

# Contribution of regulatory T cells to immune tolerance and association of microRNA-210 and Foxp3 in preeclampsia

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**Abstract.** Increasing evidence suggests that an exaggerated maternal systemic inflammatory response may play a central role in the pathogenesis of preeclampsia (PE). Considering the growing evidence on microRNAs (miRNAs) and tissue-specific regulators of gene expression, we investigated the potential association of miR-210 and forkhead box p3 (Foxp3) in preeclamptic patients. Serum levels of the cytokines interleukin (IL)-6, IL-10, IL-17, and transforming growth factor- $\beta$ 1 were detected with ELISA. Reverse-transcription-quantitative polymerase chain reaction was performed to detect mRNA expression for maternal placenta retinoic acid-related orphan receptor C, Foxp3 and miRNA (miR)-210. Foxp3 protein expression was evaluated by western blot analysis. Serum levels of cytokines IL-10 were significantly lower in preeclamptic patients than in normal pregnant women. mRNA expression of Foxp3 was significantly lower in placenta of PE. mRNA expression of miR-210 was significantly increased in PE. Results of western blot analysis indicated that Foxp3 protein expression was lower in PE than in normal pregnant women. Our data suggest that PE manifests as a decreased number of regulatory T cells (Tregs), which regulate maternal tolerance of the fetus. In placenta from women with PE, compared with normal pregnant women, mRNA expression of Foxp3 was significantly decreased, and expression of miR-210 was significantly increased.

## Introduction

Preeclampsia (PE) is characterized by hypertension and proteinuria. The condition increases the risk for maternal and

perinatal morbidity and mortality, with worldwide incidence of approximately 4-6% (1). Despite extensive research, the etiology and pathogenesis of PE are not completely understood. Previous research has shown that placental tissue but not the fetus is necessary for the development of PE. The condition is cured upon delivery of the placenta, which underscores the indispensable role of this tissue in the pathophysiology of PE (2).

The consensus reached at a recent workshop sponsored by the National Institute of Child Health and Human Development/National Institutes of Health is that preeclampsia is a multifactorial disease whose pathogenesis is not solely vascular, genetic, immunologic, or environmental but a complex combination of factors (3). In PE, excessive activation of peripheral blood leukocytes is associated with exaggerated innate and adaptive immune responses, which may interfere with normal progression of a pregnancy (4).

Regulatory T cells (Tregs) are a specialized subpopulation of T cells that act as potent suppressors of inflammation to prevent autoimmunity and graft rejection (5). Tregs prevent activation of maternal T cells against fetal cells; this protection of the fetus from the maternal immune system has been widely reported in both mice and humans (6-8). A reduction in maternal Treg populations could prevent immunological tolerance of the fetus and has been associated with obstetrical complications, such as miscarriage, PE and preterm labor (9-14).

Tregs comprise Th1 and Th2 cells. Tregs expressing forkhead box p3 (Foxp3) help to maintain immune tolerance and homeostasis. At the steady-state level, transforming growth factor- $\beta$  1 (TGF- $\beta$ 1) produced in the immune system induces Foxp3<sup>+</sup> regulatory T cells and maintains self-tolerance (15). However, in the inflammatory insult, by the activated innate immune system IL-6 will suppress the generation of TGF- $\beta$ 1-induced Treg cells and induce a pro-inflammatory Th17 cell response (15). CD4<sup>+</sup>CD25<sup>+</sup> Tregs play a critical role in maternal tolerance in mice and in humans (6,9,16). CD4<sup>+</sup>CD25<sup>+</sup> Tregs play a critical role in PE. This immunological disturbance seems to result from overactivation of the maternal inflammatory response, which involves overproduction of pro-inflammatory cytokines such as interferon- $\gamma$  (IFN- $\gamma$ ), interleukin (IL)-1 $\beta$ , IL-6 and tumor necrosis factor- $\alpha$

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(TNF- $\alpha$ ) (14-16) and lower concentrations of regulatory cytokine IL-10 (17,18). Th17 cells are a lineage of CD4<sup>+</sup> T cells that differentiate in the presence of IL-6 and TGF- $\beta$ . These cells express transcription factor retinoic acid-related orphan receptor C (RORc) and produce cytokine IL-17 (19).

Previous researchers found a decrease in Tregs and an increase in Th17-profile cells in the peripheral blood of women with PE compared to normal pregnant women. This imbalance may underlie activation of the inflammatory response in women with PE (20,21).

Tregs are also considered to play a crucial role in the implantation of embryos and the maintenance of maternal immune tolerance to the fetus (22). Zhang *et al* demonstrated that the percentage of CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>-</sup> cells among the CD4<sup>+</sup> T cells was significantly lower in patients with severe PE compared to healthy pregnant women (23). Numerous researchers have sought to establish a protocol for identifying Tregs. Intracellular Foxp3 staining cannot be used to isolate the subpopulation of Tregs for functional tests, as it requires that cells be fixed and permeabilized. Published reports have shown that simultaneous high expression of CD25 and low expression of CD127 correspond with increased expression of intracellular factor Foxp3. This profile allows researchers to identify T cells with suppressor activity (24,25).

A correlation between CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>-</sup> cells and CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells has been confirmed in healthy individuals (26). No significant difference was found between the number of CD25<sup>+</sup>CD127<sup>-</sup> cells among the CD4<sup>+</sup> T cells with and without permeabilization in the same NHDs (27).

