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ORIGINAL ARTICLE

Evaluation of Exhausted Regulatory T Cells in Preeclampsia

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ABSTRACT

Background: The development of a maternal immune response to fetal antigens and deficiency in regulatory T-cells (Tregs) may lead to preeclampsia. A plausible explanation for the reduced Treg cell function in women with preeclampsia is the presence of exhausted Treg cells which express CD279 or programmed cell death receptor-1 (PD-1), a negative regulatory molecule associated with limited proliferative capacity and reduced immune suppression. **Objective:** To assess the number of Treg $CD4^+$ $CD25^{high}$ and exhausted Treg $CD4^+$ $CD25^{high}$ $CD279^+$ cells in women with preeclampsia (PE group) and healthy pregnant women (HP group) during the third trimester of pregnancy. **Methods:** Three-color flow cytometry was used to determine the proportion of Treg and exhausted Treg cells in 40 women in the PE group and 37 women in the HP group. Participants' blood samples were placed in EDTA blood collection tubes. Peripheral mononuclear cells were separated from the samples and stained with flurochrome-conjugated antibodies against human CD4, CD25 and CD279 markers, and subsequently analyzed by flow cytometry. **Results:** The PE group had fewer Tregs compared to the HP group (p=0.011). There was a significant increase in the percentage of exhausted PD-1^+(CD279) Tregs (p=0.035) in the PE group comparisons with the HP group. **Conclusion:** The increased number of PD-1 (CD279) molecules on the Treg cells may play a role in preeclampsia, hence it recommendation as a therapeutic target for the disease.

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Keywords: Exhausted Regulatory T Cells, Flow cytometry, PD-1, Preeclampsia

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INTRODUCTION

One of the severe complications in human pregnancy is preeclampsia (PE) which occurs in the third trimester after 20 weeks of pregnancy (1). Preeclampsia and eclampsia, with no known definitive causes, are the most common reasons for maternal and fetal mortality in 2%-10% of all pregnancies worldwide (2). Moreover, PE has a complex pathophysiology and is the consequence of abnormal placenta formation (3). Regulatory T-cells (Tregs) are a subpopulation of $CD4^+$ T-cells detected by the presence of $CD4^+$ and $CD25^{high}$ markers on their surfaces. These T-cells have a role in the maintenance of immune system homeostasis and development of tolerance to self-antigens (4). Tregs are involved in the regulation and function of B, T, natural killer (NK), NKT, and antigen presenting cells (4-6). Tregs perform their regulatory function through cell-to-cell contact and with soluble mediators such as CTLA-4, IL-10, TGFβ, and IL-35 (7,8). Exhausted and nonfunctional Treg phenotypes have been reported in infectious and chronic diseases (9). Exhausted Tregs express CD279 [programmed cell death-1 (PD-1)] – a negative regulatory molecule and programmed cell death factor. Treg exhaustion is associated with a gradual loss of proliferation and effector cytokine production (10), along with the co-expression of the inhibitory receptors T-cell immunoglobulin, mucin-domain containing-3 (Tim-3), and lymphocyte activation gene-3 (LAG-3) (11). CD274 or programmed cell death ligand-1 (PDL-1) is a ligand for CD279 on Tregs. Both PD-1 and its ligand are receptors involved in T-cell exhaustion (12). Tregs play an essential role in immune response regulation and tolerance against the fetus (13). During a normal pregnancy, Tregs increase in the blood and maternal decidua (14). The reduced number of Tregs may worsen systemic inflammation in patients with PE, and the prevalence of these cells is lower in the peripheral blood of women with PE compared to HP. Studies on women with PE have shown a reduced Treg population in the peripheral blood and uterine decidua (15,16). Certain studies, on the other hand, have observed similar numbers of Tregs in both patients and controls (2,17). Alterations in Tregs are possibly able to contribute to the development of PE. In this study, we sought to clarify the role of exhausted Tregs in PE and HP women in their third trimester of pregnancy. Further conducted were flow cytometry analyses, investigating the number of Treg $CD4^+$ CD25high and exhausted Treg $CD4^+$ CD25high $CD279^+$ cells in both PE and HP groups.

