Journal of Reproductive Immunology 133 (2019) 43–51

Contents lists available at ScienceDirect

Journal of Reproductive Immunology

journal homepage: www.elsevier.com/locate/jri

Analysis of the frequencies and functions of $CD4^+CD25^+CD127^{low/neg}$, $CD4^+HLA\text{-}G^+$, and $CD8^+HLA\text{-}G^+$ regulatory T cells in pre-eclampsia

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ARTICLE INFO

Keywords:
$CD4^+CD25^+CD127^{low/neg}$ regulatory T cells
$CD4^+HLA\text{-}G^+$ regulatory T cells
$CD8^+HLA\text{-}G^+$ regulatory T cells
Pre-eclampsia
Pregnancy

ABSTRACT

Most of the investigations on regulatory T cells (Treg) have focused on $CD4^+CD25^+Foxp3^+$ Treg cells. Although new subsets of these cells such as $CD4^+CD25^+CD127^{low/neg}$, $CD4^+HLA\text{-}G^+$, and $CD8^+HLA\text{-}G^+$ Treg cells have been introduced, documents regarding these populations are limited or controversial in case of pregnancy and pre-eclampsia (PE). Here, we investigated the frequencies of the three aforementioned Treg cell subsets in the peripheral blood of non-pregnant (n = 15), healthy pregnant, and preeclamptic women (n = 17 in each group) using flow cytometry. We also assessed the ability of the isolated $CD4^+CD25^+CD127^{low/neg}$ and $CD4^+HLA\text{-}G^+$ Treg cells to suppress responder T cells proliferation and cytokine secretion using CFSE dye dilution and ELISA technique. Our results showed that the frequency of $CD4^+CD25^+CD127^{low/neg}$ Treg cells was significantly lower in preeclamptic women ($p = 0.001$). Also, this subset negatively correlated with both systolic ($R = -0.401$, $p = 0.004$) and diastolic ($R = -0.541$, $p = 0.001$) blood pressures. Regarding $CD4^+HLA\text{-}G^+$ and $CD8^+HLA\text{-}G^+$ Treg cells, the mean percentages of these cells were significantly higher in the context of normal pregnancy ($p < 0.01$). Finally, our results in the functional assay experiments did not show statistically significant differences between groups ($p \geq 0.05$), but they reveal a shift toward the lower suppressive capacity of $CD4^+CD25^+CD127^{low/neg}$ and $CD4^+HLA\text{-}G^+$ Treg cells in preeclamptic patients which might be clinically important. In conclusion, a significant decrease in the frequency of Treg cell subsets and also a shift toward the lower suppressive capacity of these cells in preeclamptic patients may lead to immunological maladaptation in the context of PE.

1. Introduction

Pre-eclampsia (PE) is one of the important multi-organ pregnancy-related disorders and a critical risk factor for adverse outcomes of pregnancy such as feto-maternal death and developmental disabilities in the fetus (Mol et al., 2016; Duley, 2009). PE is responsible for about 12–25% and 15–20% of all fetal growth restriction and preterm birth, respectively (Jeyabalan, 2013). Although the precise etiology of this pregnancy complication is unknown yet, it seems that different mediators of the immune system such as complement system, auto-antibodies, cytokines, immune receptors, and immune cells have important roles in disease pathogenesis (Martinez-Varea et al., 2014; Laresgoiti-Servitje et al., 2010; Saito, 2010; Molvarec et al., 2015).

Regulatory T cells (Treg) are one of the most intriguing cells in the immune system which are important in pregnancy, mediating tolerance toward the semi-allogeneic fetus (Martinez-Varea et al., 2014). Apart from the well-known $CD4^+CD25^+Foxp3^+$ and $CD8^+CD25^+Foxp3^+$ Treg cells which are crucial in the context of pregnancy and pre-eclampsia (Hosseini et al., 2018; Yu et al., 2017; Toldi et al., 2015, 2012), there are also additional subtypes of regulatory T cells that are important for maintenance of tolerance in the body (Ligocki and Niederkorn, 2015). $CD4^+CD25^+CD127^{low/neg}$ cells are one of these important regulatory T cell subsets. These cells, which are mostly $Foxp3^+$, can effectively suppress the proliferation of $CD4^+CD25^-$ responder T cells (Yu et al., 2012). Different studies have been conducted to assess the importance of these cells in case of pregnancy and pre-eclampsia, but there is no consistency regarding the results (Steinborn et al., 2012; Salazar Garcia et al., 2018; Santner-Nanan et al., 2009; Boij et al., 2015). In this regard, some experiments found a decrease in the frequency or function of these cells in pre-eclampsia compared to

Abbreviations: CFSE, carboxyfluorescein succinimidyl ester; DI, division index; FMO, fluorescence minus one; HP, healthy pregnancy; NP, non-pregnant; PBMC, peripheral blood mononuclear cell; PE, pre-eclampsia; Treg, regulatory T cells; Tresp, responder T cells

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https://doi.org/10.1016/j.jri.2019.06.002
Received 14 March 2019; Received in revised form 17 April 2019; Accepted 15 June 2019

normal pregnancy (Steinborn et al., 2012; Salazar Garcia et al., 2018), while others reported no change (Santner-Nanan et al., 2009; Boij et al., 2015)

HLA-G$^+$ regulatory T cell subset is an additional population that was first described by Feger et al (Feger et al., 2007). These cells are thymic-derived, hypo-proliferative, and do not express CD25 and Foxp3 molecules. Both CD4$^+$ and CD8$^+$ T cells can express HLA-G molecule (Feger et al., 2007). These cells are studied in transplantation, multiple sclerosis, and HIV-1 infection (Pankratz et al., 2014; Huang et al., 2009; Li et al., 2013; Vigano et al., 2017), but there are only few experiments regarding the frequency of CD4$^+$HLA-G$^+$ Treg cells in the context of pregnancy and pre-eclampsia (Hsu et al., 2014). To our knowledge, there is no study regarding the frequency of CD8$^+$HLA-G$^+$ cells in pregnancy and its complications. Also, there is no experiment that compares functional properties of CD4$^+$HLA-G$^+$ cells in the context of pre-eclampsia.

