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RESEARCH ARTICLE

The altered PD-1/PD-L1 pathway delivers the 'one-two punch' effects to promote the Treg/Th17 imbalance in pre-eclampsia

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The programmed cell death-1 (PD-1)/PD-ligand 1 (PD-L1) pathway is critical for normal pregnancy by promoting regulatory T (Treg) cell development and inhibiting the Th17 response. However, the relationship between the PD-1/PD-L1 pathway and the Treg/Th17 imbalance in pre-eclampsia (PE) is an enigma. In this study, decreased PD-1 and PD-L1 expression and a Treg/Th17 imbalance were observed at the maternal-fetal interface in PE. The regulatory effects of the PD-1/PD-L1 pathway on the Treg and Th17 cell quantities were determined *in vitro* by targeting T-cell proliferation, differentiation and transdifferentiation. First, decreased PD-1 expression might contribute to a higher Th17 cell frequency by promoting proliferation in PE. Second, the percentages of Treg but not Th17 cells differentiated from peripheral naive $CD4^+$ T cells were increased by PD-L1 Fc administration. This effect was accompanied by decreased PI3K/AKT/m-TOR and increased PTEN mRNA expression and was completely reversed by PD-1 blockade. Finally, the percentage of IL-17-producing Treg cells increased and was positively associated with the Th17 cell frequency in PE. Increased RORγt and IL-17 but not Foxp3 and IL-10 mRNA expression by Treg cells was observed with PD-1 blockade. Similar findings occurred when Treg cells were exposed to IL-6/IL-23/IL-1β and were reversed by PD-L1 Fc. Taken together, our findings indicate that the PD-1/PD-L1 pathway contributes to the Treg/Th17 imbalance via 'one-two punch' approaches: (i) promoting Th17 cell proliferation, (ii) inhibiting Treg cell differentiation and (iii) enhancing Treg cell plasticity into Th17 cells in PE. The therapeutic value of PD-L1 Fc for PE treatment will be explored in the future.
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Keywords: differentiation; proliferation; Th17 cell; transdifferentiation; Treg cell

INTRODUCTION

Pre-eclampsia (PE) affects 5–8% of all pregnancies worldwide and is traditionally diagnosed by the combined presentation of high blood pressure and proteinuria.[1–3] The diagnostic criteria for PE were changed by the International Society for the Study of Hypertension in Pregnancy (ISSHP) in 2014.[4] As proteinuria is no longer required in the new definition, the new definition of PE determined by the ISSHP requires the presence of hypertension together with evidence of systemic disease (such as thrombocytopenia, elevated levels of liver transaminases, renal insufficiency, pulmonary edema and visual or cerebral disturbances).[4,5] When left untreated, PE can be lethal, and in low-resource settings, this disorder is one of the main causes of maternal and child mortality.[1,2] Simple preventive measures, such as low-dose aspirin, calcium and diet and lifestyle interventions, show potential but marginal benefits.[4] Severe complications can occur in both the mother and the fetus, and there is no effective method of treatment.[5] Moreover, mothers and children who have undergone a PE pregnancy will suffer from an increased long-term cardiovascular risk.[6] Therefore, understanding the enigmatic etiology of PE is critical.

Pregnancy constitutes a major challenge for the maternal immune system. During pregnancy, the maternal immune system is challenged by the allogeneic fetus, and maternal immune adaptation occurs systemically and locally. However, maternal immune tolerance toward the allogeneic fetus during

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normal pregnancy (NP) is compromised in pregnancy complications such as pre-term birth[7] and PE.[8] An important feature of immune intolerance in PE is the absence of the regulatory T (Treg) skewness characteristic of NP, leading to a pre-dominance of Th17 immunity.[9,10] Treg cells promote maternal-fetal tolerance; for instance, the rate of fetal resorption was increased during allogeneic gestation in Treg cell-deficient mice.[11] Furthermore, Treg cell transplantation not only rescued pregnancy in abortion-prone mice, but also reduced the increased abortion rate associated with IL-17 in the CBA/J × BALB/c model.[12] Therefore, the Treg/Th17 balance promotes NP, whereas a lower than normal Treg cell frequency and/or excessive Th17 immunity may contribute to the exaggerated systemic inflammation in PE.[9,10,13] Thus, factors that subtly alter the balance between Treg and Th17 cellular functions serve as important regulators of susceptibility to PE.

Peripheral immune tolerance is maintained by multiple mechanisms. The programmed cell death-1 (PD-1)/PD-ligand 1 (PD-L1) pathway has emerged as an important mediator in terminating the immune response and inducing immune tolerance by promoting Treg cell development and inhibiting effector T (for example, Th17) cell responses.[14] PD-1 is a cell surface receptor belonging to the CD28 family. PD-1 inhibits signaling through the T-cell receptor (TCR), resulting in reduced proliferation, cytokine production and cytotoxic activation of T cells.[15,16] PD-1 contributes to the maintenance of immune tolerance, whereas PD-1 deficiency causes autoimmunity in mice. Apart from autoimmune disorders, the PD-1/PD-L1 pathway also participates in the establishment of maternal-fetal tolerance by promoting the Treg/Th17 balance.[17] Our previous findings showed that PD-L1 Fc exerted a protective effect in the L-NAME-induced pre-eclamptic murine model by reversing the Treg/Th17 imbalance in the periphery and at the maternal-fetal interface.[9] However, the precise role of the PD-1/PD-L1 pathway in PE is unclear.

In this study, we investigated the relationship between the PD-1/PD-L1 pathway and the Treg/Th17 imbalance in PE. Then, the regulatory effect of the PD-1/PD-L1 pathway on the Treg/Th17 paradigm in PE was explored by targeting T-cell proliferation, differentiation and transdifferentiation.

MATERIALS AND METHODS

Subjects

This study was reviewed and approved by Huazhong University of Science and Technology Clinical Trial Ethics Committee. All methods were carried out in accordance with the approved guidelines and regulations. Written informed consent was obtained from each participant prior to entering the study. All the study subjects, namely, healthy pregnant women and pre-eclamptic women, were recruited from the Department of Obstetrics and Gynecology at the Maternal and Child Health Hospital of Hubei Province, Wuhan, China. The presence of PE was assessed according to ACOG guidelines (ACOG Task Force on Hypertension in Pregnancy, 2013). All pregnancies in this study were singleton gestations, and none of the participants had active labor at the time of enrollment and blood sampling. None of the participants was affected by pre-existing clinical disorders, such as renal diseases or chronic hypertension before pregnancy, or complicated by pre-term labor, chorioamnionitis or diabetes. Normal pregnant women in the third trimester were matched with the pre-eclamptic women for age, gestational age and parity (Table 1).

Placenta sampling

Placentas were collected after cesarean section. Two pieces of tissue were obtained immediately after delivery from the center of the maternal placental surface rather than the infarction and calcification area. The sample size was 1.0 cm × 1.0 cm × 1.0 cm. One sample was fixed in 4% paraformaldehyde for hematoxylin and eosin (H&E) staining and immunohistochemistry. The other sample was stored at −80 °C for quantitative real-time polymerase chain reaction (qRT-PCR) and western blot analysis.

Blood sampling

Peripheral blood samples were collected into heparinized tubes, and peripheral blood mononuclear cells (PBMCs) were isolated using the density centrifugation technique and immediately used.

H&E staining and immunohistochemistry

The 4-μm-thick paraffin sections of placentas were stained with H&E according to the standard H&E protocol. After antigen retrieval, the paraffin-embedded placental tissue sections were processed using standard immunohistochemical techniques according to the manufacturer's instructions. The antibodies used in this part included an anti-PD-1 Ab (Bioss, Beijing, China), an anti-PD-L1 Ab (Santa Cruz, Dallas, TX, USA), an anti-Foxp3 Ab (Abcam, Cambridge, UK) and an anti-RORγt Ab (Bioss).

qRT-PCR

Total RNA was extracted and purified using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and an equal amount of total

Table 1 Clinical characteristics of women with PE ($n = 41$) and women with NP ($n = 67$)

	PE	*NP*	P-*value*
Age (years)	29.70 ± 4.27	27.60 ± 3.76	NS
Gestational age at diagnosis of PE (weeks)	37.13 ± 1.26	36.92 ± 1.24	NS
Gravidity	1.50 ± 0.85	1.46 ± 0.78	NS
Parity	0.30 ± 0.18	0.17 ± 0.38	NS
Systolic blood pressure (mmHg)	144.40 ± 10.18	119.50 ± 9.93	<0.01
Diastolic blood pressure (mmHg)	94.00 ± 8.59	74.67 ± 7.87	<0.01
Proteinuria (g/24 h)	0.81 ± 0.20	—	—
Creatinine (μmol/l)	67.92 ± 7.38	41.20 ± 3.88	<0.01

Abbreviations: NP, normal pregnancy; PE, pre-eclampsia.
Data are presented as the mean ± s.e.m.

RNA (1 μg) was used for cDNA synthesis (Takara Bio, Shiga, Japan). The primer sets used in this study were designed using Primer-BLAST software. The primers were as follows: PD-1 F: 5′-ACCCTGGTGGTTGGTGTCGT-3′, R: 5′-CCTGGCTCCTATTGTCCCTC-3′; PD-L1 F: 5′-TTTGCTGAACGCCCCATA-3′, R: 5′-TGCTTGTCCAGATGACTTCG-3′; Foxp3 F: 5′-CACTGACCAAGGCTTCATCTG-3′, R: 5′-GGAGGAACTCTGGGAATGTG-3′; RORγt F: 5′-ATGGAAGTGGTGCTGGTT-3′, R: 5′-GGGAGAAGTCAAAGATGGAG-3′; PI3K F: 5′-TGCTGTCTCCTCTAAACCCTG-3′, R: 5′-TCTTGCCGTAAATCATCCC-3′; AKT F: 5′-TTCTTTGCCGGTATCGTGT-3′, R: 5′-TGTCATCTTGGTCAGGTGGT-3′; m-TOR F: 5′CGCTGTCATCCCTTTATCG-3′, R: 5′-AGAGTCAAGTGGTCATAGTCCG-3′; PTEN F: 5′-AAGACCATAACCCACCACAGC-3′, R: 5′-CCAGTTCGTCCCTTCCAG-3′; IL-10 F: 5′- GGAGAACCTGAAGACCCT-3′, R: 5′-TGATGAAGATGTCAAACTCACT-3′; IL-17 F: 5′-TGTCACTGCTACTGCTGCT-3′, R: 5′-GGTTATGGATGTTCAGGTTG-3′; GAPDH F: 5′-GGACCTGACCTGCCGTCTAG-3′, R: 5′-GTAGCCCAGGATGCCCTTGA-3′. The cDNA (2 μl) was subjected to qRT-PCR amplification analysis using SYBR Green PCR mix (Applied Biosystems, Carlsbad, CA, USA). The qRT-PCR parameters were as follows: step one, one cycle at 95 °C for 30 s; step two, 40 cycles at 95 °C for 5 s, 58 °C for 30 s and 72 °C for 30 s; step three, 95 °C for 15 s, 60 °C for 60 s and 95 °C for 15 s (LightCycler 96 System, Roche, Basel, Switzerland). The amount of target relative to a calibrator was computed by $2^{-\Delta\Delta CT}$, and GAPDH was used for normalization.