MATERIALS AND METHODS

Sample Collection. In this case-control study, we enrolled 40 women with PE and 37 healthy pregnant women in their trimester of pregnancy (HP). Both cases and controls were between 18-35 years old and matched for age. The Ethics Committee of Ahvaz University of Medical Sciences (AJUMS) approved this study (IR.AJUMS.REC.1395.1), and all participants provided written informed consent for study participation. Inclusion criteria for PE group were systolic blood pressure ≥140 mmHg, diastolic blood pressure ≥90 mmHg, and proteinuria of 300 mg per 24 hours or 30 mg/dl in 2 random urine samples during the third trimester of pregnancy. Excluded subjects were those with chronic kidney disease, history of high blood pressure before the 20th week of pregnancy and prior to pregnancy, bleeding during pregnancy, diabetes mellitus, and autoimmune diseases before pregnancy. The HP (control) group had no history of any chronic kidney diseases, high blood pressure, bleeding, or autoimmune and infectious diseases prior to

pregnancy. Both groups, during the third trimester, provided 3-5 mL samples of blood, then placed in EDTA blood collection tubes (18) and sent to Imam Khomeini Hospital and Razi Hospital, affiliated with AJUMS.

Flow cytometry. Peripheral blood mononuclear cells were isolated by standard density gradient centrifugation for 25 minutes at 400 g and 22°C (Ficoll Paque, BaharAfshan , Iran). The cells were then washed twice with phosphate buffered saline (PBS). White blood cells were stained for 30 minutes at room temperature in the dark with monoclonal antibodies against CD4, CD25, and CD279 markers and their respective Isotype controls (eBioscience, USA). After washing with PBS, the cells were analyzed on a BD FACSCalibur flow cytometer (BD Biosciences, USA), where 100,000 cells were recorded per sample. The data were processed by FlowJo_V10 software, and further examined were $CD4^+$ $CD25^{high}$ (19) and exhausted $CD4^+$ $CD25^{high}$ $CD279^+$ Treg cells.

Statistical Analysis. The Kolmogorov-Smirnov and independent *t-tests* were used to determine the normality of the data and data comparison for both groups, respectively. p-value <0.05 were considered significant. GraphPad Prism software (version 6, USA) was used for calculations. Data are presented as mean ± SEM.

RESULTS

Table 1 lists the clinical characteristics of both groups. Tregs were defined as cells with $CD4^+$ $CD25^{high}$ markers and exhausted Tregs were considered as those containing $CD4^+$ $CD25^{high}$ $CD279^+$ markers.

Table 1. Clinical characteristics of study participants.

Characteristics	Healthy pregnant women (n=37) Mean ± SEM	Preeclamptic women (n=40) Mean ± SEM	p-value
Age (years)	26.5 ± 0.821	28.5 ± 0.925	0.226
Gestational age (weeks)	34 ± 0.322	32 ± 0.534	0.075
Systolic blood pressure (mmHg)	117.5 ± 1.212	160 ± 3.325	0.022*
Diastolic blood pressure (mmHg)	70 ± 1.341	90 ± 3.021	0.031*
Proteinuria	_	≥1+	0.045*

Blood pressure ≥140 mm Hg systolic or ≥90 mm Hg diastolic and proteinuria +1 or higher in the beginning of the twentieth week of pregnancy and the following weeks are diagnostic criteria for preeclampsia.

Figure 1 shows the gating strategy for Tregs and exhausted Tregs and the percentages of Tregs and exhausted Tregs. The results showed a significant decrease in the percentage of $CD4^+$ $CD25^{high}$ Tregs in the PE group compared to the HP group (p=0.011; Table 2). The comparison of exhausted $CD4^+$ $CD25^{high}$ $CD279^+$ Tregs between the two groups showed significantly increased numbers of exhausted Tregs ($CD279^+$) in the PE group (p=0.035; Table 2).