Thus, the present study was conducted to investigate the frequency of the three aforementioned Treg cell subsets and compare the suppressive capacity of isolated CD4$^+$CD25$^+$CD127$^{low/neg}$ and CD4$^+$HLA-G$^+$ isolated Treg cells in normal pregnancy and pre-eclampsia.

2. Materials and methods

2.1. Subjects

In this study, 17 pregnant women with de-novo hypertension and proteinuria were included as the preeclamptic patient group (PE). Pre-eclampsia diagnosis was according to a) blood pressure (BP) higher than 140/90 mm Hg at admission, and b) significant proteinuria in the absence of urinary tract infection ($>$ 0.3 g in a 24 -h urine sample or a dipstick reading of $>$ 1$^+$ (Roberts et al., 2013). A total of 17 healthy pregnant (HP) (age- and gestational month-matched to PE patients) were also considered. They had at least one previous successful pregnancy without any complication. As pregnancy per se alters the immune system, 15 non-pregnant (NP) women without a history of pregnancy at the proliferative phase of the menstrual cycle were also included in our study as the control of pregnancy. These subjects were age-matched with the two previous groups. Exclusion criteria of the study were chronic hypertension, gestational diabetes, HELLP syndrome, cancer, active infection, or autoimmune disorders. Prior to the whole blood collection, written informed consents approved by a local Ethics Committee of Shiraz University of Medical Sciences (number: 1395.S144) were obtained from all of the participants. PE patients and healthy pregnant subjects were chosen from women who referred to Hafez and Zeynabiye Hospitals, Shiraz University of Medical Sciences, Shiraz, Iran. Clinical features of the participants are presented in Table 1.

Table 1
Clinical features of the non-pregnant, healthy pregnant and preeclamptic women.

	Non-pregnant (n = 15)	Healthy pregnant (n = 17)	Preeclamptic (n = 17)	*P* value
Age (Mean ± SD)	29.7 ± 1.8	29.6 ± 2.7	31.2 ± 2.4	0.120
Gestational week (Mean ± SD)	–	35.1 ± 03.7	33.9 ± 3.5	0.327
Gestational week at delivery (Mean ± SD)	–	39.0 ± 0.7	35.3 ± 3.6	0.001
Birth weight (gr) (Mean ± SD)	–	2992.5 ± 771	2240 ± 775	0.009
Gravidity (Mean ± SD)	–	2.4 ± 0.7	1.7 ± 0.8	0.021
Parity (Mean ± SD)	–	1.3 ± 0.4	0.6 ± 0.8	0.004
Systolic BP [a] (Mean ± SD)	108.4 ± 7.0	111.6 ± 8.8	141.0 ± 8.8	< 0.001 [b]
Diastolic BP [a] (Mean ± SD)	71.3 ± 5.0	71.6 ± 6.0	91.5 ± 5.7	< 0.001 [b]
Urine dipstick protein test	N.A	Neg	1$^+$ to 3$^+$	–

ANOVA and independent samples *t*-test were used for data analysis. *P* values less than 0.05 were considered to be statistically significant. [a]Blood pressure was measured in the morning (7–11 A.M). [b]The differences were between the preeclamptic group and the two other groups. BP: blood pressure, N.A: not assessed, Neg: negative, PE: pre-eclampsia.

2.2. PBMC isolation and cryopreservation

Peripheral blood mononuclear cells (PBMCs) were isolated from 40 to 50 ml of whole peripheral blood by density centrifugation using Lymphoprep™ (Axis-Shield, Oslo, Norway) and washed via RPMI 1640 media (Gibco by Thermo Fisher Scientific, New York, USA). One part of the PBMCs (7×10^6 cells) was transferred to cryovials containing freezing media and preserved in liquid nitrogen tank until cell frequency analyses experiments. The preservation period was adjusted between samples in the three studied groups. The remaining part of the cells was used for fresh isolation of regulatory and responder T cells (Tresp).

2.3. Flow cytometry and cell frequency analyses

At the time of the experiments, cells were thawed and washed twice using the RPMI 1640 media. Cell viability was determined using trypan blue exclusion test and more than 90% of the cells were viable. Human serum was added to the cell suspension (10% v/v) in order to block Fc receptors (15 min at 4°C). To analyze the regulatory T cells frequencies, PBMCs (7×10^5 cells) were surface-stained with specific antibodies against CD3-PerCP, CD4-APC, CD8-APC, CD25-FITC, CD127-PE (all from Biolegend, San Diego, California, USA), HLA-G-FITC, (Exbio, Praha, Czech Republic). Also, biotinylated anti-human HLA-G (Exbio, Praha, Czech Republic) and Streptavidin-PE (Biolegend, San Diego, California, USA) were used in the experiments. The ratio between biotinylated Ab and Streptavidin-PE was determined by titration. Tubes which were incubated only with PE-conjugated streptavidin served as control to check unspecific binding. Fluorescence minus one (FMO) controls were used as gating control and the positive gates were defined so that less than 0.2% of the cells were included in the FMO control gate. The stained cells were then run with BD FACS Calibur and BD FACS Aria III flow cytometers (BD Biosciences, San Jose, California, USA) and the data were analyzed using FlowJo software, version 7.6.1.

2.4. Regulatory and responder T cells purification

Human CD4$^+$CD25$^+$ regulatory T cell isolation kit, CD127 microbead kit, anti-biotin microbead (all from Miltenyi Biotec, Bergisch Gladbach, Germany), and biotinylated anti-Human HLA-G (Exbio, Praha, Czech Republic) were used to obtain responder T cells, CD4$^+$CD25$^+$CD127$^{low/neg}$ and CD4$^+$HLA-G$^+$ Treg cells. Isolation of the aforementioned cell populations, purity, and yield of the magnetic-activated cell sorting process (MACS) are described in *Supplementary Document.*

2.5. Inhibition of responder T cell proliferation

In order to assess the suppressive capacity of CD4$^+$CD25$^+$CD127$^{low/neg}$ or CD4$^+$HLA-G$^+$ Treg cells, isolated CD4$^+$ responder T cells (5×10^6 in 1 ml PBS/1% FBS) were labeled via 5 μM of carboxyfluorescein succinimidyl ester (CFSE; Invitrogen by Thermo Fisher Scientific, CA, USA) and subsequently quenched with room-temperature FBS and medium congaing 10% FBS (CM10).