Western blot analysis

Following SDS/PAGE, transfer and blocking, the PVDF blots were incubated with primary antibodies. The membranes were washed and incubated with a secondary antibody, followed by electrochemical luminescence detection. Relative protein levels were quantified by scanning densitometry and analyzed using ImageJ software (National Institutes of Health). Protein detection was carried out using anti-GAPDH (Abcam), anti-PD-1 (Bioss), anti-PD-L1 (Santa Cruz), anti-Foxp3 (Abcam) and anti-RORγt (Millipore, Darmstadt , Germany) Abs.

Flow cytometry

A total of 2×10^6 PBMCs were resuspended and stained with anti-CD4-FITC monoclonal antibodies (mAbs), anti-CD25-PE mAbs, anti-CD127-Percp-Cy5.5 mAbs and anti-PD-1-APC mAbs for surface antigens (eBioscience, San Diego, CA, USA), in accordance with the manufacturer's instructions. For intracellular cytokine detection, 2×10^6 PBMCs were stimulated with 2 μl of Cell Stimulation Cocktail and 2 μl of Protein Transport Inhibitor Cocktail (eBioscience) for 4 h. All the cells were stained with fluorescein-labeled mAbs for surface antigens before culture. The cultured cells were then permeabilized with permeabilization/fixation buffer (eBioscience) and then stained with anti-Ki67-PE-Cy7 mAbs (Ki67 was used as a marker of cell proliferation), anti-Foxp3-APC mAbs, anti-IL-17A-PE mAbs or anti-IL-17A-PE-Cy7 mAbs (eBioscience). Finally, the cells were resuspended in 300 μl of PBS for subsequent flow cytometric analysis. All data were acquired using a FACScalibur (BD Biosciences, San Jose, CA, USA), and processed using the CellQuest program (Becton Dickinson, Franklin Lakes, NJ, USA).

Cell isolation, activation and culture

Naive $CD4^+$ T and Treg cells were isolated from PBMCs of women with NP and of women with PE by multi-step magnetic sorting using a human Naive $CD4^+$ T Cell Isolation Kit II and a human $CD4^+CD25^+$ Regulatory T Cell Isolation Kit (Miltenyi Biotec, Auburn, CA, USA), respectively, with a Midi&Mini MACS instrument (Miltenyi Biotec) in accordance with the manufacturer's instructions. Sorted naive $CD4^+$ T cells (1×10^6/well in 24-well plates) were then cultured at 37 °C in a moist atmosphere containing 5% CO_2 in complete RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml), 2 mM L-glutamine, 10 mM HEPES, 0.1 mM non-essential amino acids and 1 mM sodium pyruvate (all from Sigma, Oakville, ON, Canada). The purity of the sorted cells was generally >95%. All the cells were cultured under Th0- (anti-CD3 mAbs (5 μg/ml)/anti-CD28 mAbs (5 μg/ml)) (eBioscience), Th17- (anti-CD3 mAbs (5 μg/ml)/anti-CD28 mAbs (5 μg/ml)/TGF-β (5 ng/ml)/IL-1β (5 ng/ml)/IL-6 (20 ng/ml)/anti-IFN-γ mAbs (2 μg/ml)/anti-IL-4 mAbs (2 μg/ml)) (PeproTech, Rocky Hill, NJ, USA) and Treg-prone (anti-CD3 mAbs (5 μg/ml)/anti-CD28 mAbs (5 μg/ml)/TGF-β (5 ng/ml) /anti-IFN-γ mAbs (2 μg/ml)/anti-IL-4 mAbs (2 μg/ml)) conditions with or without PD-L1 Fc (10 μg/ml) (R&D, Minneapolis, MN, USA) or anti-PD-L1 mAbs (10 ng/ml) (eBioscience) for 3 days. For each study case, nine groups were established as shown in the following scheme (Table 2). Sorted Treg cells (1×10^5/well in 96-well plates) from women with NP were then cultured at 37 °C in a moist atmosphere containing 5% CO_2 in complete RPMI 1640 medium under rh IL-2 (0.5 U/ml) (PeproTech)/anti-CD3 mAbs (5 μg/ml)/anti-CD28 mAbs (5 μg/ml) condition with or without anti-PD-L1 mAbs (10 ng/ml), or under pro-inflammatory conditions (rh IL-2 (0.5 U/ml)/anti-CD3 mAbs (5 μg/ml)/anti-CD28 mAbs (5 μg/ml)/IL-1β (10 ng/ml)/

Table 2 Nine culture conditions for Treg and Th17 cell differentiation from naïve $CD4^+$ T cells in NP and in PE

NO.	*Culture conditions for naive $CD4^+$T cells from NP*	*Culture conditions for naïve $CD4^+$T cells from PE*
1	Th0-prone condition+PBS	Th0-prone+PBS
2	Th0-prone+PD-L1 Fc	Th0-prone+PD-L1 Fc
3	Th0-prone+anti-PD-L1 mAbs	Th0-prone+anti-PD-L1 mAbs
4	Th17-prone+PBS	Th17-prone+PBS
5	Th17-prone+PD-L1 Fc	Th17-prone+PD-L1 Fc
6	Th17-prone+anti-PD-L1 mAbs	Th17-prone+anti-PD-L1 mAbs
7	Treg-prone+PBS	Treg-prone+PBS
8	Treg-prone+PD-L1 Fc	Treg-prone+PD-L1 Fc
9	Treg-prone+anti-PD-L1 mAbs	Treg-prone+anti-PD-L1 mAbs

Abbreviations: NP, normal pregnancy; PE, pre-eclampsia.

IL-6 (20 ng/ml)/IL-23 (20 ng/ml)) (PeproTech) with or without PD-L1 Fc for 5 days.

Statistical analysis

Statistical analyses were performed using the Statistical Package for Social Science (SPSS) for Windows (Version 18.0 software, SPSS Inc., Chicago, IL, USA) and GraphPad Prism software, version 5 (GraphPad, San Diego, CA, USA). Differences between two groups were analyzed using Student's *t*-test or Mann-Whitney *U*-test as applicable. Spearman's correlation test was used to assess the correlation between PD-1 expression and T-cell proliferation capacity, and the correlation between Treg and Treg cells, as well as that between the percentages of IL-17$^+$ Treg and Th17 cells. The differences between multiple groups were analyzed by one-way ANOVA and χ^2-test. In all experiments, the n number of independent experiments is provided in the figure legend. P-values less than 0.05 were considered significant.

RESULTS

Decreased PD-1, PD-L1 and Foxp3 expression and increased RORγt expression in placentas from pre-eclamptic women

The nuclear transcription factors Foxp3 and RORγt play crucial roles in the quantities and functions of Treg and Th17 cells,

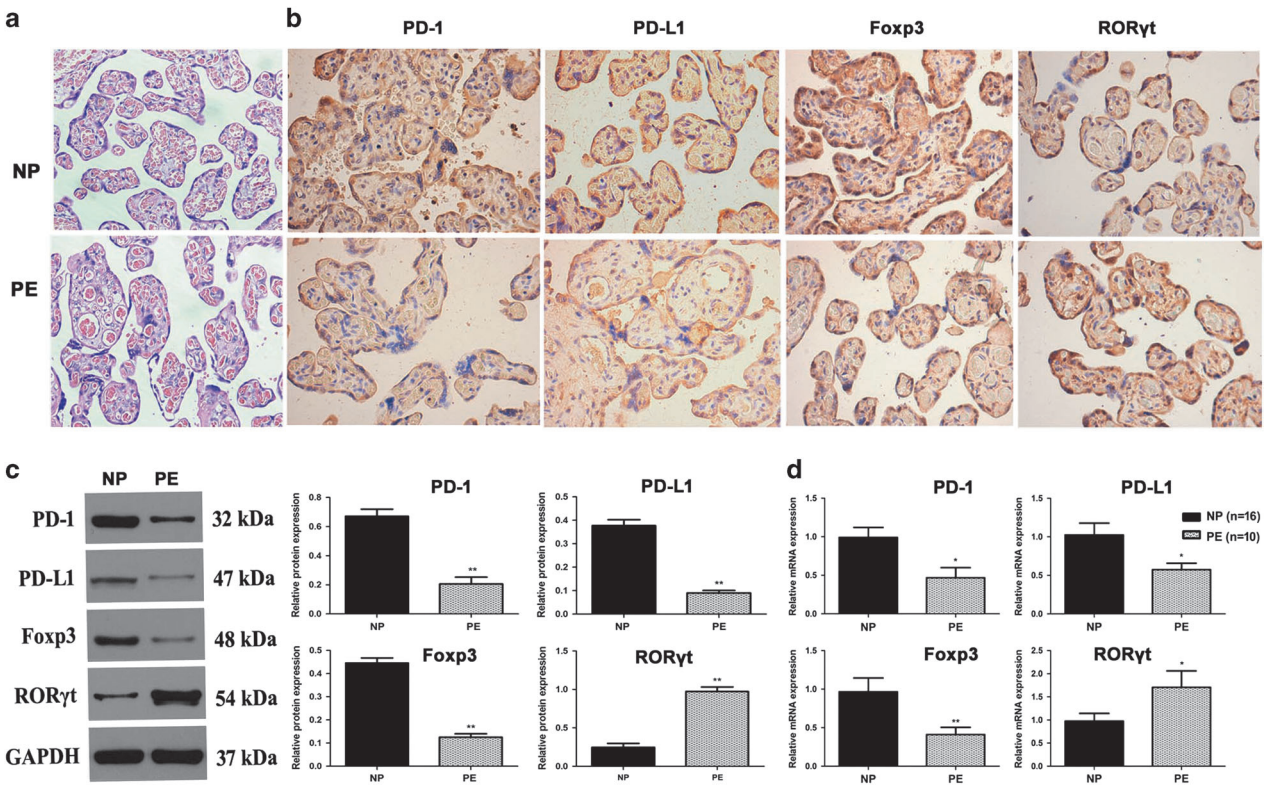
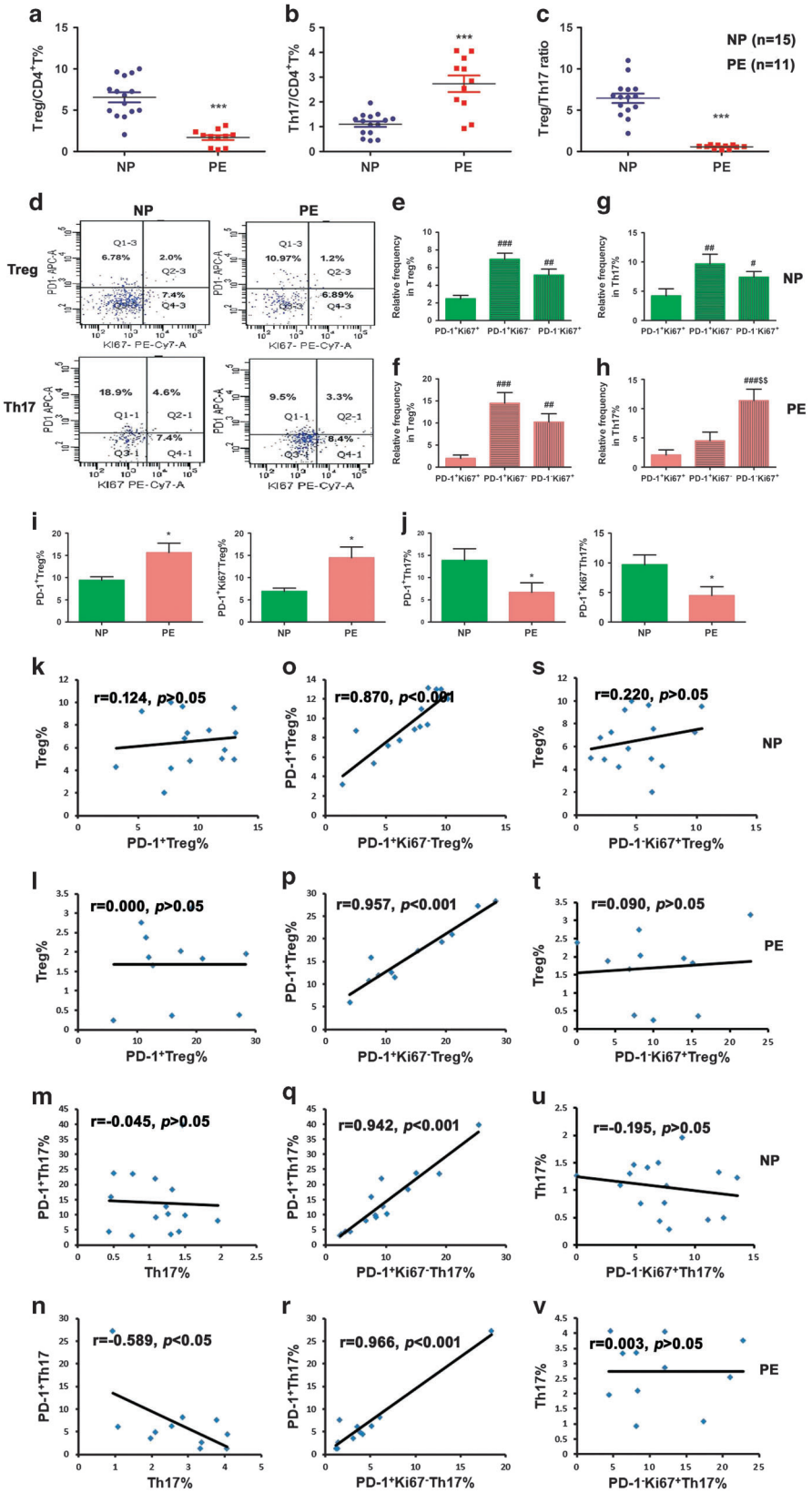