MicroRNAs (miRNAs) are a major class of tissue-specific regulators of gene expression (28). Numerous studies have shown that the miRNAs expressed primarily during pregnancy are clustered in chromosomal regions, suggesting control by the same promoters, and overlap in target genes (29). Many miRNAs have been shown to tightly control placental development, which is a complex physiological process that involves numerous cell types (30). miRNAs are exported from human placental syncytiotrophoblasts into the maternal circulation via exosomes, where they regulate trophoblast cell invasion, placental immune activation and platelet aggregation (31,32).

Alterations in placental miRNA expression have been associated with in utero exposure and adverse pregnancy outcomes (33-36). Zhao *et al* (37) reported significantly higher levels of miR-210 in CD4<sup>+</sup> T cells of patients with psoriasis, compared with controls. The same authors confirmed Foxp3 as a target gene regulated by miR-210. After overexpression of miRNA-210, CD4<sup>+</sup> T cells from healthy controls showed decreased expression of Foxp3 and impaired Treg immune suppressor function.

The present study aimed to explore the profile of Th cytokines and Treg/Th17 cells in healthy non-pregnant, normal pregnant and preeclamptic women. The aim of the present study was to characterize the evolution of Treg subsets (defined as CD4<sup>+</sup>CD25<sup>+</sup>, CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>-</sup> T cells) in peripheral blood in patients with PE. To investigate the mechanisms underlying Treg/Th17 activity in patients with PE, we also measured mRNA expression of Foxp3, retinoic acid-related orphan receptor C (RORc) and miR-210 in placenta from patients with PE.

## Materials and methods

A total of 66 women (age, 24-38 years) were included in this study: 29 patients with late-onset preeclampsia ( $\geq 36$  weeks of gestation), 27 pregnant women with normal uncomplicated pregnancies ( $\geq 36$  weeks of gestation) and 10 healthy non-pregnant women. Serum levels of cytokines (IL-6, IL-10, IL-17 and TGF- $\beta$ 1) were detected using enzyme-linked immunosorbent assay (ELISA). Levels of CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>low/-</sup> Tregs in peripheral blood were evaluated by flow cytometry (FCM). The study included 29 patients with PE with placenta as the experimental group and 27 women with normal pregnancy placenta as a control group. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed to detect mRNA expression of maternal placenta retinoic acid-related orphan receptor C (RORc), Foxp3 and miR-210. Expression of inflammation-related protein Foxp3 was evaluated by western blot analysis. Samples from the same patient were used to quantify the levels of Tregs, FOX-3 and miR-210.

*Study patients.* Study participants were enrolled at the Department of Obstetrics and Gynecology, Shenzhen Longhua District Central Hospital (Shenzhen, China). Exclusion criteria were multifetal gestation, hypertension, diabetes, autoimmune or vascular disease, infection (HIV, syphilis or hepatitis B or C) and renal disorder.

Pregnant women were excluded if they had signs of infection. None of the pregnant women were in active labor, and none had rupture of the amniotic membrane. Serum samples were collected at the time of delivery via Cesarean section. Placental tissue was collected during Cesarean section for testing Foxp3, RORc mRNA and miR-210 expression. We also recruited sequential healthy non-pregnant women who were attending routine annual well-woman examinations. None of these women had received hormonal contraception, which may influence peripheral blood lymphocytes (38).

The clinical diagnosis of PE was based on the development of hypertension [blood pressure (BP)  $\geq 140 \times 90$  mmHg] and proteinuria ( $\geq 300$  mg/24 h) during week 20 of gestation or later (39). Late onset PE ( $\geq 34$  weeks) was defined as those patients who displayed gestational hypertension and proteinuria without a history of hypertension.

All women included in the study were recruited during the period from July 2016 to March 2017. The Ethics Committee of the Shenzhen Longhua District Central Hospital approved the study protocol. All patients provided written informed consent prior to the participation in the study.

*Measurement of cytokine levels in serum and placenta.* Whole blood was collected in sterile EDTA tubes and centrifuged at 3,000 x g for 15 min to separate the plasma. Plasma samples were stored at -80°C for subsequent analysis. Cytokine concentrations in plasma were determined by enzyme-linked immunosorbent assay (ELISA). All ELISA kits were purchased from Milbio Co., Ltd. (Shanghai, China), including: IL-6 (cat. no. m19028583), IL-17 (cat. no. m19028599), IL-10 (cat. no. m19027436) and TGF- $\beta$ 1 (cat. no. m16025653). Assay sensitivity limits for IL-6, IL-10, TGF- $\beta$ 1 and IL-17 (Th17) were 1.5, 1.25, 7.5 and 10 pg/ml, respectively. Absorbance was measured at 450 nm.

**Flow cytometry.** EDTA anticoagulated whole blood samples were collected and processed within 24 h. Briefly, peripheral blood mononuclear cells (PBMCs) were obtained with standard Ficoll-Hypaque technique. PBMCs ( $5 \times 10^5$ ) were stained with the following antibodies: Fluorescein isothiocyanate-conjugated CD127 (cat. no. 560549; BD Pharmingen; BD Biosciences, San Jose, CA, USA); phycoerythrin-conjugated CD25 (cat. no. 557138; BD Pharmingen; BD Biosciences) and allophycocyanin-conjugated CD4 (cat. no. 551980; BD Pharmingen; BD Biosciences) at 4°C in dark for 30 min. Following washing with FACs buffer (PBS with 2% fetal bovine serum (FBS, cat. no. 10099141; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 1 mM EDTA), cells were analyzed using a BD FACSAria II cell sorter with BD FACSDiva software (Version 6.1; BD Biosciences); a subpopulation of CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>+</sup> cells was identified.