Then, responder T cells (1×10^5 cells per well) were stimulated with 1 μg/ml soluble anti-CD3/anti-CD28 antibodies (Invitrogen by

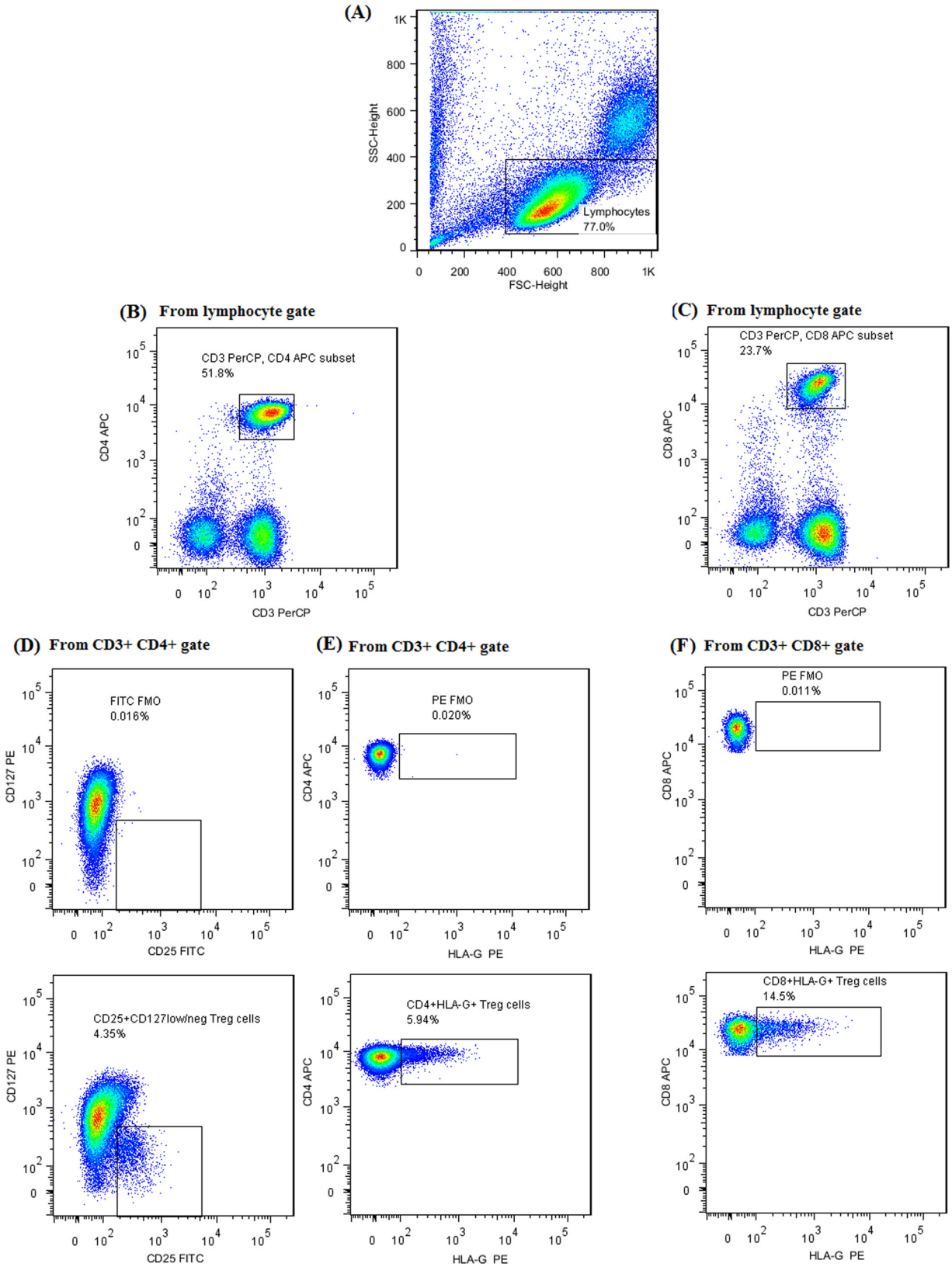

Fig. 1. Four color flow cytometric analysis and the gating strategy used for Treg cells detection in the peripheral blood. For the gating strategy, A) lymphocytes were gated based on the forward & side scatter parameters, B) then $CD3^+CD4^+$ or C) $CD3^+CD8^+$ T cells were determined. D) CD25 and CD127 markers were plotted against each other and the frequency of $CD3^+CD4^+CD25^+CD127^{low/neg}$ Treg cells was determined. E and F) $CD4^+HLA\text{-}G^+$ and $CD8^+HLA\text{-}G^+$ Treg cells frequencies were determined in the pool of $CD4^+$ or $CD8^+$ T cells, respectively. Fluorescence minus one (FMO) controls were used to plot appropriate gates.

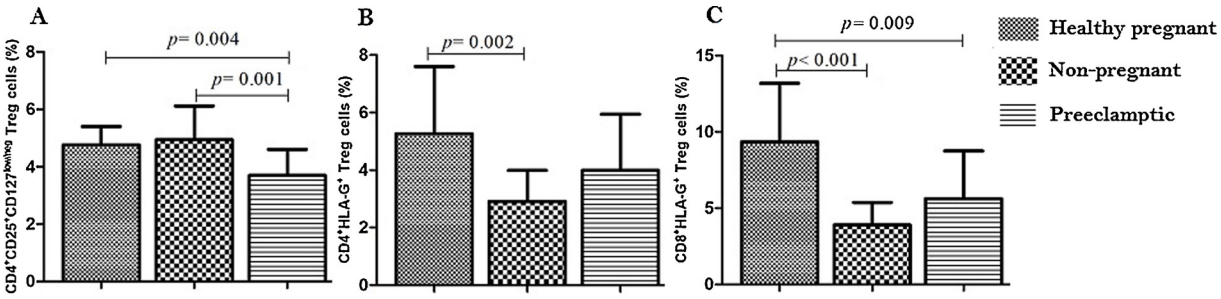

Fig. 2. Frequencies of $CD4^+CD25^+CD127^{low/neg}$, $CD4^+HLA\text{-}G^+$, and $CD8^+HLA\text{-}G^+$ regulatory T cells in the peripheral blood of healthy pregnant (n = 17), non-pregnant (n = 15), and preeclamptic women (n = 17). A) Preeclamptic patients had the lowest frequency of $CD4^+CD25^+CD127^{low/neg}$ Treg cells in the peripheral blood compared to healthy pregnant and non-pregnant women. B) Healthy pregnant women had the highest frequency of $CD4^+HLA\text{-}G^+$ Treg cells in the peripheral blood. C). Healthy pregnant women had the highest level of $CD8^+HLA\text{-}G^+$ Treg cells in the peripheral blood compared to two other groups. Graphs show mean ± SD. ANOVA test (post-hoc: Teukey's test) was used for data analysis. P value < 0.05 was considered statistically significant.