Figure 1 PD-1, PD-L1, Foxp3 and RORγt expression in placentas from women with NP and women with PE. The placentas were collected from women with NP ($n=16$) and women with PE ($n=10$). (**a**) Structural changes in the placentas were observed by H&E staining (×200). (**b**) PD-1 and PD-L1, Foxp3 and RORγt expression in placentas was analyzed by immunohistochemistry (×200). (**c**) The PD-1 (32 kDa), PD-L1 (47 kDa), Foxp3 (48 kDa) and RORγt (54 kDa) protein levels in the placenta were analyzed by western blotting. (**d**) The PD-1, PD-L1, Foxp3 and RORγt mRNA levels in the placenta were analyzed by qRT-PCR. The data are presented as the mean ± s.e.m. *$P<005$, **$P<001$ versus NP. NP, normal pregnancy; PE, pre-eclampsia.

the percentages of IL-17$^+$

Figure 2 PD-1 and Ki67 expression in peripheral Treg and Th17 cells and their potential associations. Peripheral blood mononuclear cells were obtained from women with NP ($n=15$) and women with PE ($n=11$), and PD-1 and Ki67 expression in Treg and Th17 cells was detected by flow cytometry. (**a**) The percentages of Treg (CD4$^+$CD25^{high}CD127$^{low/-}$) cells, (**b**) the percentages of Th17 (CD4$^+$IL-17A$^+$) cells and (**c**) the Treg/Th17 cell ratios were analyzed. (**d**) Dot plot of PD-1 and Ki67 detected on Treg and Th17 cells. (**e** and **f**) The percentages of PD-1$^+$Ki67$^-$, PD-1$^+$Ki67$^+$ and PD-1$^-$Ki67$^+$ Treg cell subsets. (**g** and **h**) The percentages of PD-1$^+$Ki67$^-$, PD-1$^+$Ki67$^+$ and PD-1$^-$Ki67$^+$ Th17 cell subsets. (**i**) The percentages of PD-1$^+$ Treg and PD-1$^+$Ki67$^-$ Treg cells between NP and PE. (**j**) The percentages of PD-1$^+$ Th17 and PD-1$^+$Ki67$^-$ Th17 cells between NP and PE. (**k** and **l**) Correlation analysis between the percentages of Treg and PD-1$^+$ Treg cells. (**o** and **p**) Correlation analysis between the percentages of PD-1$^+$ Treg cells and PD-1$^+$Ki67$^-$ Treg cells. (**s** and **t**) Correlation analysis between the percentages of Treg and PD-1$^-$Ki67$^+$Treg cells. (**m** and **n**) Correlation analysis between the percentages of Th17 cells and PD-1$^+$ Th17 cells. (**q** and **r**) Correlation analysis between the percentages of PD-1$^+$ Th17 and PD-1$^+$Ki67$^-$ Th17 cells. (**u** and **v**) Correlation analysis between the percentages of Th17 and PD-1$^-$Ki67$^+$Th17 cells. The data are presented as the mean ± s.e.m. * versus NP, # versus PD-1$^+$Ki67$^+$ subset, $ versus PD-1$^+$Ki67$^-$ subset, $^{*/\#}P<005$, $^{**/\#\#/\$\$}P<001$, $^{***/\#\#\#}P<0001$ versus NP. NP, normal pregnancy; PE, pre-eclampsia.

respectively, and their expression levels match the percentages of the corresponding subsets. To investigate the relationship between the PD-1/PD-L1 pathway and the Treg/Th17 imbalance during pregnancy, we first evaluated PD-1, PD-L1, Foxp3 and RORγt expression in placentas from women with PE and from women with NP. As expected, PD-1, PD-L1,



Foxp3 and RORγt were detected in placentas in NP and in PE (Figures 1a and b). The protein and mRNA levels of PD-1, PD-L1 and Foxp3 were decreased in PE compared with NP, whereas the RORγt expression levels were increased (Figures 1c and d). These results suggested a potential correlation between the PD-1/PD-L1 axis and the Treg/Th17 imbalance at the maternal-fetal interface in PE.

PD-1 deficiency enhances Th17 proliferation and may directly contribute to expansion of the Th17-cell pool in the peripheral blood of pre-eclamptic women

Engagement of PD-1 by PD-L1 can block T-cell proliferation, cytokine production and cytolytic functions and impair T-cell survival.[14] Therefore, we hypothesized that the decreased percentage of Treg cells and the increased Th17 numbers in PE might be associated with their differential proliferation capacities, which might be influenced by the PD-1/PD-L1 pathway. To test this hypothesis, we first evaluated PD-1 and Ki67 expression in Treg and Th17 cells in NP and in PE (Table 2). Herein, Treg and Th17 cells were defined as the $CD4^+CD25^{high}CD127^{low/-}$ and $CD4^+IL\text{-}17^+$ cell populations, respectively. We found an inverse correlation between the proportions of Treg and Th17 cells in PE versus NP. In PE, the percentage of Treg cells was decreased (Figure 2a) and the percentage of Th17 cells was increased (Figure 2b). In addition, the Treg/Th17 cell ratios were decreased in PE (Figure 2c). The PD-1 and Ki67 expression levels in Treg and Th17 cells were then determined (Figure 2d). The percentage of the $PD\text{-}1^+Ki67^-$ subset was higher than the percentages of the $PD\text{-}1^+Ki67^+$ and $PD\text{-}1^-Ki67^+$ subsets in the Treg cells in the two groups (Figures 2e and f), but no differences were found between the single-positive subsets. Similar results were found in Th17 cells in NP, whereas the percentage of the $PD\text{-}1^-Ki67^+$ subset was higher than the other Th17 cell subsets in PE (Figures 2g and h). Furthermore, we found similar variation tendencies in the $PD\text{-}1^+$ and $PD\text{-}1^+Ki67^-$ Treg and Th17 cell subsets in NP and in PE. Thus, the percentages of $PD\text{-}1^+$ Treg and $PD\text{-}1^+Ki67^-$ Treg cells (Figure 2i) were increased in PE, and the percentages of $PD\text{-}1^+$ Th17 and $PD\text{-}1^+Ki67^-$ Th17 cells (Figure 2j) were decreased in PE. Nearly all the PD-1-positive cells were Ki67-negative (Table 3). Therefore, PD-1 might determine the numbers of Treg and Th17 cells by inhibiting cell proliferation. Next, a correlation analysis was performed. No significant correlation was found between the percentages of Treg and $PD\text{-}1^+$ Treg cells in either NP or PE (Figures 2k and l), whereas a positive correlation was found between the percentages of $PD\text{-}1^+$ Treg and $PD\text{-}1^+Ki67^-$ Treg cells in the two groups (Figures 2o and p). No correlation was found between the percentages of $PD\text{-}1^-Ki67^+$ Treg and Treg cells in NP or PE (Figures 2s and t). These results indicated that PD-1 inhibited the proliferation capacity of the Treg cells, although this effect did not significantly influence the cell numbers in NP and in PE. Compared with NP, the percentage of Th17 cells was negatively correlated with the percentage of $PD\text{-}1^+$ Th17 cells in PE (Figures 2m and n), and the percentage of $PD\text{-}1^+$ Th17 cells was positively associated with the percentage of $PD\text{-}1^+Ki67^-$ Th17 cells in the two groups (Figures 2q and r). Furthermore, no correlation was found between the percentages of $PD\text{-}1^-Ki67^+$ Th17 and Th17 cells in NP or PE (Figures 2u and v). Therefore, the inhibition of proliferation by PD-1 was more evident in Th17 cells than in Treg cells, particularly in PE.

