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Immune imbalance is associated with the development of preeclampsia

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Abstract

Preeclampsia (PE) is characterized by hypertension and proteinuria. It affects about 5% to 8% of pregnancies and causes maternal and perinatal mortality and morbidity. The immune imbalance and excessive inflammatory response play vital roles in the pathogenesis of PE.

In this study, we performed a case-control study to investigate the levels of cytokines, chemokines and adhesion molecules in serum and placenta of normal pregnant and PE women by Bio-Plex multiplex immunoassay and immunohistochemistry. In addition, we explored the phenotypes of monocyte and macrophage in peripheral blood and placentas in 2 groups by using flow cytometry analysis and immunohistochemistry.

Our results show that pro-inflammatory factors, including interleukin-1β (IL-1β), IL-6, IL-7, IL-8, IL-17a, monocyte chemotactic protein 1 (MCP -1), and macrophage inflammatory protein 1β (MIP-1β) were significantly increased in serum of women with PE compared with controls. In addition, we detected that IL-1β, IL-6, and MCP-1 were also increased in placentas of women with PE. We further revealed that peripheral blood monocytes showed a pro-inflammatory M1-like phenotype in women with PE. Consistently, M1 macrophage infiltration was increased in placenta of women with PE compared to that of normal pregnant women.

Our results demonstrated that immune imbalance promotes an inflammatory state during PE and it may be a potential therapeutic possibility for the management of PE.

Abbreviations: ALT = alanine transaminase, Arg1 = arginase 1, AST = aspartate transaminase, AT1-AA = autoantibodies to the Angiotensin II type 1 receptor, BMI = body mass index, ET-1 = endothelin-1, IL = interleukin, MCP-1 = monocyte chemotactic protein 1, MIP-1β = macrophage inflammatory protein 1β, PBMC = peripheral blood monocyte cell, PE = preeclampsia, ROS = reactive oxygen species, TNF-α = tumor necrosis factor α.

Keywords: cytokines, inflammation, macrophage, monocytes, preeclampsia

1. Introduction

Preeclampsia (PE) is a pregnancy-specific syndrome leading to maternal and perinatal mortality, which is characterized by new onset hypertension and proteinuria after the 20th week of pregnancy. PE appears to progress in 2 states: the first stage is called poor placentation, which arises from failure of trophoblast invasion and remodeling of the spiral arteries, leading to poor blood supply to the placenta. The second stage is a maternal syndrome, which is associated with the systemic inflammatory response caused by the placenta, leading to the signs of PE.[1,2]

Maternal immune system adapts to the semiallogeneic fetus from the beginning of a healthy pregnancy. Accordingly, many changes have been observed both in the innate and adaptive immune systems. Normal pregnancy is a state that immune system is activated and pregnancies affected by PE are regarded to involve more complex immunological processes.[3] The improper immune response is associated with the imbalance of pro-inflammatory and anti-inflammatory immune cells and cytokines,[4] which leads to the pathophysiology associated with PE, including the production of reactive oxygen species (ROS), increased endothelin-1 (ET-1) and autoantibodies to the Angiotensin II type 1 receptor (AT1-AA) expression.[5]

During normal pregnancy, there is an increase in systemic immune cell activation, especially monocyte.[6] Monocyte is macrophage precursor. When infiltrated into tissues, circulating monocytes will differentiate to steady state macrophages.[7] Macrophage can be classified into 2 major subtypes: classically activated M1 macrophage and alternatively activated M2 macrophage.[8] These subsets are distinct in surface receptor, cytokine and chemokine expression.[9] Macrophage polarization occurs under different pathophysiological conditions and surrounding microenvironments. M1 phenotype expresses CD11c and CD38, producing pro-inflammatory cytokines, such as IL-1β, IL-6, and tumor necrosis factor α (TNF-α). Whereas M2 phenotype expresses CD163, CD206, and CD209, anti-inflammatory factors like IL10 and Arginase 1 (Arg1).[10] The macrophage classification for M1 and M2 can be extended to

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human peripheral blood monocytes in several diseases such as type 2 diabetes, sepsis and acute pancreatitis.[11–13] In the present study, we investigated inflammatory factors level in serum and placenta of PE pregnancies compared with healthy pregnancies. We also studied the phenotypic distinction of peripheral blood monocyte and placental macrophages between 2 groups in order to explore the role of immune imbalance in the development of PE and highlight novel therapeutic target for PE.

2. Methods

2.1. Ethics statement

This human study was approved by the Ethics Committee of Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, China and conducted in accordance with the Declaration of Helsinki. All study participants provided written informed consent for the collection of blood samples and tissues.