**mRNA expression of *Foxp3* and *RORc*.** Placentas from a total of 56 women were included in this study. The women consented to the collection of samples at the time of birth. A full-thickness placental biopsy was obtained after delivery, avoiding the periphery and areas of obvious infarction. Samples obtained were flash frozen in liquid nitrogen and stored at -80°C prior to analysis. RT-qPCR was performed to measure mRNA expression of ROR, *Foxp3* and miR-210 in maternal placenta.

mRNA expression levels of transcription factors associated with Treg/Th17 in placenta were measured by RT-qPCR. miRNA was extracted from 30-50 mg fresh tissue with the Qiagen miRNeasy Mini kit (cat. no. 217004; Qiagen GmbH, Hilden, Germany). Total RNA was extracted from 30-50 mg fresh tissue using TRIzol<sup>®</sup> Reagent (cat. no. 15596026; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions. cDNA was synthesized with PrimeScript RT Master Mix (Takara Bio, Inc., Shiga, Japan), as described in the manufacturer's instructions. qPCR was performed with PowerUp SYBR Green Master Mix (cat. no. a25780; Thermo Fisher Scientific, Inc.). Table II shows all PCR primer pairs. qPCR was carried out in duplicate wells using a LightCycler Real-Time PCR System (Roche Diagnostics, Indianapolis, IN, USA). Quantitation cycle (Cq) values for target genes and the housekeeping gene GAPDH were calculated. Levels of mRNA and miRNA expression were normalized to GAPDH and U6 respectively, and relative expression of indicated genes in PE were compared to that in normal pregnant women using the  $2^{-\Delta\Delta C_q}$  method (40).

**Western blotting of *Foxp3*.** For protein extraction, 30-50 mg placentas were homogenized, washed with PBS, and lysed for 30 min in ice-cold radioimmunoprecipitation assay lysis buffer supplemented with 10 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Protein concentrations were determined by Pierce BCA Protein Assay kit, according to the manufacturer's instructions (cat. no. 23225; Thermo Fisher Scientific, Inc.). Proteins samples (~30 μg) were separated by 10% SDS-PAGE and electrotransferred to polyvinylidene fluoride (PVDF) membranes (EMD Millipore, Billerica, MA, USA). PVDF membranes were blocked using 5% non-fat dried milk dissolved in TBS (20 mM Tris-HCl,

150 mM NaCl, pH 7.6) supplemented with 0.1% (v/v) Tween-20 (TBST) at room temperature for 1 h. Following washing with TBST, the PVDF membranes were incubated with 1:500 diluted anti-FOXP3 antibody (cat. no. ab4728; Abcam, Cambridge, MA, USA) overnight at 4°C. Membranes were washed with TBST and subsequently incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (1:5,000; cat. no. ab205718; Abcam) for 1 h at room temperature; protein bands were visualized with the Enhanced Chemiluminescence (ECL) detection reagents (Millipore). Membranes were then stripped and reblotted with 1:5,000 diluted anti-β-actin antibody (cat. no. A5316; Sigma-Aldrich; Merck KGaA) at room temperature for 1 h. Following TBST washing, blots were incubated with HRP-conjugated goat anti-mouse secondary antibody (1:5,000; cat. no. ab205719; Abcam) for 1 h at room temperature; the blots were visualized with the ECL detection reagents. Protein expression levels were calculated as the relative band density to that of β-actin using ImageJ software (National Institute of Health, Bethesda, MD, USA).

**Statistical analysis.** Continuous variables are presented as mean ± standard deviation. For continuous variables, inter-group differences were compared with Student's t-test (for 2 groups) or one-way analysis of variance (ANOVA; for ≥3 groups). For significant differences in ANOVA, multiple comparisons were conducted with the Dunnett's t-test. For categorical variables, inter-group differences were compared with the  $\chi^2$  test or the Fisher's exact probability test. P<0.05 was considered to indicate a statistically significant difference.

## Results

**Patient characteristics.** The clinical characteristics of the study participants are presented in Table I. The percentage of primiparas was similar in all study groups. Maternal age was significantly higher among preeclamptic patients compared with normal pregnant women. Gestational age was significantly lower in preeclamptic patients than in normal pregnant women. Systolic and diastolic blood pressures were significantly higher in both preeclamptic groups as compared to normal pregnant women. All other clinical features presented in Table I differed significantly among groups. Fetal growth restriction was absent in normal pregnant women but observed in 41.4% of preeclamptic women.

**Cytokine levels.** Regulatory T cells are well known to suppress effector activity of T cells. We then aimed to determine cytokine production in the patients with PE. Plasma production of IL-6, IL-17 and TGF-β1 was significantly higher in preeclamptic patients compared with these levels in normal pregnant women (P=0.0021, P=0.015 and P=0.019, respectively; Fig. 1A-C). Accordingly, levels of Treg cytokines such as IL-10 were significantly lower in preeclamptic women than in normal pregnant women (P=0.0012; Fig. 1D). Levels of serum IL-17, TGF-β1 and IL-10 were similar in healthy non-pregnant and normal pregnant women (P=0.085, P=0.163 and P=0.248, respectively; Fig. 1B-D). However, levels of serum IL-6 were significantly higher in healthy non-pregnant women than in normal pregnant women (P=0.008; Fig. 1A).