PD-L1 Fc inhibits Th17 differentiation but promotes Treg development and anti-PD-L1 mAb administration interrupts the immune balance by facilitating Th17 development in the peripheral blood of women with NP and women with PE

Treg and Th17 cells are derived from the same precursor cells, namely, naive $CD4^+$ T cells. This process is closely and distinctively regulated by the PD-1/PD-L1 pathway, which is favorable for Treg development from naive $CD4^+$ T cells and inhibits Th17 differentiation.[14] We hypothesized that the differentiation of Treg and Th17 cells might also be regulated by the PD-1 pathway in NP and in PE. To test this hypothesis, we isolated the peripheral naive $CD4^+$ T cells from women with NP and women with PE and stimulated them under Th0-, Th17- and Treg-prone conditions in the presence or absence of PD-L1 Fc or an anti-PD-L1 mAbs for 3 days. Then, the percentages of Treg and Th17 cells were determined by flow cytometry (Figures 3a and b). The Treg-prone condition contributes to Treg development, whereas the Th17-prone condition facilitates Th17 differentiation.[18] As expected, the percentages of Treg cells that developed from naive $CD4^+$ T cells increased following PD-L1 Fc treatment, even under the Th17-prone condition in NP (Figure 3c), whereas the anti-PD-L1 mAbs treatment decreased the percentages of Treg cells, even under the Treg-prone condition in PE (Figure 3f). No significant differences were found in the percentages of Th17 cells among the nine groups in NP (Figure 3d). The percentages of Th17 cells differentiated from naive $CD4^+$ T cells decreased with anti-PD-L1 mAbs administration, even under the Treg-prone condition in PE (Figure 3g). PD-L1 Fc had no effect on Treg/Th17 cell ratios in NP and in PE, whereas the anti-PD-L1 mAbs decreased the Treg/Th17 cell ratios in the two groups (Figures 3e and h). Thus, PD-L1 Fc selectively promotes Treg development during T-cell differentiation and suppresses Th17 differentiation in human pregnancy *ex vivo*.

PD-L1 Fc promotes peripheral Treg cell differentiation by inhibiting PI3K/AKT/m-TOR signaling and enhancing PTEN expression

To more clearly illustrate the regulatory effect of the PD-1/PD-L1 pathway on Treg and Th17 cell differentiation, the potential molecular mechanisms were investigated. Isolated peripheral naive $CD4^+$ T cells from NP and from PE samples were stimulated with or without PD-L1 Fc or an anti-PD-L1 mAb under Th0-, Th17- and Treg-prone conditions for 3 days. Next, the mRNA levels of potential genes were determined by qRT-PCR. The PD-1/PD-L1 pathway has been reported to promote the development of $Foxp3^+$ Treg cells by blocking AKT/m-TOR signaling and augmenting PTEN expression (Figure 4a).

Table 3 Prevalence of PD-1 and Ki67 expression on Treg and Th17 cells in women with PE ($n = 11$) and women with NP ($n = 15$)

Subset	*Marker*	*PE*	*NP*	P-*value*
Treg %[a]	$CD4^+CD25^{high}CD127^{low/-}/CD4^+$	1.69 ± 0.28	6.56 ± 0.59	< 0.001
Th17%[a]	$CD4^+IL\text{-}17A^+/CD4^+$	2.73 ± 0.32	1.05 ± 0.11	< 0.001
Treg/Th17 cell ratio[a]	$CD4^+CD25^{high}CD127^{low/-}$ / $CD4^+IL\text{-}17A^+$	0.57 ± 0.22	6.44 ± 2.22	< 0.001
PD-1^+Treg %[b]	PD-$1^+CD4^+CD25^{high}CD127^{low/-}/CD4^+CD25^{high}CD127^{low/-}$	15.80 (6.00–28.32)	9.12 (3.17–13.11)	< 0.01
PD-1^+Ki67$^-$Treg %[b]	PD-1^+Ki67$^-CD4^+CD25^{high}CD127^{low/-}/CD4^+CD25^{high}CD127^{low/-}$	11.51 (4.00–28.32)	7.88 (1.42–10.18)	< 0.05
PD-1^+Ki67$^-$Treg/PD-1^+Treg cell/ratio[b]		0.89 (0.48–1.00)	0.75 (0.29–0.91)	> 0.05
PD-1^+Th17%[b]	PD-$1^+CD4^+IL\text{-}17A^+/CD4^+IL\text{-}17A^+$	4.90 (1.32–27.20)	10.20 (3.00–39.70)	< 0.05
PD-1^+Ki67$^-$Th17%[b]	PD-1^+Ki67$^-CD4^+IL\text{-}17A^+/$ $CD4^+IL\text{-}17A^+$	3.50 (1.19–18.40)	8.30 (2.40–25.40)	< 0.01
PD-1^+Ki67$^-$Th17/PD-1^+Th17 cell/ratio[b]		0.77 (0.21–1.00)	0.77 (0.42–1.00)	> 0.05

Abbreviations: NP, normal pregnancy; PE, pre-eclampsia.
[a]Data are presented as the mean ± s.e.m.
[b]Data are presented as the median (range).

In line with published reports, our data showed that Foxp3 mRNA expression increased with PD-L1 Fc administration and decreased with anti-PD-L1 mAb treatment in both NP and PE (Figures 4b and c). PD-L1 Fc decreased RORγt expression in NP and in PE, even under the Th17-prone condition, and increased it in the presence of the anti-PD-L1 mAbs in the two groups (Figures 4d and e). This phenomenon was accompanied by decreased PI3K/AKT/m-TOR mRNA expression (Figures 4f–k) and increased PTEN expression (Figures 4l and m) in the presence of PD-L1 Fc. Higher PI3K/AKT/m-TOR expression (Figures 4f–k) and lower PTEN expression (Figures 4l and m) were observed with the anti-PD-L1 mAbs in both NP and PE. Thus, increased PTEN expression and decreased PI3K/AKT/m-TOR expression underlie the promotion of Treg cell differentiation by the PD-1 pathway.

PD-L1 prevents the induction of Treg cell transdifferentiation into Th17 cells by pro-inflammatory cytokines in peripheral blood from NP

The plasticity of the production of the pro-inflammatory cytokine IL-17 by Treg cells has been demonstrated under normal and pathogenic conditions, such as autoimmune disorders (for example, rheumatoid arthritis, inflammatory bowel disease and systemic sclerosis).[19,20] The observed plasticity of Treg cells has raised the possibility that $Foxp3^+$ Treg cell transdifferentiation into Th17 cells might play a potential pathogenic role at inflammatory sites. Additionally, the induction of IL-17 expression in Treg cells under inflammatory conditions is usually accompanied by an impaired or lost immune suppressive capacity.[19,20] To date, whether Treg cell plasticity is significant in human pregnancy is unclear. Thus, the percentage of IL-17^+ Treg cells was measured by flow cytometry in NP and in PE (Figure 5a and Table 4). According to previous reports,[19,20] Treg and Th17 cells were, respectively, defined as $CD4^+Foxp3^+$ and $CD4^+IL\text{-}17^+$ when analyzing T-cell plasticity. We found that the percentage of IL-17^+ Treg cells was higher in PE compared with NP (Figure 5b). Additionally, the IL-17^+ Treg/Treg cell ratios and IL-17^+ Treg/Th17 cell ratios were higher in PE than in NP (Figure 5b). Therefore, increased numbers of IL-17-producing Treg cells might diminish the Treg cell pool and promote Th17 cell expansion. As expected, no correlation was found between the percentages of Treg and IL-17^+ Treg cells in the two groups (Figure 5c). The percentage of IL-17^+ Treg cells and the IL-17^+ Treg/Treg cell ratio was positively associated with the Th17 cell frequency in the two groups (Figure 5c). PD-L1 Fc administration promotes the maintenance and suppressor functions of Treg cells by enhancing Foxp3 expression.[14] To explore whether Treg cell stability was regulated by the PD-1 pathway, Treg cells isolated from women with NP were cultured in the presence or absence of pro-inflammatory cytokines (IL-6/IL-1β/IL-23) with or without PD-L1 Fc or an anti-PD-L1 mAbs for 5 days, and the mRNA expression levels of potential genes were determined by qRT-PCR (Figure 5d). Our results showed that the Foxp3 mRNA levels were significantly decreased