Thermo Fisher Scientific, CA, USA) in the presence or absence of the two aforementioned Treg cell subsets. Different ratios of Treg/Tresp cells were tested in set up experiments (1/2, 1/4, 1/8, and 1/16). Eventually, the 1/4 ratio was selected for the final tests, as the suppressive capacity was obvious and it was more similar to the physiologic ratio of these cells in the body. After 4 days, proliferation was measured using flow cytometer and percentage of suppression was calculated by the following formula (McMurchy and Levings, 2012):

Percentage of suppression = 100 – [(Division index of Tresp in the presence of Treg/Division index of Tresp in the absence of Treg) × 100]

2.6. Inhibition of responder T cell cytokine secretion

To assess the percentage of cytokine secretion inhibition, 100 µl of the cell culture supernatants were collected from the wells (duplicate) containing responder T cells in the presence or absence of $CD4^+CD25^+CD127^{low/neg}$ or $CD4^+HLA\text{-}G^+$ on day 4 of culture and stored at -70°C until the time of experiments. The levels of IFN-γ, TNF-α, IL-17A (homodimer), and IL-4 cytokines were determined using ELISA Ready-SET-Go! Kits from eBioscience (San Diego, CA, USA) following the manufacturer's guidelines and the percentage of cytokine secretion inhibition was calculated.

2.7. Statistical analysis

Statistical analyses were performed using SPSS software (version 18). Levene's and Kolmogorov-Smirnov tests were used to evaluate homogeneity of variances and normality of data, respectively. Statistical comparison of the frequency of different Treg cell subsets and also the percentage of inhibition between different studied groups were performed using ANOVA or Kruskal-Wallis tests. Comparison of the division index and cytokine secretion in the presence or absence of Treg cell subsets was performed using Wilcoxon rank sum test. The correlations between the frequencies of different subsets of regulatory T cells and continuous variables were measured using Pearson's correlation coefficient. A value of $p < 0.05$ was considered statistically significant. GraphPad Prism software (version 5) was used for plotting the graphs.

3. Results

3.1. Frequencies of $CD4^+CD25^+CD127^{low/neg}$, $CD4^+HLA\text{-}G^+$, and $CD8^+HLA\text{-}G^+$ Treg cells

We used 4 color flow cytometry to define the frequencies of regulatory T cell subsets. Fig. 1 represents our gating strategy and flow cytometry analysis regarding the three subsets of regulatory T cells.

The percentage of $CD4^+CD25^+CD127^{low/neg}$ Treg cells was determined in the peripheral blood of non-pregnant, healthy pregnant, and preeclamptic women. Our results revealed that the mean percentage of this population was significantly lower in preeclamptic group when compared to both non-pregnant and healthy pregnant women (3.96 ± 0.90, 4.94 ± 1.17, and 4.76 ± 0.63, respectively; $p = 0.001$; Fig. 2A). Regarding $CD4^+HLA\text{-}G^+$ Treg cells, there was a significant difference between the studied groups ($p = 0.009$), as healthy pregnant women had the highest level of this subset (5.26 ± 2.33) in the peripheral blood, followed by preeclamptic (3.99 ± 1.95) and non-pregnant ones (2.91 ± 1.07) (Fig. 2B). It should be noted that according to the phenotypic analysis, $CD4^+HLA\text{-}G^+$ regulatory T cells are a complete distinct regulatory subset which are $CD127^+$ and $CD25^-$ *(Supplementary Document)*. Moreover, $CD8^+HLA\text{-}G^+$ Treg cells frequency was significantly higher in healthy pregnant women compared to two other groups ($p < 0.001$). The mean percentage of the $CD8^+HLA\text{-}G^+$ regulatory T cells in the peripheral blood of participants was as follows: 9.35 ± 3.83 in healthy pregnant women, 5.63 ± 3.12 in preeclamptic women, and 3.90 ± 1.74 in non-pregnant ones (Fig. 2C).

3.2. Correlation between the frequencies of Treg cell subsets and clinical characteristics of subjects

Pearson's correlation test was used to assess the correlation between the frequencies of different subsets of regulatory T cells and variables such as systolic BP, diastolic BP, and age. There was no correlation between different subsets of regulatory T cells and age. As shown in Fig. 3, $CD4^+CD25^+CD127^{low/neg}$ regulatory T cells had significant negative correlations with both systolic ($R = -0.401$, $p = 0.004$) and diastolic ($R = -0.541$, $p = 0.001$) BPs. Regarding $CD4^+HLA\text{-}G^+$ and $CD8^+HLA\text{-}G^+$ regulatory T cell subsets, there were no significant correlations with systolic and diastolic blood pressures (Fig. 3).

3.3. Proliferation inhibition assay using $CD4^+CD25^+CD127^{low/neg}$ or $CD4^+HLA\text{-}G^+$ Treg cells

We also investigated the suppressive capacity of MACS isolated Treg cells in a co-culture suppression assay. After isolating Treg cells, the CFSE labeled Tresp cells were cultured with/without the sorted Treg cells (Treg/Tresp = 1/4) in the presence of soluble anti-CD3/CD28 antibodies, and the proliferation of responder T cells was assessed on day 4 using CFSE dilution peaks (Fig. 4A).