Table I. Clinicopathological characteristics of the patients used in the present study.

Clinicopathological characteristic	Patients with preeclampsia (n=29)	Normal pregnant women (n=27)	Healthy non-pregnant women (n=10)	ANOVA F value; P-value
Maternal age at delivery (years)	31±7 <sup>a,b</sup>	26±6	27±7	4.251; 0.015
BMI at blood draw (kg/m <sup>2</sup> )	28.7±6.3 <sup>a,b</sup>	26.0±5.9 <sup>b</sup>	21.6±1.6	4.912; 0.001
Gestational age at delivery (weeks)	37±2.1 <sup>a</sup>	39±2.2	n.a.	2.376; <0.001
Primiparous women (%)	17 (58.6%)	16 (59.25%)	n.a.	0.002; 0.961
Systolic blood pressure (mmHg)	155±49 <sup>a,b</sup>	119±30	115±36	6.814; 0.001
Diastolic blood pressure (mmHg)	100±36 <sup>a,b</sup>	77±26	74±33	4.593; 0.010
Fetal birth weight (g)	2,932±378 <sup>c</sup>	3,212±212	n.a.	2.563; 0.013
Fetal growth restriction (%)	12 (41.4%) <sup>a</sup>	0	n.a.	<0.001

<sup>a</sup>P<0.001 vs. normal pregnant women; <sup>b</sup>P<0.001 vs. healthy non-pregnant women; <sup>c</sup>P<0.05 vs. normal pregnant women. Data are presented as the mean ± standard deviation for continuous variables and as number (percentage) for categorical variables. ANOVA, analysis of variance; BMI, body mass index; n.a., not applicable.

Table II. Primer sequences used for RT-qPCR.

Gene	qPCR primer sequence (5'→3')
Foxp3	F: GTGGCCCCGATGTGAGAAG R: GGAGCCCTTGTCGGATGATG
RORc	F: GAAGTGGTGCTGGTTAGGATGTG R: GCCACCGTATTTGCCCTTCAA
GAPDH	F: GCACCGTCAA-GGCTGAGAAC R: TGGTGAAGACGCCAGTGGA
U6	F: TCGGGGTGCTCGCTTCGGCAGC R: CCAGTGCAGGGTCCGAGGTAT
miR-210	F: CGGCGGTCTGTGCGTGTGACAGC R: CCAGTGCAGGGTCCGAGGTAT

Gene	RT primer sequence (5'→3')
U6 RT	GTCGTATCCAGTGCAGGGTCCGAGG TATTCGCACTGGATACGACAAAATAT GGAAC
miR-210 RT	GTCGTATCCAGTGCAGGGTCCGAGG TATTCGCACTGGATACGACTCAGCC

F, forward; Foxp3, forkhead box p3; miR, microRNA; qPCR, quantitative polymerase chain reaction; R, reverse; RORc, retinoic acid-related orphan receptor C; RT, reverse transcription.

**Decreased frequency of Tregs in preeclampsia patient.** Tregs are key players in successful pregnancy and their deficiencies are implicated in complications in pregnancy such as PE, but the results are inconsistent among studies. Although the percentage of CD4<sup>+</sup>CD25<sup>+</sup> cells among the CD4<sup>+</sup> T cells was notably higher in the normal pregnancy group compared to the percentage in the healthy non-pregnant women (46.27±1.11 vs. 42.71±1.05%), the difference was not significant; the percentage of CD4<sup>+</sup>CD25<sup>+</sup> cells among the CD4<sup>+</sup> T cells were significantly

lower in women with PE compared with normal pregnant subjects and healthy non-pregnant subjects (both P<0.01; Fig. 2A). The percentage of CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>-</sup> cells among the CD4<sup>+</sup> T cells was analyzed in peripheral blood of patients, and the percentage of CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>-</sup> cells among the CD4<sup>+</sup> T cells was significantly lower in the peripheral blood of patients with PE compared with normal pregnant women (6.34±0.28 vs. 9.10±0.28%; P<0.01; Fig. 2B). The percentage of CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>-</sup> cells among the CD4<sup>+</sup> T cells was significantly lower in the peripheral blood of PE compared with healthy non-pregnant women (6.34±0.28 vs. 8.75±0.54%; P<0.01). However, the proportion of CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>-</sup> cells among the CD4<sup>+</sup> T cells was similar in healthy non-pregnant and normal pregnant women, indicating that a decrease in CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>-</sup> cells was associated with PE. These findings suggest that the proportion of Tregs in peripheral blood was increased in normal pregnant subjects but decreased in women with PE. The number of CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>-</sup> T cell population decreased in peripheral blood of PE patients. The image shows the typical data of the T cell through flow cytometry (Fig. 3). The peripheral blood of patients with PE exhibited a decline in the population of CD4<sup>+</sup>CD25<sup>+</sup> T cells; typical T cell data from flow cytometry are presented in Fig. 4.