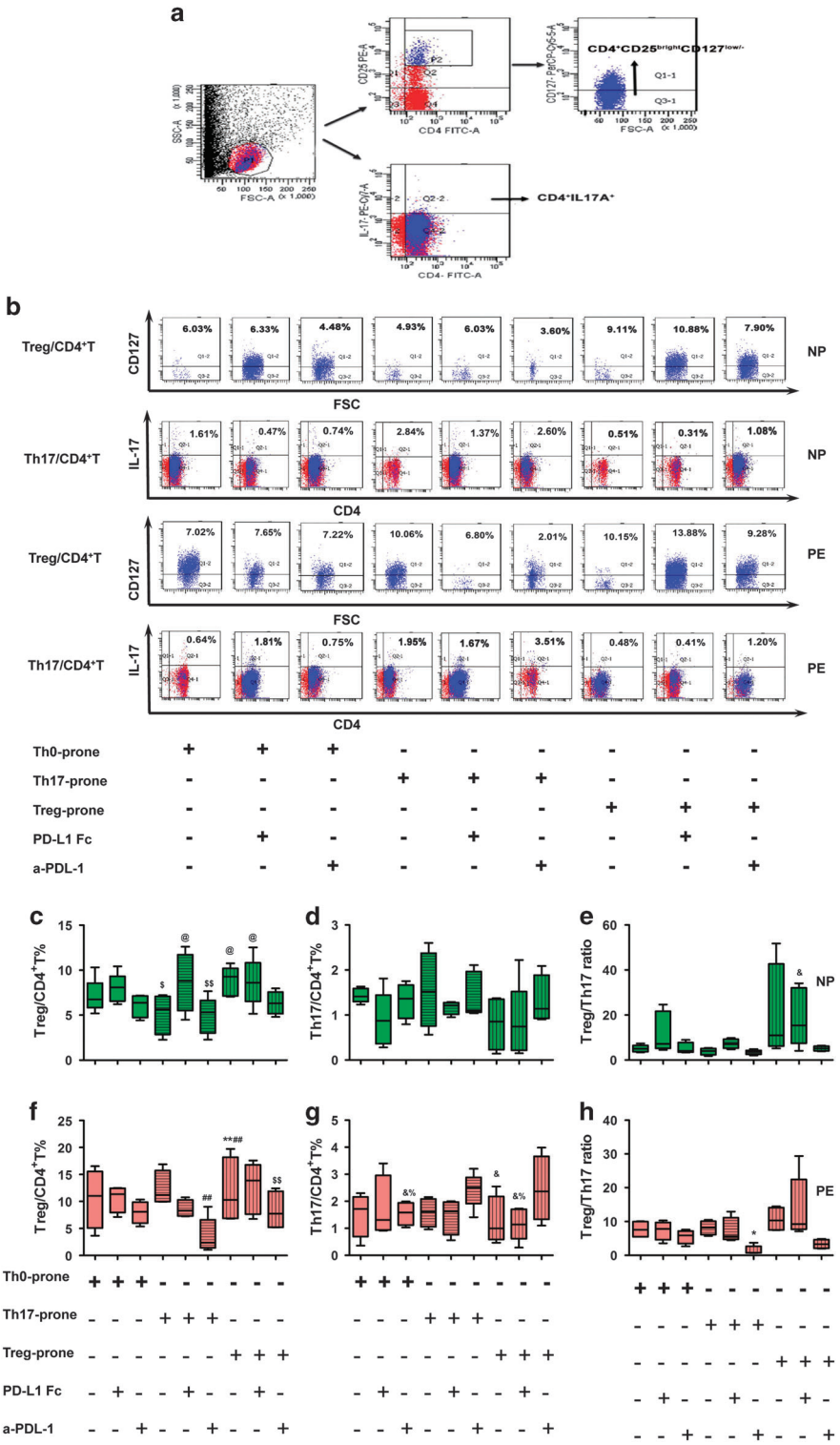


Figure 3 The percentages of Treg and Th17 cells differentiated from naive CD4$^+$ T cells under different conditions with or without PD-L1 Fc or the anti-PD-L1 mAb. Peripheral naive CD4$^+$ T cells freshly isolated from women with NP ($n=15$) and women with PE ($n=15$) were seeded at a density of 1×10^6 cells/ml per well in 24-well plates. After 3 days, the cells were washed twice with PBS. (**a**) Gating strategy for determination of CD4$^+$CD25^{high}CD127$^{low/-}$ Treg and CD4$^+$IL-17A$^+$ Th17 cells by flow cytometry. (**b**) Flow cytometry was used to determine the percentages of Treg and Th17 cells differentiated from naive CD4$^+$ T cells under Th0-, Th17- and Treg-prone conditions with or without PD-L1 Fc or an anti-PD-L1 mAb. (**c** and **f**) The percentages of Treg cells. (**d** and **g**) The percentages of Th17 cells. (**e** and **h**) The Treg/Th17 cell ratios. The data are presented as the median (range). * versus Th0-prone condition, $ versus Treg-prone condition, @ versus Th17-prone condition, # versus Th0-prone+anti-PD-L1 mAb condition, & versus Th17-prone+anti-PD-L1 mAb condition, % versus Treg-prone+anti-PD-L1 mAb condition. */$/@/&/%$P<0.05$, **/$$/##$P<0.01$. NP, normal pregnancy; PE, pre-eclampsia.

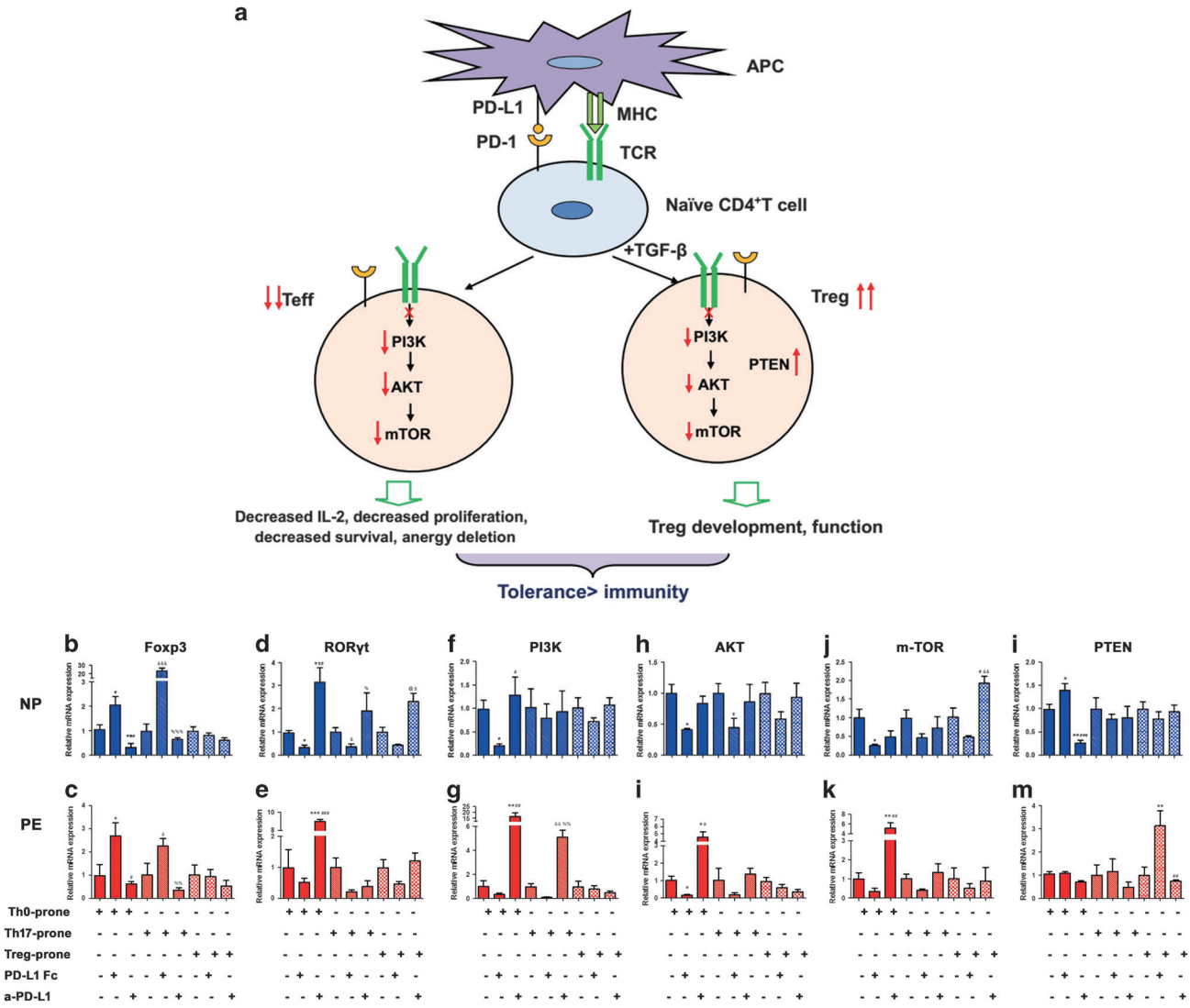


Figure 4 Expression of nuclear transcriptional factors (Foxp3 and RORγt) and potential signaling molecules involved in Treg and Th17 cell differentiation. Naïve CD4$^+$ T cells from women with NP ($n=15$) and women with PE ($n=15$) were isolated and cultured under Th0-, Treg- and Th17-prone conditions with or without PD-L1 Fc fusion protein or an anti-PD-L1 mAb for 3 days. The Foxp3, RORγt, PI3K/AKT/m-TOR and PTEN mRNA expression levels were determined by quantitative RT-PCR. (**a**) Molecular mechanism of the PD-1/PD-L1 pathway on T-cell differentiation. The PD-1 pathway inhibits downstream signaling of the PI3K/AKT signaling in naive CD4$^+$ T cells, resulting in functional inactivation of naive CD4$^+$T cells and inhibition of effector T-cell differentiation and function. In the presence of TGF-β, the PD-1 pathway attenuates the AKT-m-TOR signaling, preferentially biasing naive T-cell programming toward the development of Treg cells and suppressive capacity. (**b** and **c**) The mRNA expression of Foxp3. (**d** and **e**) The mRNA expression of RORγt. (**f**–**k**) The mRNA expression levels of PI3K/AKT/m-TOR. (**l** and **m**) The mRNA expression of PTEN. The data are presented as the mean ± s.e.m. * versus Th0-prone condition, # versus Th0-prone+PD-L1 Fc condition, & versus Th17-prone condition, % versus Th17-prone+ PD-L1 Fc condition, @ versus Treg-prone condition, $ versus Treg-prone+PD-L1 Fc condition. $^{*/\#/\&/\%/@/\$}P<0.05$, $^{**/\#\#/\&\&/\%\%/@@/\$\$}P<0.01$, $^{***/\#\#\#}P<0.001$. NP, normal pregnancy; PE, pre-eclampsia.

by the anti-PD-L1 mAbs in the absence of pro-inflammatory cytokines. Similarly, IL-10 expression was decreased by the PD-1 pathway blockade. IL-10 expression decreased following exposure to the pro-inflammatory cytokines; however, PD-L1 Fc increased IL-10 expression in the presence of the pro-inflammatory cytokines. The anti-PD-L1 mAbs significantly promoted RORγt and IL-17 expression. Although the pro-inflammatory cytokines enhanced RORγt and IL-17 expression, this effect could be inhibited by PD-L1 Fc. Therefore, PD-L1 Fc enhances the stability of Treg cells, which are more prone to revert into pro-inflammatory IL-17-producing Treg cells in PE.

DISCUSSION

In this study, we verified the regulatory effects of the PD-1/PD-L1 pathway on the Treg/Th17 paradigm in PE. We showed for the first time altered PD-1 and PD-L1 expression on Treg and Th17 cells at the maternal-fetal interface in PE. Furthermore,