As shown in Fig. 4B, the percentage of proliferating cells was significantly lower in the presence of $CD4^+CD25^+CD127^{low/neg}$ and $CD4^+HLA\text{-}G^+$ regulatory T cells, which means that both of these regulatory T cell subsets were able to exert their inhibitory capacity in vitro. Then, the percentage of suppression was calculated in different groups based on the division index using the aforementioned formula (McMurchy and Levings, 2012). Regarding both

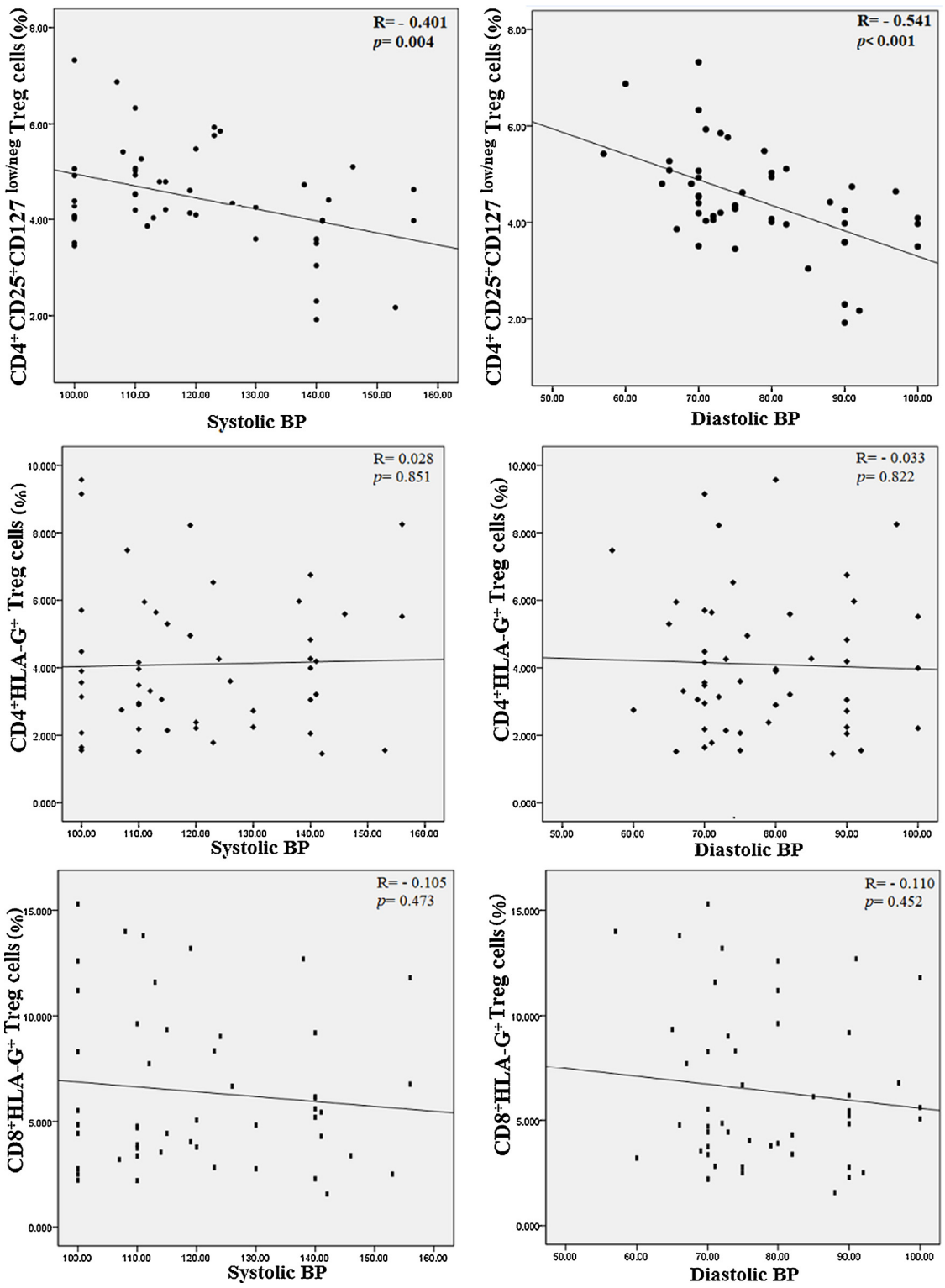

Fig. 3. Correlations between the percentages of Treg cell subsets and blood pressure. $CD4^+CD25^+CD127^{low/neg}$ regulatory T cells had negative correlations with both systolic and diastolic BPs. $CD4^+HLA\text{-}G^+$ and $CD8^+HLA\text{-}G^+$ Treg cell subsets had no significant correlation with systolic and diastolic BPs. Pearson's correlation coefficient was used for data analysis. P value < 0.05 was considered statistically significant.

$CD4^+CD25^+CD127^{low/neg}$ and $CD4^+HLA\text{-}G^+$ regulatory T cells, the mean percentage of suppression was lower in preeclamptic patients compared to non-pregnant and healthy pregnant women, but the difference was not statistically significant between the studied groups (Fig. 4C).

3.4. Cytokine secretion inhibition assay using $CD4^+CD25^+CD127^{low/neg}$ or $CD4^+HLA\text{-}G^+$ Treg cells

We also assessed the capacity of isolated Treg cells to suppress cytokine secretion by responder T cells. Our results revealed that $CD4^+CD25^+CD127^{low/neg}$ regulatory T cells were suppressive, as these