2.2. Subjects

We totally recruited 52 women: 22 women with established PE and 30 normotensive pregnant women in Renji Hospital, Shanghai Jiao Tong University between December 2015 and November 2016. PE was defined as hypertension (systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg after 20 weeks of gestation in previously normotensive women) with proteinuria (≥300 mg total protein in a 24-hour urine specimen or 2 readings of dipstick measurement of 1+ or more) according to the American College of Obstetricians and Gynecologists guidelines.[14] The exclusion criteria were: diabetes mellitus, cardiovascular disease, renal disease, autoimmune disease, malignancies or women who had recent trauma or surgery. Selected control women had normal blood pressure without obstetrical and medical complications. As presented in Table 1, there are no significant variations in maternal age, gestational weeks at entry, and prepregnancy BMI between PE pregnancies and healthy pregnancies.

2.3. Serum cytokine profile

Blood samples from 2 groups were collected using BD vacutainer tube. After centrifugation at 825 ×g for 10 min at 4°C, Serum specimens were separated from the clot. The supernatant obtained was then aliquoted and kept at −80°C until analyzed. The samples were assayed according to the manufacturer's instructions. Cytokine levels in serum samples from PE and normal pregnant women were measured using Bio-Plex multiplex immunoassay according to the manufacturer' protocol. Data were acquired using the Bio-PlexTM 200 system (Bio-Rad Laboratories).

Table 1

Clinical and laboratory characteristics of the study population.

	Control (n=30)	Preeclampsia (n=22)	*P* Value
Maternal age, yr	28.8±4.1	30.7±4.6	.12
Gestational weeks at entry, wk	28.8±4.0	28.4±4.2	.70
Gestational weeks at delivery, wk	39.4±1.1	33.4±3.5	<.0001
Prepregnancy BMI, kg/m^2	21.5±3.7	23.2±3.4	.08
Nulliparity, n (%)	25 (83.3)	18 (81.8)	1.0
Systolic blood pressure, mmHg	120.2±7.0	163.5±20.2	<.0001
Diastolic blood pressure, mmHg	75.1±5.8	101.0±11.7	<.0001
Proteinuria, g/24 h	–	5.53±4.6	–
Birth weight, g	3412±407.2	1919±956.7	<.0001
Creatinine, umol/L	40.0±7.0	49.4±10.1	.0002
Fasting glucose, mmol/L	4.4±0.4	4.4±0.7	0.85
ALT, U/L	21.5±14.4	22.0±13.9	.91
AST, IU/L	20.4±6.7	21.3±6.3	.61

Values are presented as mean±SD or n (%). BMI=body mass index; ALT=alanine transaminase; AST=aspartate transaminase.

2.4. Immunohistochemistry

Placenta tissues were obtained from women (PE group and normal pregnant group, n=6 per group) who were hospitalized in the Department of Gynecology and Obstetrics of Shanghai Jiao Tong University School of Medicine. Placenta tissue blocks (1 cm^3) from central parts of the maternal side were collected after the placentas delivered, and immediately washed in ice-cold phosphate-buffered saline (PBS) 2 or 3 times to clear the blood. Then the placenta tissues (1 cm^3) were fixed in 4% paraformaldehyde. After a 72-hour fixation, embedded in paraffin and the 5-μm-thick sections were processed. Placenta slices were deparaffinized, rehydrated and followed by antigen retrieval. Then the slices were blocked in 10% normal goat serum for 30 minutes and incubated with primary antibodies against CD11b (BD Biosciences, San Jose, CA), Arg1 (Santa Cruz, Dallas, TX), iNOS (CST, Danvers, MA), IL-1β (Boster, Wuhan, China), IL-6 (Boster, Wuhan, China), and MCP-1 (Boster, Wuhan, China) at 4°C overnight. Subsequently, slices were stained with fluorescent secondary antibodies. Nuclei were stained with DAPI. Sections were observed and imaged using a laser scanning confocal microscope (LSM 710; Carl Zeiss, Germany).

2.5. Flow cytometry analysis

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood by Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden) density gradient centrifugation method. Briefly, Diluted blood sample (4 ml) was layered on Ficoll-Paque PLUS (3 ml) and centrifuged at 400 ×g for 30 minutes at 20°C. Mononuclear cell layer was transferred to a clean centrifuge tube, subsequently, washed by 3 volumes of RPMI 1640 medium (Gibco, USA) and centrifuged at 100 ×g for 10 minutes. After washed one more time with RPMI 1640, cells were resuspended in flow cytometry staining buffer solution (eBioscience, San Diego, CA) and stained with fluorescent antibodies against CD14 (APC), CD11c, and CD163 (BD Biosciences, Heidelberg, Germany) to differentiate M1 from M2 macrophages. The stained cells were assayed by flow cytometry on a FACS CaliburTM flow cytometer (BD Biosciences, Germany) and data were analyzed with FlowJo software.