**Expression of Foxp3, RORc and miR-210 in placenta.** The mRNA expression levels of transcription factors associated with Treg/Th17 cells were analyzed in placentas from patients with PE and normal pregnant women. miR-210 was previously reported to target Foxp3 and inhibit Treg cell function in patients with psoriasis (37). In the present study, significantly higher expression of miR-210 was observed in patients with PE compared with normal pregnant women (1.744±0.153 vs. 0.578±0.744; P<0.01; Fig. 5A). In addition, mRNA expression levels of RORc were significantly higher in women with PE compared with normal pregnant women (0.599±0.069 vs. 0.380±0.046; P=0.0127; Fig. 5B). Higher expression of RORc may promote enhanced production of inflammatory cytokines (20). The results demonstrated that mRNA expression of Foxp3 was significantly lower in preeclamptic women compared with normal pregnant women (0.713±0.088 vs.

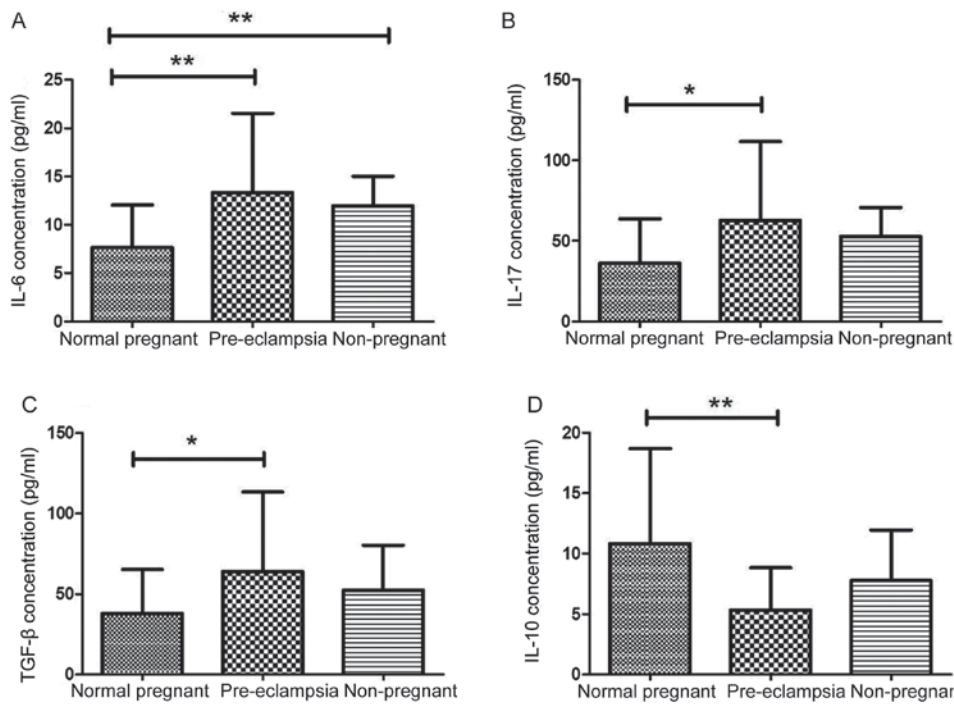


Figure 1. Expression of pro-inflammatory cytokines were varied in plasma of patients with PE. (A-D) Concentrations of (A) IL-6, (B) IL-17, (C) TGF-β1 and (D) IL-10 were determined by ELISA in the plasma of normal pregnant women (n=27), patients with PE (n=29) and non-pregnant women (n=10). Levels of pro-inflammatory cytokines (A) IL-6, (B) IL-17 and (C) TGF-β1 were significantly higher in women with PE compared with normal pregnant women. Levels of regulatory T cell cytokine IL-10 were significantly lower in preeclamptic women compared with normal pregnant women Results are expressed as the mean ± standard deviation; \*P<0.05, \*\*P<0.01. IL, interleukin; PE, preeclampsia; TGF-β1, transforming growth factor-β1.

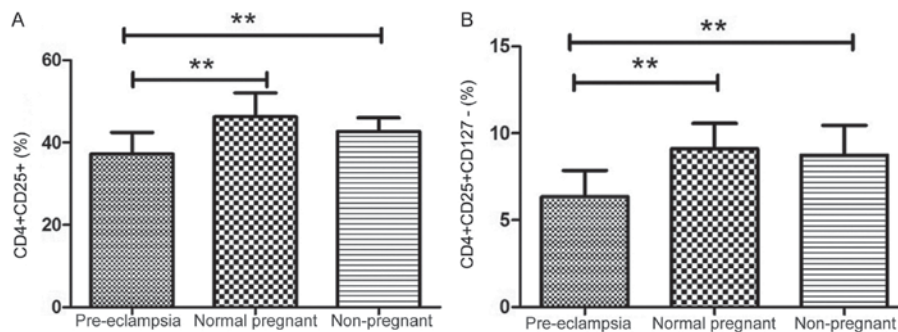


Figure 2. Different frequencies of CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>-</sup> and CD4<sup>+</sup>CD25<sup>+</sup> T cells in patients with PE, normal pregnant women and non-pregnant women. (A) Representative results of flow cytometric analysis for CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>-</sup> cells among the CD4<sup>+</sup> T cell population. (B) Relative proportion of CD4<sup>+</sup>CD25<sup>+</sup> T cells among the CD4<sup>+</sup> T cells. Values are presented as the mean ± standard deviation; \*\*P<0.01. PE, preeclampsia.

1.105±0.136; P=0.019; Fig. 5C), which was consistent with decreased Tregs in patients with PE.