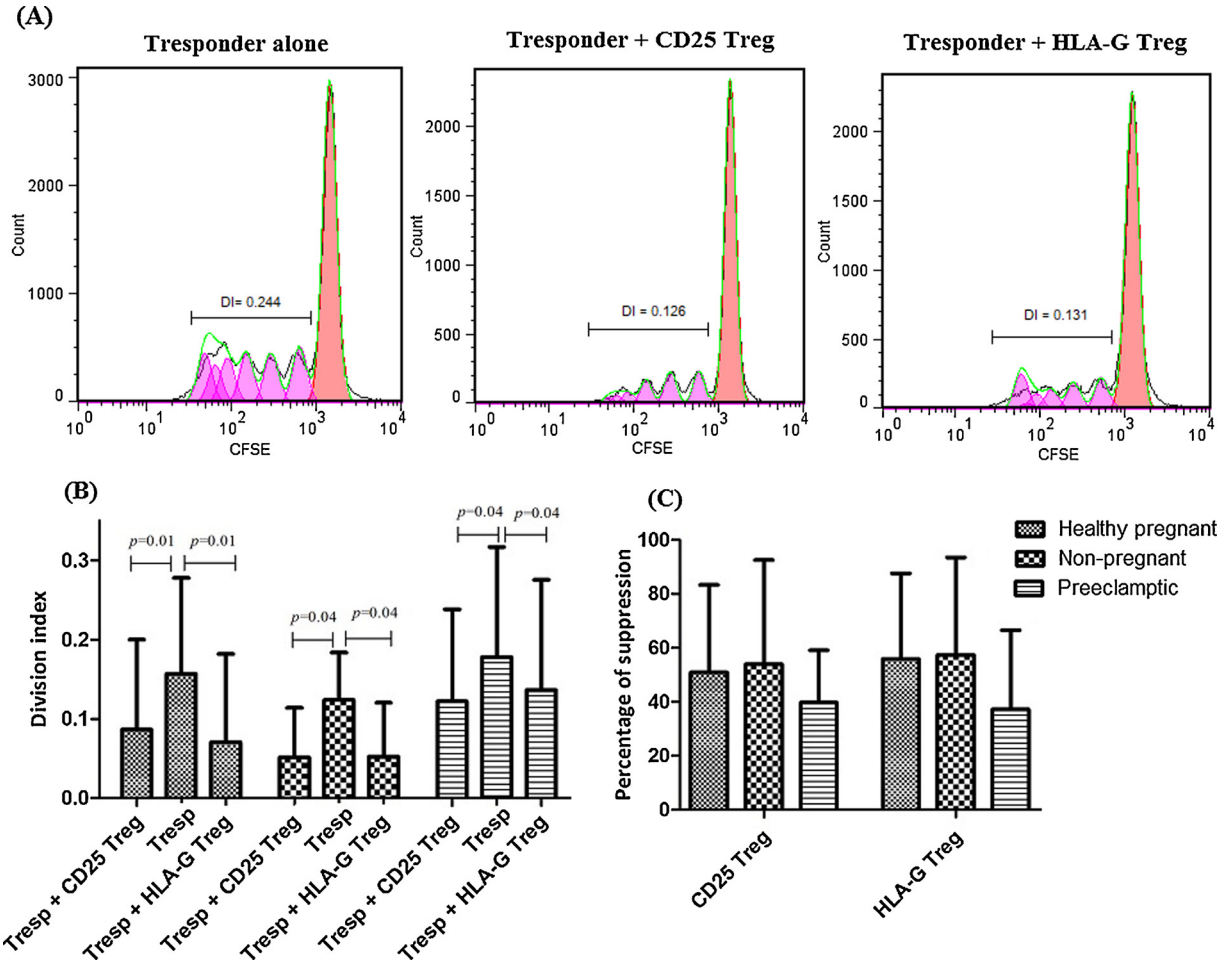

Fig. 4. Analysis of the suppressive capacity of $CD4^+CD25^+CD127^{low/neg}$ and $CD4^+HLA\text{-}G^+$ Treg cells in healthy pregnant, non-pregnant, and preeclamptic women (n = 10 in each group). A) FACS plot showing the proliferation of CFSE labeled responder T cells stimulated by soluble anti-CD3/CD28 antibodies (1 μg/ml) in the presence or absence of Treg cells. Division index (DI) of proliferating cells are marked on the histogram. B) Graphical representation of the division index of CFSE labeled responder T cells in the presence or absence of the two Treg cell subsets. Both $CD4^+CD25^+CD127^{low/neg}$ and $CD4^+HLA\text{-}G^+$ Treg cells significantly suppressed proliferation of the responder T cells in all of the groups. Graphs show mean ± SD. Wilcoxon test used for data analysis. P value < 0.05 was considered statistically significant. C) Percentage of suppression was determined in studied groups for each subset of Treg cells. There was no significant difference in the suppressive capacity of Treg cells between the three groups. Graphs express mean ± SD. Kruskal-Wallis test was used for data analysis. CD25 Treg: $CD4^+CD25^+CD127^{low/neg}$ regulatory T cells, HLA-G Treg: $CD4^+HLA\text{-}G^+$ regulatory T cells, Tresp: responder T cells.

cells significantly reduced TNF-α secretion by responder T cell in all three studied groups (Fig. 5A). We next assessed the percentage of suppression for this cytokine in the three groups. The percentage of TNF-α suppression (mean ± SE) was 41.82 ± 9.26% in non-pregnant, 39.95 ± 9.63% in healthy pregnant, and 26.02 ± 5.54% in pre-eclamptic women, but these differences were not statistically significant (Fig. 5B). Also, $CD4^+CD25^+CD127^{low/neg}$ regulatory T cells significantly decreased IFN-γ secretion by responder T cells only in healthy pregnant subjects (Fig. 5C). Similar to TNF-α, there were no significant differences between the groups regarding the percentage of IFN-γ suppression (Fig. 5D). There were not any significant differences in IL-17 and IL-4 secretion by responder T cells in the presence or absence of $CD4^+CD25^+CD127^{low/neg}$ Treg cells (Fig. 5E and F). It is also worth noting to state that we did not detect IL-4 cytokine in the cell culture supernatant of non-pregnant women (Fig. 5F).

Regarding $CD4^+HLA\text{-}G^+$ regulatory T cells, the levels of TNF-α and IFN-γ cytokines were decreased in the presence of Treg cells, but was not statistically significant (Fig. 5A and C). Also, there were no significant differences regarding the percentages of cytokine secretion inhibition between the three studied groups (Fig. 5B and D).

4. Discussion

In the present study, we assessed the frequencies of $CD4^+CD25^+CD127^{low/neg}$, $CD4^+HLA\text{-}G^+$, and $CD8^+HLA\text{-}G^+$ regulatory T cells in the context of normal pregnancy and pre-eclampsia. Then, we focused on the functional properties of the peripheral blood isolated $CD4^+CD25^+CD127^{low/neg}$ and $CD4^+HLA\text{-}G^+$ Treg cells.

