25^+Foxp3^+$ cells decrease in pre-eclampsia. b) There is a positive correlation between $CD4^+$ and $CD8^+$ regulatory T cells; suggesting that these cells may work in concert to establish immune tolerance. c) $CD4^+$ and $CD8^+$ $Foxp3^+$ regulatory T cells might play a role in the pathogenesis of pregnancy hypertension. Finally, we propose that preeclamptic patients might have defects in immune tolerance mechanisms as they had lower frequency of both $CD4^+$ and $CD8^+$ regulatory T cells in their peripheral blood compared to normal pregnant women.

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