**Expression of inflammation-related protein Foxp3.** Consistent with mRNA expression results, lower protein expression levels of Foxp3 was observed in patients with PE compared with normal pregnant subjects; however, this difference was not indicated to be significant (Fig. 6).

**Discussion**

Foxp3 is a member of the fork head transcription factor family and is exclusively expressed in CD4<sup>+</sup>CD25<sup>+</sup> Tregs (41). CD4<sup>+</sup>CD25<sup>+</sup> Tregs are a subset of mature T cells that play an important role in maintaining immune homeostasis and preventing autoimmune disease. Patients

with preeclampsia (PE) have been shown to have fewer active CD4<sup>+</sup>CD25<sup>+</sup> Tregs in peripheral blood and related tissues (42).

The TGF-β1 signaling pathway is involved in the induction of Foxp3 as well as the generation of peripheral T cells and thymocytes. Previous research has revealed a reduced number of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs in the peripheral blood of women with PE compared to normal pregnant women. CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs express specific anti-inflammatory cytokines such as IL-10, which dampen an excessive effector immune response (43,44).

In the absence of any inflammatory insult, TGF-β produced in the immune system will suppress the generation of effector T cells and induce Foxp3<sup>+</sup> Tregs, thus, maintaining self-tolerance. However, upon infection or inflammation, IL-6 produced by the activated innate immune system will

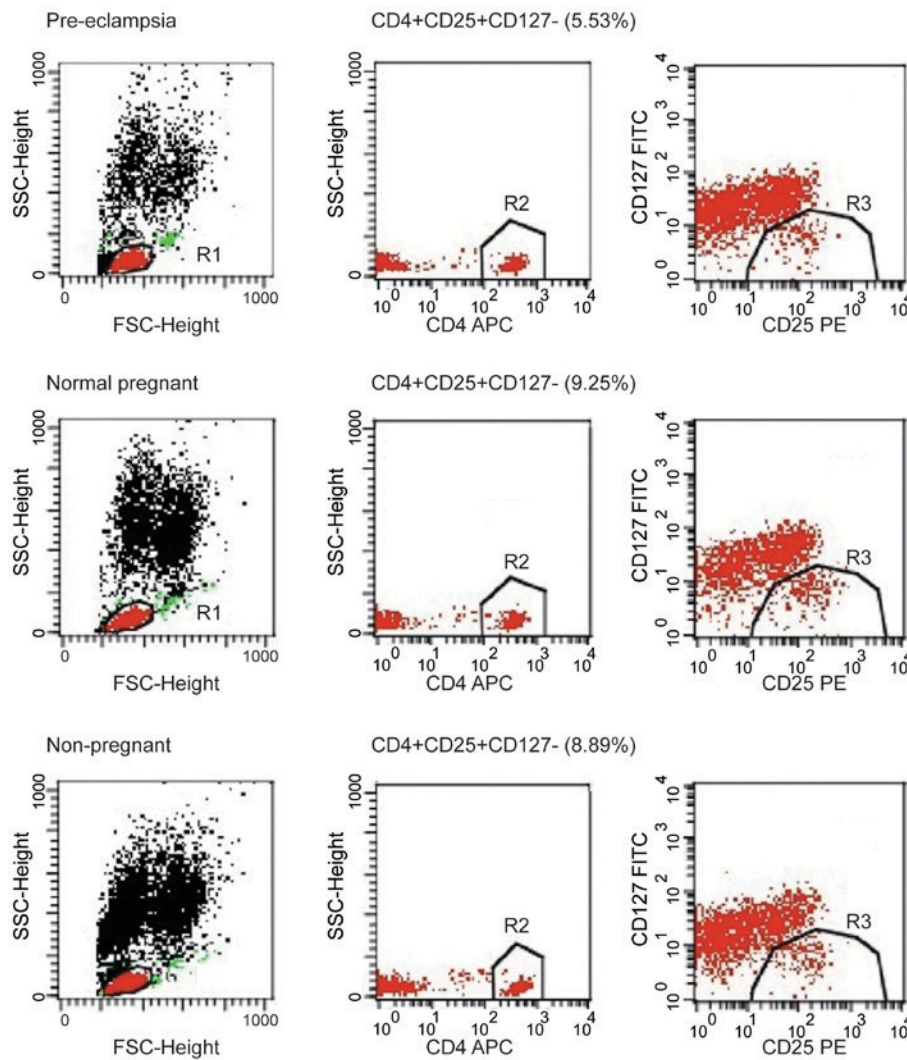


Figure 3. CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>-</sup> T cell population is decreased in peripheral blood of patients with PE. Peripheral blood mononuclear cells were purified from normal pregnant women, pregnant women with PE and non-pregnant women and stained for CD4, CD25 and CD127. Images show representative flow cytometry data for total T cells. Plots are gated on CD4<sup>+</sup> lymphocytes. APC, allophycocyanin; FITC, fluorescein isothiocyanate; FSC, forward scatter; PE, preeclampsia; SSC, side scatter.

suppress the generation of TGF- $\beta$ -induced Tregs and induce a pro-inflammatory T-cell response (predominately Th17 cells) (45). These results support the results of recent studies showing an increase in circulating levels of IL-17 in pregnant women with PE when compared to healthy pregnant women and non-pregnant women (46).