$CD4^+CD25^+CD127^{low/neg}$ cells define a subset of regulatory T cells in the peripheral blood (Yu et al., 2012), which are important players during pregnancy and pre-eclampsia. Santner-Nanan et al. and Steinborn et al. showed that the frequency of this cell subset significantly decreased in women complicated with pre-eclamsia (Santner-Nanan et al., 2009; Steinborn et al., 2012). Interestingly, in a recently published study, Salazar et al. proposed that a decrease in the frequency of $CD3^+CD4^+CD25^+CD127^{low/neg}$ Treg cells is one of the early immune biomarkers that can be used to predict pre-eclampsia (Salazar Garcia et al., 2018). In line with the previous experiments, we found that the frequency of $CD4^+CD25^+CD127^{low/neg}$ regulatory T cells significantly decreased in the peripheral blood of preeclamptic women compared to healthy pregnant and non-pregnant subjects. Also, we observed that this regulatory T cell subset had negative correlations with systolic and diastolic blood pressures. Interestingly, two experiments reported that

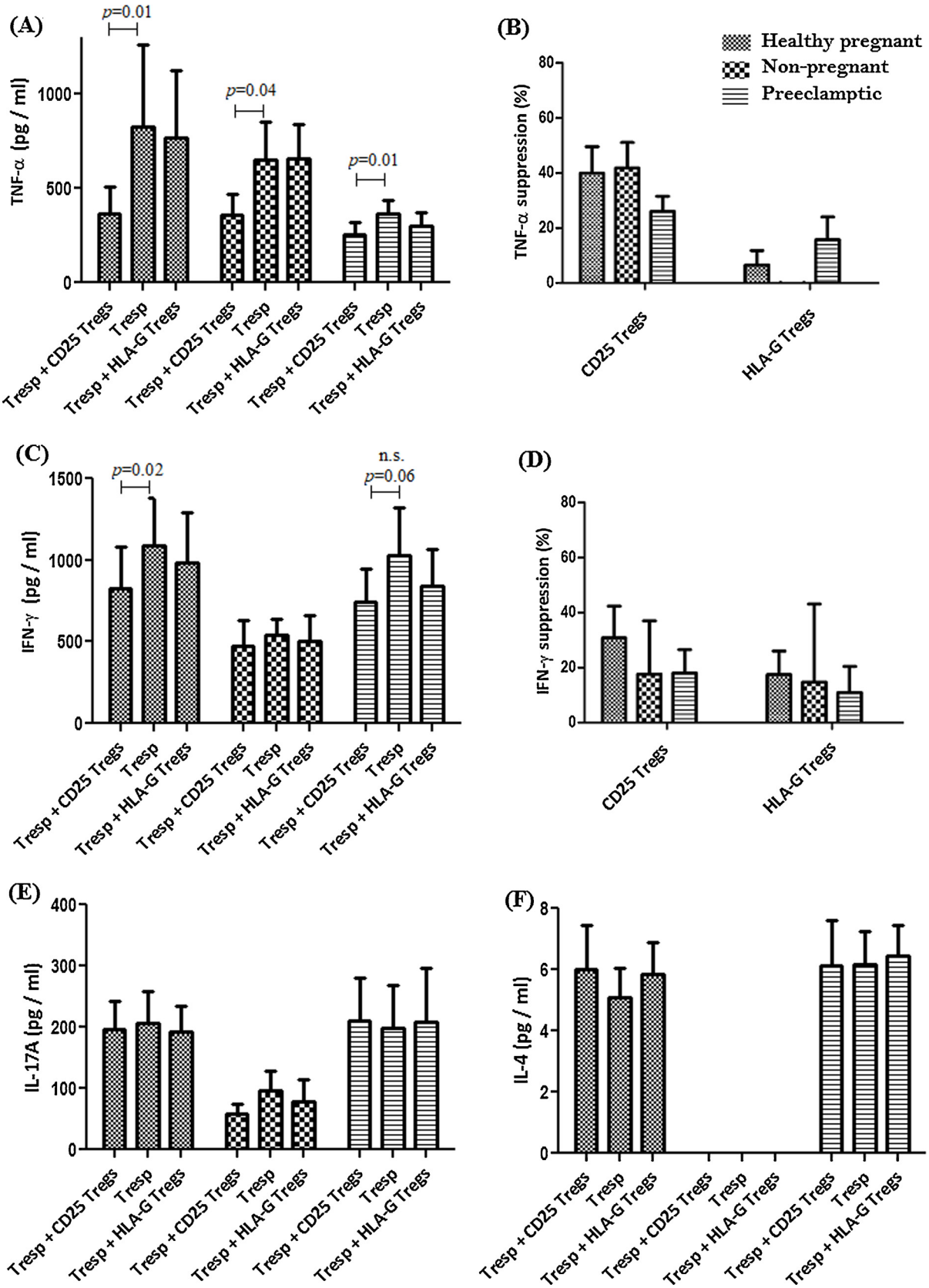

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Fig. 5. Cytokine secretion inhibition by $CD4^+CD25^+CD127^{low/neg}$ or $CD4^+HLA\text{-}G^+$ regulatory T cells in healthy pregnant, non-pregnant, and preeclamptic women. Levels of TNF-α, IFN-γ, IL-17 A, and IL-4 cytokines were measured in cell culture supernatants after 4 days. A) TNF-α production was measured in the presence or absence of the two aforementioned Treg cell subsets. $CD4^+CD25^+CD127^{low/neg}$ Treg cells significantly suppressed TNF-α production in all groups. B) Percentage of TNF-α secretion inhibition was calculated in the studied groups. Isolated Tregs from PE patients showed lower suppressive capacity, but the differences were not statistically significant. C) IFN-γ secretion was determined in the presence or absence of Treg cell subsets. $CD4^+CD25^+CD127^{low/neg}$ Treg cells significantly decreased IFN-γ production only in the healthy pregnant group. D) Percentage of IFN-γ secretion inhibition was calculated in different studied groups. Although isolated regulatory T cells from PE patients showed lower suppressive capacity, the differences were not statistically significant. E and F) Levels of IL-17A and IL-4 were measured in the presence or absence of $CD4^+CD25^+CD127^{low/neg}$ or $CD4^+HLA\text{-}G^+$ Treg cells. There were no significant differences in IL-17 and IL-4 secretion by responder T cells in the presence or absence of both Treg cell subsets. Graphs show mean ± SEM. Wilcoxon and Kruskal-Wallis tests were used for data analysis. P value < 0.05 was considered statistically significant.