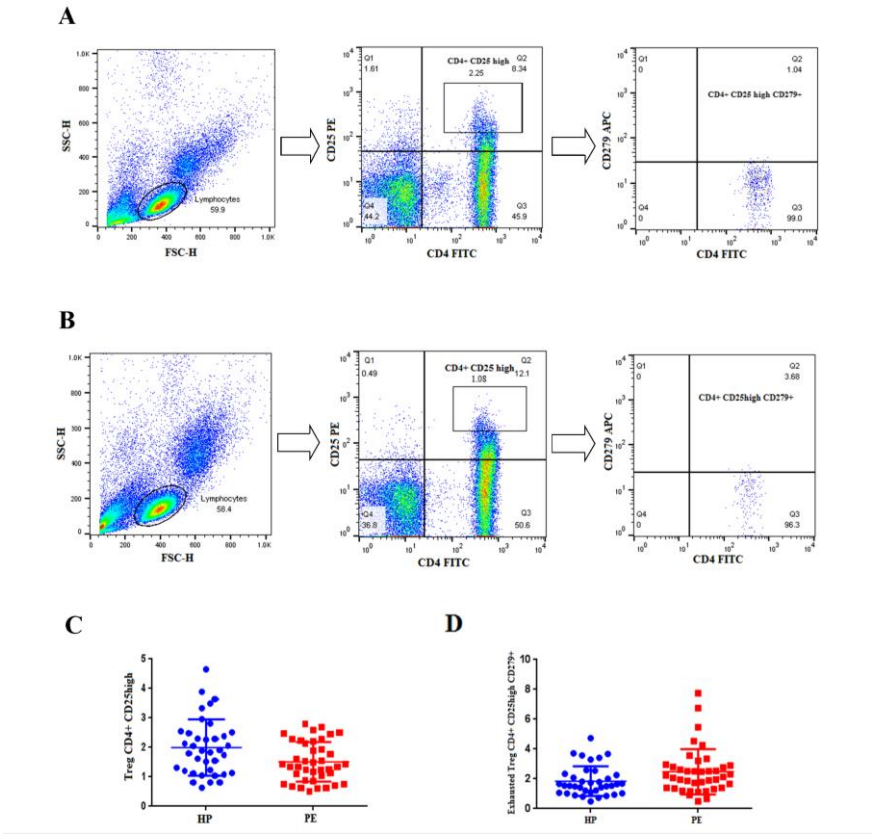

Figure 1. Gating strategies and repertoire of Treg and exhausted Treg cells in peripheral blood of the patient and control groups. (A) Representative flow cytometry analyses of peripheral blood Tregs and exhausted Tregs stained with mAbs to CD4, CD25, and CD279 in healthy pregnant women (HP). **(B)** Representative flow cytometry analyses of peripheral blood Tregs and exhausted Tregs stained with mAbs to CD4, CD25, and CD279 in preeclamptic women (PE). **(C)** The percentage of Treg cells significantly decreased in the PE group compared to the HP group. **(D)** The percentage of exhausted Tregs significantly increased in the PE group compared to the HP group.

Table 2. Prevalence of the cell subsets in women with healthy pregnant (HP) and preeclampsia (PE).

Subset	Marker	HP (n=37), Mean ± SEM	PE (n=40), Mean ± SEM	p-value
Tregs	$CD4^+$ $CD25^{high}$	1.99 ± 0.156	1.51 ± 0.106	**0.011***
Exhausted Tregs	$CD4^+$ $CD25^{high}$ $CD279^+$	1.84 ± 0.163	2.48 ± 0.366	**0.035***

DISCUSSION

In this study, the number of Tregs and exhausted Tregs was evaluated in women with PE versus HP women. There was a reduction in the ratio of $CD4^+$ $CD25^{high}$ Tregs in the PE group compared to the HP group. Although numerous studies have reported lower number of Tregs in women with PE compared to HP,(15,20,21) certain studies have observed