2.6. Statistical analysis

Data analysis was carried out using SPSS version 20.0 and the GraphPad Prism 5 software (San Diego, CA); Data were presented as mean ± SD or median (interquartile range). Normally distributed continuous variables were analyzed using Student t-test. Skewed variables were analyzed using Mann–Whitney *U* test. *P* values <.05 were considered significant.

3. Results

3.1. Characteristics of the study population

A total of 22 women with PE and 30 normal pregnant women were enrolled in our study. The characteristics of the study population, including maternal age, gestational weeks at entry,

gestational weeks at delivery, prepregnancy body mass index (BMI), nulliparity, blood pressure, proteinuria, birth weight, creatinine, fasting glucose, alanine transaminase (ALT), and aspartate transaminase (AST) are shown in Table 1. There were no statistically significant differences in maternal age, gestational weeks at entry, prepregnancy BMI, percentage of nulliparity fasting glucose, ALT and AST between 2 groups. However, compared to normal pregnant women, blood pressure (systolic pressure and diastolic pressure), birth weight, and level of creatinine were significantly higher in women with PE. Whereas, women with PE were more likely to have a small for gestational age baby compared to normal controls.

Flow cytometric analysis of monocytes in normal pregnant and PE women.

In comparison with that in the controls, proportion of CD14+CD11c+CD163- M1 monocyte was significantly increased in PE ($P<.05$; Fig. 1B). On the other hand, proportion of CD14+CD163+CD11c- M2 monocyte was markedly decreased ($P<.01$; Fig. 1C).

3.2. Serum pro-inflammatory cytokines levels are increased in PE

As summarized in Table 2, IL-1β, IL-6, IL-7, IL-8, IL-17a, MCP-1, and MIP-1β levels were much higher in serum of women with PE than normal controls ($P<.05$). However, there were no significant differences for IL-2, IL-4, IL-5, IL-10, IL-12p70, IL-13, granulocyte-colony stimulating factor (G-CSF) and interferon-γ (IFN-γ) between 2 groups. Granulocyte-macrophage colony stimulating factor (GM-CSF) and TNF-α were not detectable in most of the samples.

3.3. Pro-inflammatory factors are increased in placentas of women with PE

We detected expression of several typical inflammatory factors as IL-1β, IL-6 and MCP-1 in placentas of 2 groups. Immunohistochemistry showed strong reactivity for pro-inflammatory cytokines IL-1β, IL-6 and chemokines MCP-1 in placentas of women with PE (Fig. 2), which were in accordance with the result in maternal serum.

3.4. Macrophage displays a M1 phenotype in placentas of women with PE

CD11b is highly expressed on the surface of macrophages. Arg1 and iNOS, which both utilize L-arginine as a common substrate, represent the M2 and M1 phenotype, respectively. Therefore, we examined the expression of Arg1 and iNOS in placentas. There was a significantly lower proportion of CD11b and Arg1 co-labelled cells and higher proportion of CD11b and iNOS co-labelled cells in preeclamptic placentas compared to the normal placentas (Fig. 3).

4. Discussion

PE, characterized by systemic immune activation, affects the function and metabolism of multiple organs and leads to