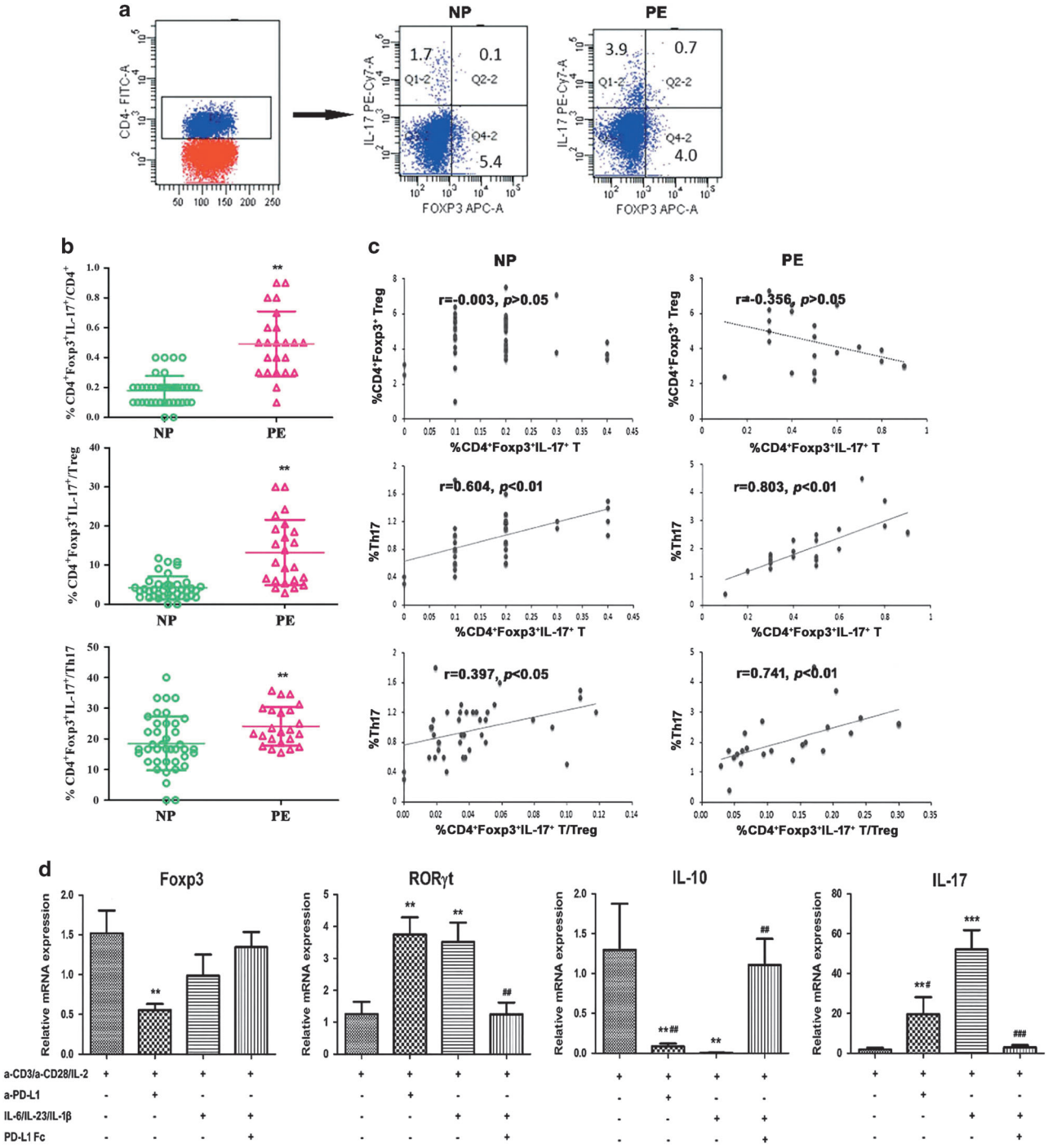


Figure 5 PD-L1 Fc promoted Treg cell stability by preventing Treg cell transdifferentiation into Th17 cells. PBMCs were isolated from women with NP ($n=40$) and women with PE ($n=23$) and stained with antibodies against CD4, IL-17A and Foxp3 to assess IL-17 expression on $CD4^+Foxp3^+$ Treg cells by flow cytometry. (**a**) The percentage of IL-17-producing Treg cells was determined by flow cytometry. (**b**) The percentage of $IL\text{-}17^+$ Treg, $IL17^+$ Treg/Treg cell ratios and $IL\text{-}17^+$ Treg/Th17 cell ratios. (**c**) Correlation analysis between the percentage of Treg cells and the IL-17-producing Treg cell frequency, between the percentage of Th17 cells and the $IL\text{-}17^+$ Treg cell frequency, and between the percentage of Th17 cells and the $IL\text{-}17^+$ Treg/Treg cell ratio. Further investigations were performed to explore the relationship between the PD-1 pathway and Treg cell stability in PE. The regulatory effect of the PD-1/PD-L1 pathway on Treg cell plasticity was studied *in vitro*. Peripheral $CD4^+CD25^+$ Treg cells freshly isolated from women with NP ($n=20$) were seeded at a density of 1×10^5 cells/well in 96-well plates in the presence or absence of IL-6/IL-23/IL-1β with or without PD-L1 Fc or an anti-PD-L1 mAb. After 5 days, the cells were collected, and the Foxp3, RORγt, IL-10 and IL-17 mRNA levels were determined by RT-PCR. (**d**) The Foxp3, RORγt, IL-10 and IL-17 mRNA levels were determined. The data are presented as the mean ± s.e.m. * versus anti-CD3 mAb/anti-CD28 mAb/IL-2 condition, # versus IL-6/IL-23/IL-1β condition. $^{*/\#}P<0.05$, $^{**/\#\#}P<0.01$, $^{***/\#\#\#}P<0.001$. NP, normal pregnancy; PE, pre-eclampsia.

Table 4 Prevalence of IL-17-producing Treg cells in PE ($N=23$) and in NP ($N=40$)

Subset	*Marker*	*PE*	*NP*	P-*value*
IL-17$^+$Treg %	CD4$^+$Foxp3$^+$IL-17$^+$/CD4$^+$	0.50±0.22	0.18±0.10	<0.01
IL-17$^+$Treg/Treg cell ratio %	CD4$^+$Foxp3$^+$IL-17$^+$/CD4$^+$Foxp3$^+$	24.11±6.32	18.54±8.75	<0.01
IL-17$^+$Treg/Th17 cell ratio %	CD4$^+$Foxp3$^+$IL-17$^+$/CD4$^+$IL-17$^+$	13.20±8.39	4.20±2.89	<0.01

Abbreviations: NP, normal pregnancy; PE, pre-eclampsia.
Data are presented as the mean±s.e.m.

we demonstrated that the PD-1/PD-L1 pathway promoted the Treg/Th17 balance by preferentially inhibiting Th17 proliferation, promoting Treg cell development and sustaining Treg cell stability during pregnancy. However, a dysfunctional PD-1/PD-L1 axis might disrupt these regulatory pathways, thereby contributing to the Treg/Th17 imbalance systemically and locally, leading to the development of PE.

The PD-1/PD-L1 pathway has emerged as a critical regulator in immune homeostasis by promoting Treg immunity and inhibiting effector T (for example, Th17) cell responses.[14] Previous evidence confirmed that PD-1/PD-L1 pathway blockade resulted in failure of maternal-fetal tolerance with Treg cell deficiency and Th17 cell hyperactivity.[17] Our previous findings suggested that altered PD-1 and PD-L1 expression might lead to a peripheral Treg/Th17 imbalance in women with PE.[9] PD-L1 Fc also had protective effects in PE-like models by reversing the Treg/Th17 imbalance.[9] Consistent with earlier reports,[9,21,22] we confirmed the occurrence of a Treg/Th17 imbalance and altered PD-1 and PD-L1 expression in the placentas of women with PE. However, the potential mechanism underlying the regulation of the Treg/Th17 imbalance by the PD-1/PD-L1 pathway has not been illustrated in PE. Because T-cell proliferation, differentiation and survival were associated with the PD-1/PD-L1 axis, we speculated that the Treg and/or Th17 cell pools were associated with cell proliferation, differentiation and transdifferentiation, which might be influenced by the PD-1/PD-L1 pathway in PE.

PD-1 has been proposed as a marker of T-cell exhaustion.[23] PD-1 cross-linked with PD-L1 inhibited effector T-cell proliferation and cytokine production. The Ki67 protein is a cellular marker for proliferation, which is present during all active phases of the cell cycle but is absent from resting cells. Therefore, higher PD-1 expression would be accompanied by lower Ki67 expression. Interestingly, we observed significant changes in PD-1 and Ki67 expression levels in Treg and Th17 cells from women with PE. These differences were cell-specific (that is, PD-1 expression was increased in Treg cells and decreased in Th17 cells). These findings were in agreement with our former results[9] and the results published by Toldi *et al.*,[10] who suggested that higher PD-1 expression might account for the lower Treg cell proportion in PE. Similarly, the percentage of PD-1$^+$Ki67$^-$ Treg cells was higher and the percentage of PD-1$^+$Ki67$^-$ Th17 cells was lower in PE. Moreover, nearly all the Treg and Th17 cells with PD-1 expression lost Ki67 protein expression. Therefore, PD-1 expression might also block T-cell proliferation during pregnancy. Next, a correlation analysis was performed to clarify the relationship between PD-1 expression and the T-cell proliferation capacity. Although no correlation was found between PD-1 expression and the Treg cell numbers, the frequencies of PD-1$^+$ Treg cells were positively associated with PD-1$^+$Ki67$^-$ Treg cells in NP and in PE. This finding indicated that higher PD-1 expression most likely inhibited Treg cell proliferation but might have a relatively weak impact on the total Treg cell numbers in both NP and PE. Compared with NP, lower PD-1 expression was negatively associated with higher Th17 cell numbers in PE. However, the percentage of PD-1$^+$ Th17 cells was negatively associated with the Th17 cell proliferation capacity in both NP and PE. Lower PD-1 expression had a weak inhibitory effect on Th17 cell proliferation in PE, contributing to a higher Th17 cell abundance. Therefore, in contrast to hepatitis C virus infection,[24] PD-1 showed a more profound inhibitory effect on Th17 cell proliferation than Treg cells in human term-pregnancy, particularly in PE.

Treg and Th17 cells may derive from the same precursors under distinct cytokine conditions.[25] TGF-β is a critical differentiation factor for the generation of Treg cells. The differentiation of pathogenic Th17 cells from naive T cells was induced in the presence of IL-6 and completely inhibited the generation of Treg cells induced by TGF-β. However, the differential fates of naive T cells were regulated by the PD-1/PD-L1 pathway. In the presence of TGF-β, PD-L1 Fc can induce a profound increase in the *de novo* generation of Treg cells from naive CD4$^+$ T cells.[26] Moreover, PD-L1 deficiency resulted in minimal Treg cell differentiation, highlighting the essential role of PD-L1 during Treg cell induction. As expected, PD-L1 Fc induced a significantly higher percentage of Treg cells from naive CD4$^+$ T cells even in the presence of IL-6, IL-23 and IL-1β, and this effect was more significant in NP. The PD-1 blockade promoted Th17 but not Treg cell development even under Treg-prone conditions and was more profound in PE. Therefore, PD-L1 Fc selectively promoted Treg cell development during T-cell differentiation and suppressed Th17 cell differentiation in NP, but this process might be disrupted in PE.

The reasons for the differential effects of PD-L1 on Treg and Th17 cells remain unclear. However, the differences in these effects might be due to intrinsic differences that govern the metabolic activity of Treg and Th17 cells.[27] The PI3K/AKT/m-TOR axis controls Th17 cell differentiation by regulating the nuclear translocation of RORγt.[28] Blockade of the PI3K/AKT/m-TOR pathway and truncation of TCR signaling increased

Foxp3 expression.[29] PD-1 suppressed the activation of the PI3K/AKT axis, eventually leading to the attenuation of Th17 cell differentiation.[30] Moreover, the PD-1/PD-L1 pathway promoted Treg cell differentiation by blocking the AKT/m-TOR pathway and augmenting PTEN expression.[26] We found that PD-L1 Fc decreased PI3K/AKT/m-TOR expression and enhanced PTEN expression, with higher Foxp3 expression and lower RORγt expression levels. A completely opposite situation occurred with anti-PD-L1 mAb intervention (that is, PI3K/AKT/m-TOR expression increased and PTEN expression decreased, with lower Foxp3 expression and higher RORγt expression). These findings were consistent with our former *in vivo* results.[9] Therefore, our results demonstrated that PD-1/PD-L1 regulated the Treg/Th17 balance by facilitating Treg instead of Th17 cell differentiation in human pregnancy. PD-1/PD-L1 pathway dysfunction might disrupt the Treg/Th17 balance and ultimately lead to PE development.