similar numbers of Tregs between the patient and control groups (2,17). Despite years of research, the etiology of PE remains unknown. Researchers propose that dysregulated systemic and placental immunity conduce to impaired angiogenesis and the onset of preeclampsia (22). Tregs impose a suppressive effect through cell-to-cell contact and cytokine production (23). Interleukin-10 (IL-10), a key immunosuppressive cytokine produced by Tregs, increases during early pregnancy and remains elevated until the onset of labor (24). IL-10 and its receptor (IL-10R) are expressed on placental trophoblasts, decidual stromal cells, macrophages, and uterine NK cells, which are located at the maternal-fetal interface in both mice and humans (25). Studies show increased percentages of Tregs during normal pregnancy (13,14), indicating that the reduced numbers of Tregs in the peripheral blood of women with PE may be the reason for the decreased fetal tolerance. In the present study, a significant decrease was seen in $CD4^+$ $CD25^{high}$ Tregs in the PE group compared to the HP group (19,26-29). The PD-1/PDL-1 pathway is one of the important maintainers of immune homeostasis in the development of Tregs. This pathway inhibits the development of effector T-cells such as Th17. The interaction between PD-1 (CD279) and the CD274 ligand (PD-L1) plays a crucial role in immune response regulation, and is responsible for peripheral tolerance (30). Changes in the PD-1/PD-L1 pathway may be a function of an imbalanced Treg/Th17 ratio during pregnancy (31). Tian *et al.* studied the imbalanced Treg/Th17 cell ratio and the changes in PD-1 and PD-L1 expressions of Treg and Th17 cells in PE women. They observed an inverse correlation between the percentage of Treg and Th17 cells in the control group versus the PE group. In their study, there occurred a significant reduction in the percentage of Tregs in the PE group and an increase in the percentage of Th17 cells. Since PD-1/PD-L1 pathway is essential for the development and function of immune cells, Tian *et al.* have suggested that the reduced number of Tregs in patients with PE might be associated with changes in the expression or function of the PD-1/PD-L1 axis. Their data showed that the percentage of $PD1^+$ Tregs in patients with PE was higher compared with the control group, while $PD\text{-}L1^+$ Tregs did not differ between the two groups. Further seen was a correlation between the expression of PD-1/PD-L1 and the ratio of Treg/Th17 in women with PE compared to HP women. In addition, they noted that these regulatory effects were mediated by the inhibition of PI3K/AKT/mTOR signaling and PTEN expression (32). Our findings on $PD1^+$ ($CD279^+$) Treg supported the results reported by Tian *et al.*, in which $CD4^+$ $CD25^{high}$ $CD279^+$ Treg cells increased in the PE group compared to the HP group, and $CD4^+$ $CD25^{high}$ Tregs were lower in the PE group. PD-1 expression could be considered as a marker of exhausted T-cells (33). PD-L1 binding to PD-1 has been shown to suppress NFκB factor by inhibiting PI3K activity and downstream activation of the AKT/mTOR pathway (34). The PD-1/PD-L1 pathway might be a therapeutic strategy for autoimmune diseases and PD-1 binding with PD-L1 conduces to the maintenance of pregnancy by adjusting the Treg/Th17 ratio (31). Zhang *et al.* compared $PD\text{-}1^+$ Tregs between PE and HP women, where the former had a reduced percentage of Tregs and increased percentage of Th17 cells. Moreover, there was a higher PD-1 expression in Tregs and a reduced PD-1 expression regarding Th17 cells in the PE group (35). Our results are in line with the findings of these studies. FoxP3, as a marker for Tregs, was not employed in the present research, hence a limitation. However, a number of studies have only used CD4 and CD25 markers for these cells (19,26-29). We only investigated peripheral blood from the PE and HP groups, which was another study limitation. Future studies should investigate the expression of exhausted Tregs on uterine decidua cells, and assess their activity and signaling. In conclusion, we observed that the

number of Tregs identified by the $CD4^+$ $CD25^{high}$ marker decreased in the PE group, but the exhausted Treg cells identified by the $CD4^+$ $CD25^{high}$ $CD279^+$ marker increased in the PE group. Our data demonstrated that the presence of PD-1 (CD279) on the surface of Tregs, as an apoptosis factor, can a reason for the reduced number and function of Tregs. This study could be a basis for the designation of suitable therapeutic options for preeclampsia. The increased PD-1 ($CD279^+$) observed on Tregs might be involved in the pathogenesis of PE, hence the fact that we recommend that it be used as a therapeutic target for this disorder.

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