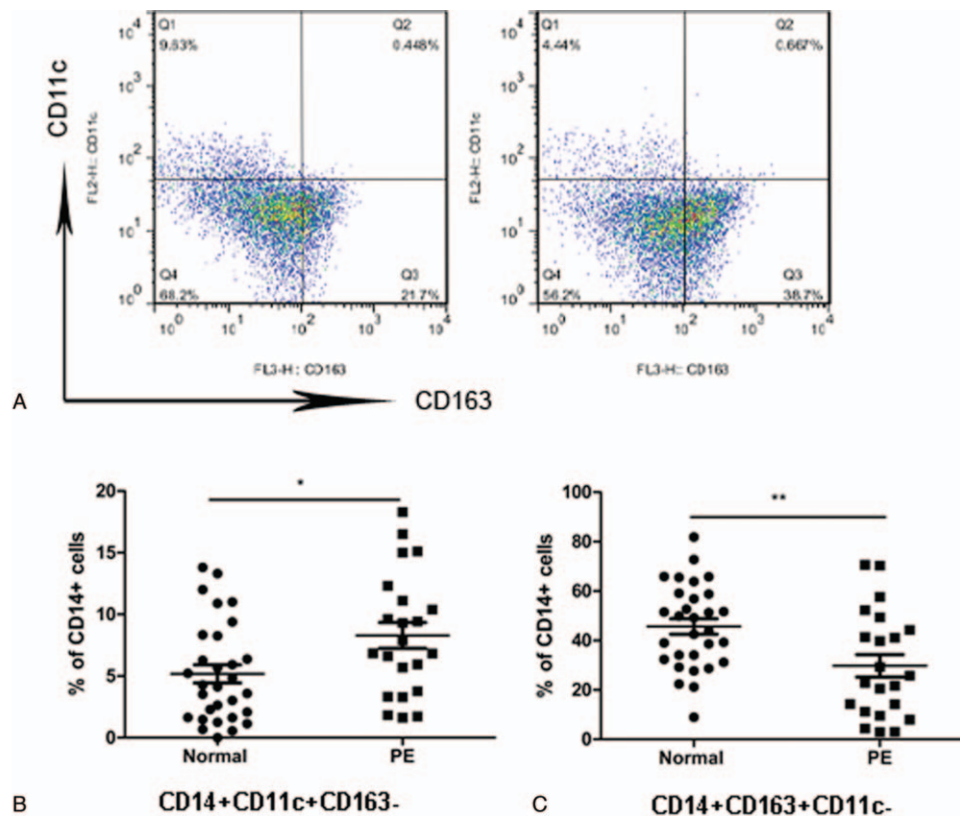

Figure 1. Subsets of peripheral monocytes in control (normal pregnant) women and women with PE. (A). Flow cytometric analysis showed CD14+ monocytes were analyzed for M1 subtype (CD11c+) and M2 subtype (CD163+). (B). Quantification of flow cytometric analysis of CD14+CD11c+CD163- monocytes between 2 groups. $^{*}P<.05$. (C). Quantification of flow cytometric analysis of CD14+CD163+CD11c- monocytes. $^{**}P<.01$.

Table 2

Comparison of serum inflammatory factors in 2 groups.

Parameter	Control (n=19)	Preeclampsia (n=20)	*P* Value
IL-1β	0.45 (0.45;0.50)	0.55 (0.41;0.61)	.02
IL-2	0.31 (0.05;1.13)	0.31 (0.18;0.79)	.88
IL-4	0.14 (0.05;0.23)	0.14 (0.05;0.23)	.89
IL-5	2.10 (1.67;2.10)	1.67 (1.23;2.10)	.17
IL-6	1.50 (0.68;2.27)	2.60 (1.89;4.99)	.0002
IL-7	3.27 (2.63;3.71)	4.26 (3.09;5.03)	.03
IL-8	1.92 (1.70;2.24)	5.02 (2.61;9.14)	<.0001
IL-10	1.66 (0.70;3.27)	2.06 (1.29;4.09)	.77
IL-12p70	9.76 (5.30;11.92)	11.93 (5.88;20.67)	.24
IL-13	1.50 (1.15;1.50)	1.50 (1.15;2.11)	.66
IL-17a	4.39 (2.98;5.86)	6.76 (4.39;11.79)	.038
G-CSF	2.89 (1.98;5.80)	4.08 (1.98;5.18)	.64
GM-CSF	–	–	ND
IFN-γ	18.06 (3.97;26.82)	3.97 (3.97;18.06)	.55
MCP-1	18.93 (17.43;25.51)	25.51 (17.35;46.74)	.016
MIP-1β	389.1 (285.8-463.1)	580.8 (344.3;915.8)	.0047
TNF-α	–	–	ND

Values are presented as median (interquartile range). IL=interleukin, G-CSF=granulocyte-colony stimulating factor, GM-CSF=granulocyte-macrophage colony stimulating factor, IFN-γ=interferon-γ, MCP-1=monocyte chemotactic protein 1, MIP-1β=macrophage inflammatory protein 1β, TNF-α=tumor necrosis factor-α, ND=not detectable.

pathophysiology during pregnancy. In this study, we investigated the detailed expression profiles of inflammatory factors in maternal circulation as well as monocyte/macrophage polarization in women with PE.