The plasticity of Treg cells means that they are not static and can transform into Th17 cells.[31] This phenomenon was first found in mice, wherein IL-6 was shown to convert Treg cells into Th17 cells in the absence of TGF-β,[32] and was later confirmed in humans.[20] Therefore, Foxp3$^+$ Treg cells with immune suppression can be converted into inflammatory cytokine-producing cells in a specific inflammatory microenvironment, gradually lose Foxp3 expression, and finally transdifferentiate into Th17 cells, which potentially contribute to disease pathogenesis.[33,34] We were the first group to find IL-17-producing Treg cells in human pregnancy. Moreover, the percentage of IL-17$^+$ Treg cells was higher in PE. The percentage of IL-17$^+$ Treg cells was positively associated with the Th17 rather than the Treg cell frequency in the two groups. These results indicated that Treg cells under inflammatory conditions might convert to IL-17-producing cells, particularly in PE, and thus potentially contribute to the pathogenesis of the disorder by enriching the Th17 cell pool. Among the various pro-inflammatory cytokines, IL-6, IL-23 and IL-1β had a profound capacity to endow Treg cells with the ability to produce IL-17.[19] Interestingly, increased serum IL-6, IL-1β and IL-23 levels were detected in women with PE.[35–37] Therefore, the suppression capacity of Treg cells might be impaired because they are transformed into Th17 cells in response to the increased pro-inflammatory cytokine production in PE. PD-L1 engagement enhanced Foxp3 expression and the suppressive function of established Treg cells.[26] Therefore,

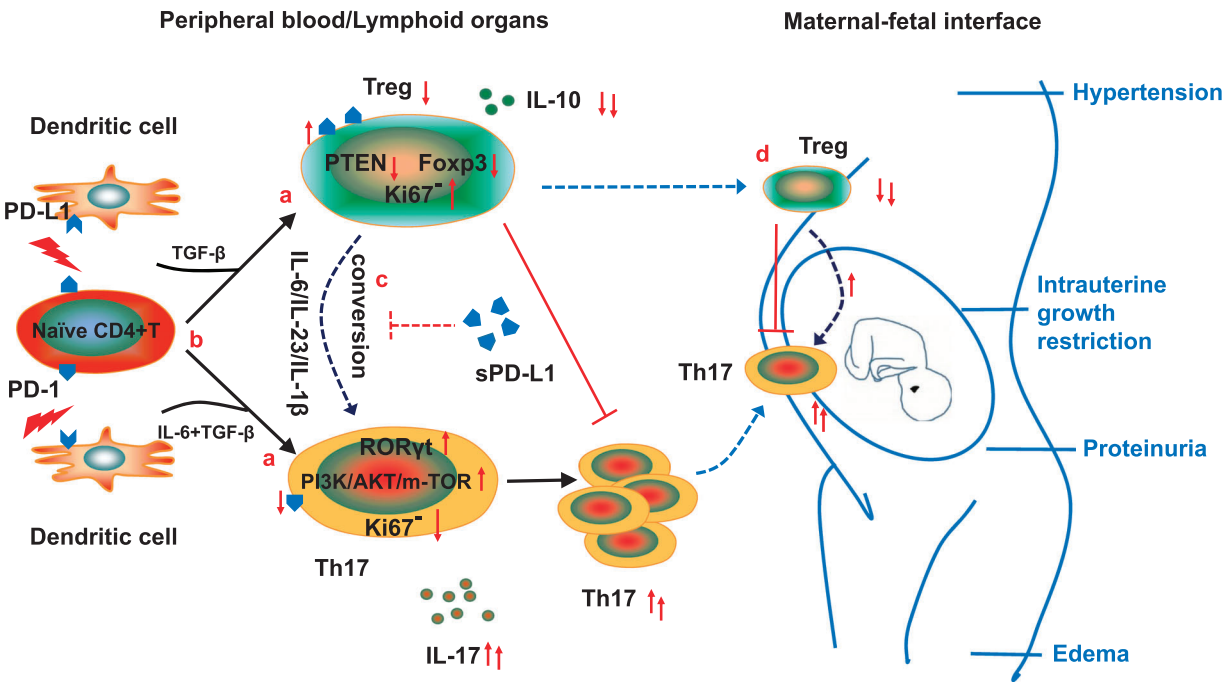


Figure 6 The PD-1/PD-L1 pathway regulated the dynamics of Treg and Th17 cells in PE. This figure illustrates the consequences of these interactions in the peripheral blood or lymphoid organs and the maternal-fetal interface in women with PE. (**a**) Decreased PD-1 expression in Th17 cells enhanced Ki67 expression, resulting in a higher proliferation capacity, whereas the opposite findings were observed in Treg cells. (**b**) With PD-1/PD-L1 pathway dysfunction, more Th17 cells were induced from naïve CD4$^+$T cells, with increased PI3K/AKT/m-TOR expression. Correspondingly, Treg cells derived from naive CD4$^+$T cells were decreased, with lower PTEN expression. (**c**) In the presence of IL-6/IL-23/IL-1β, Treg cells were more prone to transdifferentiation into IL-17-producing Treg cells and eventually transformed into Th17 cells. Furthermore, PD-1/PD-L1 crosstalk blockade promoted the conversion of Treg cells into Th17 cells. PD-L1 Fc administration reversed this conversion. (**d**) Thus, more peripheral Th17 than Treg cells migrated to the maternal-fetal interface. The conversion of Treg into Th17 cells might also occur locally. These changes might contribute to a local and systemic Treg/Th17 imbalance in PE and promote the appearance of clinical symptoms such as hypertension, proteinuria, edema and intrauterine growth restriction.

PD-1/PD-L1 pathway dysfunction might contribute to Treg cell instability in PE. Some reports have shown that immunosuppressive factors, such as IL-10, have a profound effect on Treg-mediated suppression,[38] although the absence of IL-10 does not affect the suppression capacity of Treg cells.[39] Therefore, the IL-10 mRNA level could reflect the suppression capacity of Treg cells. As expected, IL-6/IL-23/IL-1β preferentially promoted Th17-related gene expression and impaired Treg suppression but did not inhibit Foxp3 expression. A similar phenomenon occurred with anti-PD-L1 mAb administration in the absence of IL-6/IL-23/IL-1β, which decreased Foxp3 expression, indicating that the survival of Treg cells was inhibited. However, PD-L1 Fc treatment significantly reversed the effect induced by the IL-6/IL-23/IL-1β cytokines. These findings indicated that PD-L1 Fc facilitated Treg cell stability, which would be lost with PD-1 signaling blockade, especially in a pro-inflammatory microenvironment such as PE. A conflict might exist between higher PD-1 expression and lower stability in Treg cells in PE, but this possibility is beyond the scope of this study. Because PD-1 is indisputably an attenuator of antigen receptor signaling, PD-1-abundant Treg cells may receive weaker TCR stimulation *in vivo* and thus never undergo conversion into Foxp3-negative cells due to the strong immune response in PE. This discrepancy was explained in a report by Ellestad *et al.*,[40] who proposed that PD-L1 modulated Treg cell conversion not by interacting with PD-1 but instead with B7-1. Therefore, further investigations are needed to clarify this issue.

However, Treg cell markers were not consistent in our study. Treg cells were defined as $CD4^+CD25^{high}CD127^{low/-}$, $CD4^+Foxp3^+$ and $CD4^+CD25^+$ in different parts of our study. In fact, the definition of Treg cell markers has long been a consistent issue. First, Treg cells have been identified as a CD4 T-cell subset exhibiting *in vitro* suppressive properties and expressing high levels of CD25.[41–43] However, the inducible nature of CD25 expression during T-cell activation on conventional T cells renders this molecule unsuitable for Treg cell identification during immune activation. Shortly thereafter, the Foxp3 transcription factor was identified as an essential and specific factor for Treg cell development and function.[44,45] However, the expression of Foxp3 is also observed in some conventional $CD4^+CD25^-$ T cells upon activation.[46] Finally, it has been shown that human $CD4^+Foxp3^+CD25^{high}$ cells express lower levels of CD127, the alpha-chain of the IL-7 receptor, compared with their Foxp3 counterpart.[47,48] The combination of the CD25 and CD127 surface markers with or without intra-nuclear staining for Foxp3 expression has thereafter been widely employed to identify $CD4^+$Treg cells.[49] Furthermore, sorting of Treg cells has greatly benefited from the high CD25 and/or low CD127 expression. However, such an approach also presents drawbacks: conventional non-Treg CD4 T cells downregulate CD127 expression during activation while they upregulate CD25. It is therefore likely that CD127 and CD25 expression cannot accurately discriminate *ex vivo* Treg cells from activated T cells in situations of immune activation.[50] In conclusion, Treg cell identification in the context of chronic activation still suffers from the lack of indisputable markers that can unequivocally distinguish Treg from effector cells.[49] Thus, various identification strategies for Treg cells have been used by researchers for different objectives. $CD4^+CD25^{high}CD127^{low/-}$ was used to determine the frequency of Treg cells in our study. The sorted Treg cells were defined as $CD4^+CD25^+$ with the $CD4^+CD25^+$ regulatory T-cell isolation kit. When we analyzed Treg cell plasticity in the last subsection, Treg cells were defined as $CD4^+Foxp3^+$ according to previous publications.[32–34]

In conclusion, our data demonstrated that altered PD-1 and PD-L1 expression might be associated with a Treg/Th17 imbalance at the maternal-fetal interface in women with PE. We investigated the potential mechanism underlying the regulation of the Treg/Th17 imbalance by the PD-1/PD-L1 pathway in human pregnancy and found that the PD-1/PD-L1 pathway might regulate the Treg/Th17 imbalance via 'one-two punch' approaches (Figure 6): (i) promoting Th17 cell proliferation, (ii) inhibiting Treg cell differentiation and (iii) enhancing Treg cell transformation into Th17 cells in PE. Furthermore, our findings provided information supporting the hypothesis that PD-L1 Fc might be an ideal therapeutic target in PE. The therapeutic value of PD-L1 Fc in the treatment of PE will be explored in the future.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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1 Gathiram P, Moodley J. Pre-eclampsia: its pathogenesis and pathophysiology. *Cardiovasc J Afr* 2016; **27**: 71–78.
2 Saleem S, McClure EM, Goudar SS, Patel A, Esamai F, Garces A *et al.* A prospective study of maternal, fetal and neonatal deaths in low- and middle-income countries. *Bull World Health Organ* 2014; **92**: 605–612.
3 Chaiworapongsa T, Chaemsaithong P, Yeo L, Romero R. Pre-eclampsia part 1: current understanding of its pathophysiology. *Nat Rev Nephrol* 2014; **10**: 466–480.
4 Tranquilli AL, Dekker G, Magee L, Roberts J, Sibai BM, Steyn W *et al.* The classification, diagnosis and management of the hypertensive disorders of pregnancy: A revised statement from the ISSHP. *Pregnancy Hypertens* 2014; **4**: 97–104.
5 Mor BW, Robert CT, Thangaratinam S, Magee LA, de Groot CJ, Hofmeyr GJ. Pre-eclampsia. *Lancet* 2016; **387**: 999–1011.
6 Odegard RA, Vatten LJ, Nilsen ST, Salvesen KA, Austgulen R. Preeclampsia and fetal growth. *Obstet Gynecol* 2000; **96**: 950–955.
7 Arenas-Hernandez M, Romero R, St Louis D, Hassan SS, Kaye EB, Gomez-Lopez N. An imbalance between innate and adaptive immune cells at the maternal-fetal interface occurs prior to endotoxin-induced preterm birth. *Cell Mol Immunol* 2016; **13**: 462–473.
8 Sargent IL, Borzychowski AM, Redman CWG. Immunoregulation in normal pregnancy and pre-eclampsia: an overview. *Rep BioMed* 2006; **13**: 680–686.
9 Tian M, Zhang YH, Liu ZZ, Sun GQ, Mor G, Liao AH. The PD-1/PD-L1 inhibitory pathway is altered in pre-eclampsia and regulates T cell responses in pre-eclamptic rats. *Sci Rep* 2016; **6**: 27683.
10 Toldi G, Vasarhelyi ZE, Rigo Jr J, Orban C, Tamassy Z, Bajnok A *et al.* Prevalence of regulatory T cell subtypes in preeclampsia. *Am J Reprod Immunol* 2015; **74**: 110–115.