The results obtained for healthy non-pregnant women in this study are consistent with those of previous studies, which report a higher prevalence of peripheral blood IL-17 in preeclamptic patients (21,47,48). TGF- $\beta$ 1 may act as a potent inhibitor of T/B cell proliferation and auto-antibody production (49). TGF- $\beta$ 1 also contributes to suppression of the T cell response by regulating Foxp3<sup>+</sup> Tregs and is essential to the development of natural Tregs (nTregs) and induced Tregs (iTregs) (50). nTregs are produced in the thymus early in life, and iTregs are generated in the periphery from naive CD4<sup>+</sup> T cells (51). Tregs maintain peripheral tolerance through both cell-contact-dependent and cell-contact-independent mechanisms (52).

In the present study, we measured serum levels of cytokines (IL-6, IL -10, IL-17 and TGF- $\beta$ 1) in healthy non-pregnant

women, normal pregnant women and preeclamptic patients. The results showed that serum IL-17, IL-6 and TGF- $\beta$ 1 levels were significantly higher in preeclamptic patients than in normal pregnant women. Levels of Treg cytokines IL-10 were significantly lower in preeclamptic women than in normotensive women.

To further investigate the mechanisms resulting in deficient Tregs in PE, we measured mRNA expression levels of transcription factors that play an important role in the proliferation and differentiation of Tregs in PE. Foxp3 is known as a specific molecular marker for Tregs and is indispensable to the development and function of Tregs (53).

In the present study, the authors suggest that peripheral CD4<sup>+</sup>CD25<sup>+</sup> Tregs cannot be accurately identified and purified based on surface expression of CD127 (as an alternative to expression of transcription factor Foxp3) (54). Therefore, low expression of the CD127 molecule is not an inherent attribute of Tregs (55).

In the present study, cytofluorometry showed that the percentage of CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>-</sup> Tregs among CD4<sup>+</sup> T cells in peripheral blood was reduced in patients with PE compared

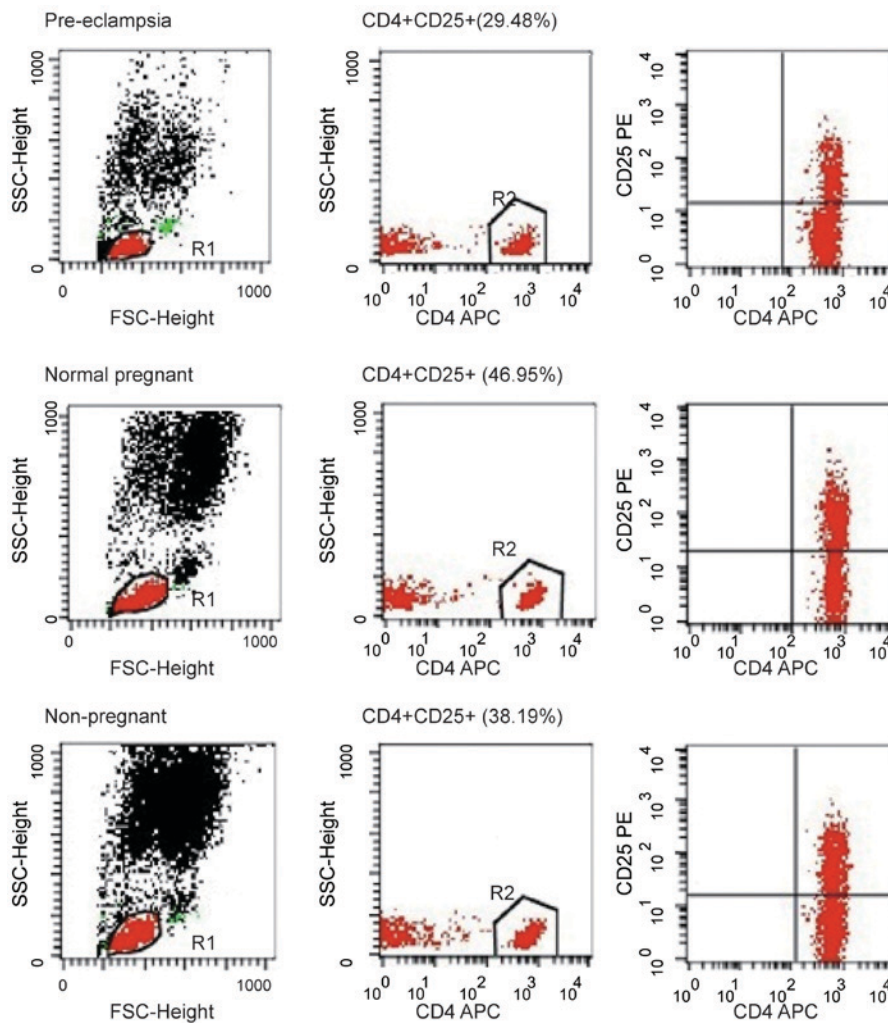


Figure 4. Decreased number of CD4<sup>+</sup>CD25<sup>+</sup> T cell population in peripheral blood of patients with preeclampsia. Images show representative data by flow cytometry for total T cells stained for CD4 and CD25. Plots are gated on CD4<sup>+</sup> lymphocytes. FSC, forward scatter; SSC, side scatter.