$Foxp3^+$ regulatory T cells play important roles in the context of other hypertension-related disorders such as coronary artery disease or pulmonary hypertension (Emoto et al., 2014; Sada and Dohi, 2016). As the majority of $CD4^+CD25^+CD127^{low/neg}$ Treg cells are also $Foxp3^+$ (Liu et al., 2006), we proposed that a decrease in the frequency of these regulatory T cells might be important in the pre-eclampsia pathogenesis and its clinical presentations, especially hypertension. On the other hand, it is also worth noting that the decreased frequency of regulatory T cells might be a result of disease itself. In this regard, increased level of soluble endoglin molecule prior to onset of preeclampsia capture circulating TGF-β (Maynard and Karumanchi, 2011) that may result in the reduced Treg cell frequency (Chen and Konkel, 2010). Considering healthy pregnant and non-pregnant women, we did not detect any significant difference between two groups regarding the frequency of $CD4^+CD25^+CD127^{low/neg}$ Treg cell subset. This finding was in line with the results of Lima et al. (Lima et al., 2017), but in contrast to the Santner-Nanan's findings (Santner-Nanan et al., 2009). Fluctuations of the percentage of Treg cells in different phases of the menstrual cycle (Arruvito et al., 2007) might be a reason for such a discrepancy. Regarding the suppressive capacity of $CD4^+CD25^+CD127^{low/neg}$ regulatory T cell subset, our results showed that isolated Treg cells from preeclamptic women had a lower suppressive capacity compared to the two other groups, but the differences were not statistically significant. Some other experiments also assessed the function of these Treg cells in the context of pre-eclampsia. Steinborn et al. reported that magnetically sorted $CD4^+CD25^+CD127^{low/neg}$ Treg cells from preeclamptic women had a significantly lower suppressive capacity compared to the normal pregnant ones (Steinborn et al., 2012). Besides, Darmochwal et al. reported that MACS isolated $CD4^+CD25^+$ Treg cells from preeclamptic patients were not able to suppress proliferation of $CD4^+CD25^-$ responder cells (Darmochwal-Kolarz et al., 2012). On the other hand, Santner-Nanan et al. and Zeng et al. showed that this Treg cell subset had comparable suppressive activity in preeclamptic and normal pregnant controls (Santner-Nanan et al., 2009; Zeng et al., 2013). We also assessed the ability of $CD4^+CD25^+CD127^{low/neg}$ regulatory T cells to suppress cytokine production by responder T cells. Our results indicated that these Treg cells significantly suppressed TNF-α and IFN-γ production by responder T cells, especially in healthy pregnant women. It could be inferred from these results that these regulatory T cells might be more competent in normal pregnant women. In line with our previous results, isolated Treg cells from preeclamptic women showed a lower suppressive capacity compared to non-pregnant and healthy pregnant subjects, although not statistically significant. We did not observe any significant difference regarding suppression of IL-17 and IL-4 cytokines. In this regard, a recent study indicated that there are T-helper like Treg cells within the population of regulatory T cells that can produce IL-17 and IL-4 cytokines (Halim et al., 2017). Hence, we hypothesized that Treg cells themselves might be a source for these cytokines production in our co-culture experiments. This issue needs to be further evaluated in future experiments.

We also assessed the frequency and function of $CD4^+HLA\text{-}G^+$ Treg cells in normal pregnancy and pre-eclampsia. In line with Amodio et al. (Amodio et al., 2013) and Hsu et al. (Hsu et al., 2014), we showed that healthy pregnant women had significantly higher frequency of $CD4^+HLA\text{-}G^+$ Treg cells in the peripheral blood in comparison to non-pregnant women. Regarding the frequency of $CD4^+HLA\text{-}G^+$ T cells in the peripheral blood of preeclamptic patients, Hsu et al. demonstrated that this cell subset was significantly lower in the patients compared to healthy pregnant women (Hsu et al., 2014). Although we detected lower frequency of this regulatory subset in preeclamptic patient compared to healthy pregnant women, the difference was not statistically significant. This discrepancy might be resulted from patient selection criteria, as half of the patients who were enrolled in the mentioned study (Hsu et al., 2014) had severe pre-eclampsia. Similar to $CD4^+CD25^+CD127^{low/neg}$ Treg cells, $CD4^+HLA\text{-}G^+$ regulatory T cells were functional and able to diminish proliferation of responder T cells. This regulatory T cell subset showed a lower suppressive capacity in preeclamptic patients compared to the two other groups, but the differences were not statistically significant between the groups. To our knowledge, there are not any experiments that assess the function of $CD4^+HLA\text{-}G^+$ regulatory T cells in the context of pre-eclampsia.

Also, we assessed the frequency of $CD8^+HLA\text{-}G^+$ Treg cells in the peripheral blood. In this regard, $CD8^+HLA\text{-}G^+$ Treg cells were only limitedly studied in HIV-1 infection (Li et al., 2013) and we investigated these cells in the context of pregnancy and pre-eclampsia for the first time. Our results indicated that healthy pregnant women had higher level of $CD8^+HLA\text{-}G^+$ Treg cells in the peripheral blood compared to non-pregnant and preeclamptic women. These data might introduce $HLA\text{-}G^+$ regulatory T cells as a distinct and important regulatory subset which is prominent in the context of pregnancy.

In conclusion, our results indicated that reduced frequencies of both $CD4^+CD25^+CD127^{low/neg}$ and HLA-G expressing regulatory T cell subsets, and a shift toward the lower suppressive capacity of Treg cells may lead to immunological maladaptation in the context of PE. In this study, we did not investigate functional properties of $CD8^+HLA\text{-}G^+$ Treg cells in pregnancy and pre-eclampsia. So, additional experiments that simultaneously investigate different immune tolerance mechanisms and regulatory cells, with increased sample size, can provide a more accurate insight in this regard.

Acknowledgments

We are really grateful to Mrs. Sedigheh Taghizadeh, Mrs. Zahra Moradi, and all of the laboratory, perinatology and labor ward staffs of Hafez and Zeynabiye Hospitals for their sincere help in blood sample collection. Also, we want to thank Mr. Ali Arabi Monfared and Mrs. Zohre Imeripour for their assistance in the technical aspects of flow cytometry. This study was extracted from the MSc thesis written by Maryam Zare and financially supported by grants no. 94-11014 and 96-14056 from Shiraz University of Medical Sciences.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jri.2019.06.002.

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