11 Aluvihare VR, Kallikourdis M, Betz AG. Regulatory T cells mediate maternal tolerance to the fetus. *Nat Immunol* 2004; **5**: 266–271.
12 Wang WJ, Liu FJ, Xin L, Hao CF, Bao HC, Qu QL *et al.* Adoptive transfer of pregnancy-induced CD4+CD25+ regulatory T cells reverses the increase in abortion rate caused by interleukin 17 in the CBA/JxBALB/c mouse model. *Hum Reprod* 2014; **29**: 946–952.
13 Quinn KH, Lacoursiere DY, Cui L, Bui J, Parast MM. The unique pathophysiology of early-onset severe preeclampsia: role of decidual T regulatory cells. *J Reprod Immunol* 2011; **91**: 76–82.
14 Gianchecchi E, Delfino DV, Fierabracci A. Recent insight into the role of the PD-1/PD-L1 pathway in immunological tolerance and autoimmunity. *Autoimmun Rev* 2013; **12**: 1091–1100.
15 Iwai Y, Terawaki S, Honjo T. PD-1 blockade inhibits hematogenous spread of poorly immunogenic tumor cells by enhanced recruitment of effector T cells. *Int Immunol* 2005; **17**: 133–144.
16 Okazaki T, Chikuma S, Iwai Y, Fagarasan S, Honjo T. A rheostat for immune responses: the unique properties of PD-1 and their advantages for clinical application. *Nat Immunol* 2013; **14**: 1212–1218.
17 D'Addio F, Riella LV, Mfarrej BG, Chabtini L, Adams LT, Yeung M *et al.* The link between the PDL1 costimulatory pathway and Th17 in fetomaternal tolerance. *J Immunol* 2011; **187**: 4530–4541.
18 Moon SJ, Park JS, Heo YJ, Kang CM, Kim EK, Lim MA *et al.* In vivo action of IL-27:reciprocal regulation of Th17 and Treg cells in collagen-induced arthritis. *Exp Mol Med* 2013; **45**: e46.
19 Wang T, Sun X, Zhao J, Zhang J, Zhu H, Li C *et al.* Regulatory T cells in rheumatoid arthritis showed increased plasticity toward Th17 but retained suppressive function in peripheral blood. *Ann Rheum Diis* 2015; **74**: 1293–1301.
20 Beriou G, Costantino CM, Ashley CW, Yang L, Kuchroo VK, Baecher-Allan C *et al.* IL-17-producing human peripheral regulatory T cells retain suppressive function. *Blood* 2009; **113**: 4240–4249.
21 Darmochwal-Kolarz D, Saito S, Rolinski J, Tabarkiewicz J, Kolarz B, Leszczynska-Gorzelak B *et al.* Activated T lymphocytes in pre-eclampsia. *Am J Reprod Immunol* 2007; **58**: 39–45.
22 Steinborn A, Haensch GM, Mahnke K, Schmitt E, Toermer A, Meuer S *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**: 401–412.
23 Sharpe AH, Wherry EJ, Ahmed R, Freeman GJ. The function of programmed cell death 1 and its ligands in regulating autoimmunity and infection. *Nat Immunol* 2007; **8**: 239–245.
24 Franceschini D, Paroli M, Francavilla V, Videtta M, Morrone S, Labbadia G *et al.* PD-L1 negatively regulates CD4+CD25+Foxp3+ Tregs by limiting STAT-5 phosphorylation in patients chronically infected with HCV. *J Clin Invest* 2009; **119**: 551–564.
25 Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M *et al.* Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* 2006; **441**: 235–238.
26 Francisco LM, Salinas VH, Brown KE, Vanguri VK, Freeman GJ, Kuchroo VK *et al.* PD-L1 regulates the development, maintenance, and function of induced regulatory T cells. *J Exp Med* 2009; **206**: 3015–3029.
27 McAlees JW, Lajoie S, Dienger K, Sproles AA, Richgels PK, Yang Y *et al.* Differential control of CD4+ T-cell subsets by the PD-1/PD-L1 axis in a mouse model of allergic asthma. *Eur J Immunol* 2015; **45**: 1019–1029.
28 Kurebayashi Y, Nagai S, Ikejiri A, Ohtani M, Ichiyama K, Baba Y *et al.* PI3K-Akt-mTORC1-S6K1/2 axis controls Th17 differentiation by regulating Gfi1 expression and nuclear translocation of RORgamma. *Cell Rep* 2012; **1**: 360–373.
29 Sauer S, Bruno L, Hertweck A, Finlay D, Leleu M, Spivakov M *et al.* T cell receptor signaling controls Foxp3 expression via PI3K, Akt, and mTOR. *Proc Natl Acad Sci USA* 2008; **105**: 7797–7802.
30 Yang L, Qiao G, Hassan Y, Li Z, Zhang X, Kong H *et al.* Program death-1 suppresses autoimmune arthritis by inhibiting Th17 response. *Arch Immunol Ther Exp* 2016; **64**: 417–423.
31 Omenetti S, Pizarro TT. The Treg/Th17 axis: a dynamic balance regulated by the gut microbiome. *Front Immunol* 2015; **6**: 639.
32 Xu L, Kitani A, Fuss I, Strober W. Cutting edge: regulatory T cells induce CD4+CD25-Foxp3- T cells or are self-induced to become Th17 cells in the absence of exogenous TGF-beta. *J Immunol* 2007; **178**: 6725–6729.
33 Zhou L, Lopes JE, Chong MM, Ivanov II, Min R, Victora GD *et al.* TGF-beta-induced Foxp3 inhibits TH17 cell differentiation by antagonizing RORgammat function. *Nature* 2008; **453**: 236–240.
34 Hovhannisyan Z, Treatman J, Littman DR, Mayer L. Characterization of interleukin-17-producing regulatory T cells in inflamed intestinal mucosa from patients with inflammatory bowel diseases. *Gastroenterology* 2011; **140**: 957–965.
35 Pinheiro MB, Gomes KB, Ronda CR, Guimaraes GG, Freitas LG, Teixeira-Carvalho A *et al.* Severe preeclampsia: association of genes polymorphisms and maternal cytokines production in Brazilian population. *Cytokine* 2015; **71**: 232–237.
36 Dong W, Yin L. Expression of lipoxin A4, TNFalpha and IL-1beta in maternal peripheral blood, umbilical cord blood and placenta, and their significance in pre-eclampsia. *Hypertens Pregnancy* 2014; **33**: 449–456.
37 Medeiros LT, Peracoli JC, Romao M, Bannwart-Castro CF, Golim MA, Borges VT *et al.* M1 Monocyte subpopulation is associated with pro-inflammatory cytokineproduction in pregnant women with preeclampsia. *Pregnancy Hypertens* 2012; **2**: 276–277.
38 Asseman C, Mauze S, Leach MW, Coffman RL, Powrie F. An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. *J Exp Med* 1999; **190**: 995–1004.
39 Takahashi T, Kuniyasu Y, Toda M, Sakaguchi N, Itoh M, Iwata M *et al.* Immunologic self-tolerance maintained by CD25+CD4+ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. *Int Immunol* 1998; **10**: 1969–1980.
40 Ellestad KK, Thangavelu G, Ewen CL, Boon L, Anderson CC. PD-1 is not required for natural or peripherally induced regulatory T cells: severe autoimmunity despite normal production of regulatory T cells. *Eur J Immunol* 2014; **44**: 3560–3572.
41 Levings MK, Sangregorio R, Roncarolo MG. Human CD25+CD4+T regulaotry cells suppress naïve and memory T cell proliferation and can be expanded in vitro without loss of function. *J Exp Med* 2001; **193**: 1295–1302.
42 Ng WF, Duggan PJ, Ponchel F, Matarese G, Lombardi G, Edwards AD *et al.* Human CD4+CD25+ cells: a naturally occuring population of regulatory T cells. *Blood* 2001; **98**: 2736–2744.
43 Dieckmann D, Plottner H, Berchtold S, Berger T, Schuler G. Ex vivo isolation and characterization of CD4+CD25+ T cells with regulatory properties from human blood. *J Exp Med* 2011; **193**: 1303–1310.
44 Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell debelopment by the transcription factor Foxp3. *Science* 2003; **299**: 1057–1061.
45 Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol* 2003; **4**: 330–336.
46 Allan SE, Crome SQ, Crellin NK, Passerini L, Steiner TS, Bacchetta R *et al.* Activation-induced Foxp3 in human T effector cells does not suppress proliferation or cytokine production. *Int Immunol* 2007; **19**: 345–354.
47 Seddiki N, Santner-Nanan B, Martinson J, Zaunders J, Sasson S, Landay A *et al.* Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. *J Exp Med* 2006; **203**: 1693–1700.
48 Liu W, Putnam AL, Xu-Yu Z, Szot GL, Lee MR, Zhu S *et al.* CD127 expression inversely correlates with Foxp3 and suppressive function of human CD4+T reg cells. *J Exp Med* 2006; **203**: 1701–1711.
49 Simonetta F, Bourgeois C. CD4+Foxp3+ regulatory T-cell subsets in human immunodeficiency virus infection. *Front Immunol* 2013; **4**: 215.
50 del Pozo Balado Mdel M, Leal M, Méndez Lagares G, Mata RC, López-Cortes LF, Viciana P *et al.* Increased regulatory T cell counts in HIV-infected nonresponders to hepatitis B virus vaccine. *J Infect Dis* 2010; **202**: 362–369.