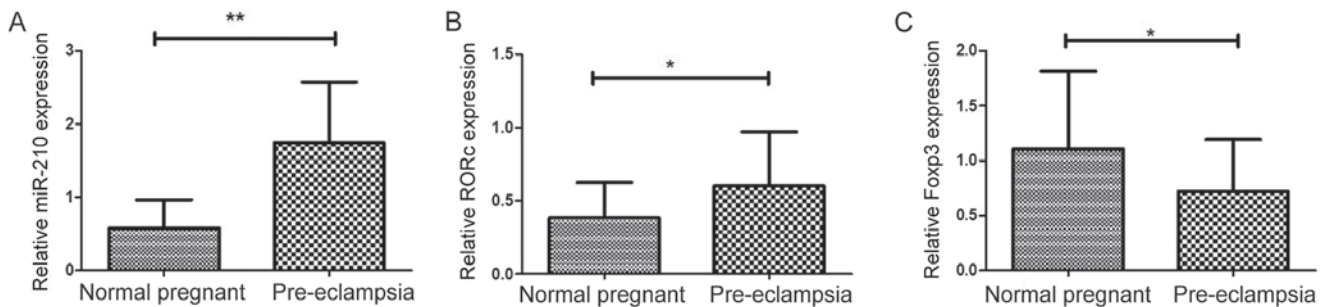


Figure 5. Expression levels of miR-210, RORc and Foxp3 in placenta. (A-C) Expression levels of (A) miR-210, (B) RORc and (C) Foxp3 in placenta attachment deciduas obtained from pregnant women with preeclampsia (n=29) and normal pregnant women (n=27) were analyzed by reverse transcription-quantitative polymerase chain reaction. Results are expressed as the mean  $\pm$  standard deviation; \*P<0.05 and \*\*P<0.01. Foxp3, forkhead box p3; miR, microRNA; RORc, retinoic acid-related orphan receptor C.

to normal pregnant women and healthy non-pregnant women. Furthermore, the percentage of CD4<sup>+</sup>CD25<sup>+</sup> Tregs was significantly lower in the peripheral blood of patients with PE compared to normal individuals.

Such tissue-specific patterns of gene expression are regulated by miRNAs. In this study, the mRNA expression of Foxp3 was significantly lower in placenta from patients with PE. The mRNA expression of miR-210 showed a significant

increase in PE. Furthermore, protein levels of Foxp3 were notably decreased in patients with PE, compared to normal pregnant subjects. These differences in miRNA expression are important as miRNAs (non-coding RNAs approximately 19-25 nucleotides in length) regulate nearly 30-50% of all genes by binding to target protein-encoding mRNAs.

In conclusion, the results from our experiments demonstrated that serum IL-10 levels are decreased in women with

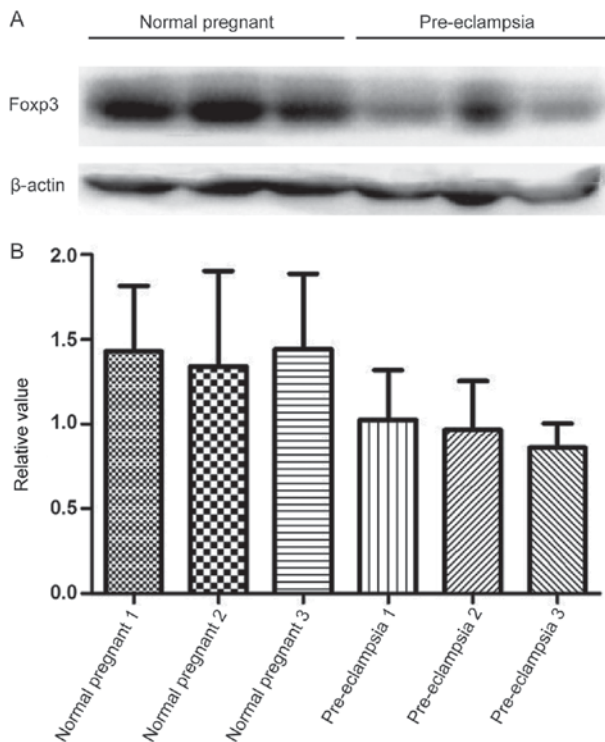


Figure 6. Protein expression levels of Foxp3 in placenta. (A) Protein expression levels of Foxp3 in placenta attachment deciduas of pregnant women with preeclampsia and normal pregnant women were detected by western blot analysis. (B) Statistical analysis of relative protein expression in (A) Foxp3, forkhead box p3.

PE. This effect may contribute to the development of excessive systemic inflammation, as is characteristic of maternal PE. The findings presented above show a decreased number of circulating  $CD4^+CD25^+CD127^-$  Tregs in women with PE compared with normal pregnant women. Placenta from patients with PE showed significantly decreased mRNA expression of Foxp3 and significantly increased expression of miR-210. The existence of a positive correlation between expression of miR-210 and Foxp3 protein in PE requires further experimental verification. Additional studies are required to confirm and extend these findings.

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#### Availability of data and materials

The data sets used and/or analysed during the current study are available from the corresponding author on reasonable request.

#### Authors' contributions

JC designed the experiments and wrote the initial draft of the manuscript. LZ, DW, YX, HG and WT conducted the experiments and analyzed the data. CW analyzed and interpreted the data, and drafted the manuscript.

#### Ethics approval and consent to participate

All experimental research on human samples followed the guidelines and approvals of Shenzhen Longhua District Central Hospital Ethics Committee (Shenzhen, China). The Ethics Committee of the Shenzhen Longhua District Central Hospital approved the study protocol. All patients provided written informed consent prior to the participation in the study.

#### Patient consent for publication

Not applicable.

#### Competing interests

The authors declare that they have no competing interest.

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