Cytokines, chemokines and adhesion molecules have been proved to play vital roles during normal pregnancy. Our data showed that pro-inflammatory cytokines IL-1β, IL-6, IL-7, IL-8, IL-17a, MIP-1β, and MCP-1 are significantly increased in the preeclamptic maternal circulation compared to normal pregnant women. Consistently, our study demonstrated that pro-inflammatory factors IL-6, IL-1β and MCP-1 are expressed higher in placenta tissues of women with PE. Previous studies have indicated that cytokines also participate in trophoblast invasion, differentiation and angiogenesis, which are hallmark features of PE.[15–17] During pregnancy, placental ischemia is thought to promote the release of cytokines as TNF-α and IL-6 from the placenta into maternal circulation. Then these cytokines lead to maternal endothelium dysfunction and hypertension. In the vasculature, the increased IL-6 and TNF-α lead to increased expression of adhesion molecules and endothelial dysfunction, which contribute to the pathogenesis of PE.[5,18] In addition, previous study has investigated that there is a positive correlation between pro-inflammatory cytokines such as IL-2, IFN-γ and TNF-α and enhanced blood pressure. [19]

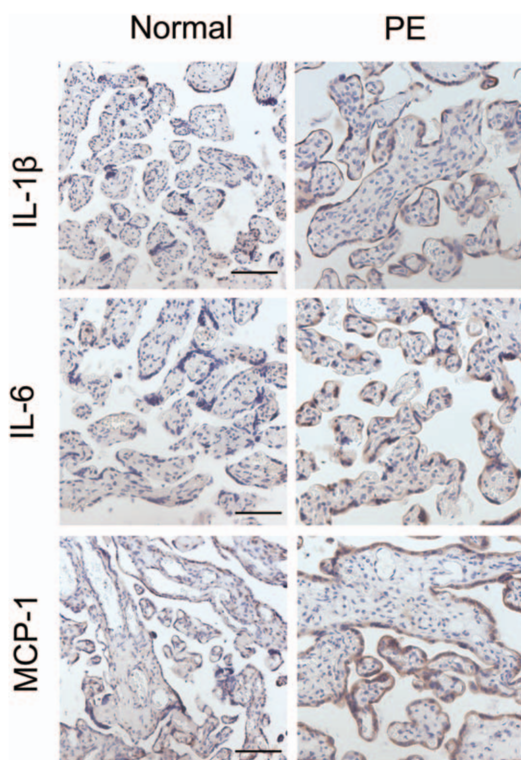

Figure 2. Placental expression of inflammatory factors in PE. Representative immunostaining of IL-6, IL-1β and MCP-1 (brown) in the placentas of control and PE groups. Scale bar: 100 μm.

Pro-inflammatory cytokines are produced by placental trophoblasts, stromal cells and macrophages, and they are also secreted by monocytes. Monocytes may represent an important source of pro-inflammatory cytokines, as maternal PBMC from women with PE produced higher levels of pro-inflammatory cytokines, such as TNF-α, IL-2, IL8, and IL1β.[20,21] Monocytes and macrophages are important immune cells and their activation is closely associated with the development of PE.[22] The mechanism of monocyte activation during pregnancy and PE is not clear. The possible reason is that maternal peripheral monocytes circulate through the placental circulation and contact with the semi-allogeneic villous syncytiotrophoblast. This notion is supported to the fact that monocytes are activated during their passage through the placenta.[22,23] Here, we investigated the subsets of monocytes/macrophages in maternal circulation and placenta. Proportion of M1 monocytes (CD14+CD11c+) is increased in preeclamptic maternal circulation, which is in line with increased M1 macrophages (CD11b and iNOS colocalized) infiltration in preeclamptic placentas. As is known that iNOS activity modulates inflammation,[24,25] and our results find increased expression of iNOS in preeclamptic placentas (M1 macrophages), suggesting that PE is the process of inflammation. Macrophage plays a vital role in homeostasis and immunity, and exist along with a continuum of functional states between 2 major subsets of M1 and M2 phenotypes with different functions dependent on microenvironment. The M1 macrophages can secrete many types of pro-inflammatory cytokines, including IL-6, IL-1β, and IL-12, and defend to foreign pathogens or injury. The M2 macrophages produce anti-inflammatory cytokines, such as IL-10, IL-4, and Arg1. The 2 subsets are different in function and inflammatory potential. The M1 macrophages specialize in phagocytosis, production of ROS, and secretion of inflammatory cytokines in response to the binding of ligands, such as LPS to extracellular Toll-like receptors.[26] It is known that ROS plays the key role in development of PE, and ROS can be induced in inflammatory states. On the other hand, normal pregnancy itself is a pro-inflammatory state[27] and in PE is the result of induced inflammation. The M2 macrophages display immunosuppressive properties and involved in biological activities, including tolerance and tissue remodeling.[9] Our data suggested that the monocytes/macrophages in women with PE tend to differentiate into M1 phenotype and displayed systemic inflammation.

A limitation of our study is that this study did not include prepregnant and postpartum data, and consequently, we are

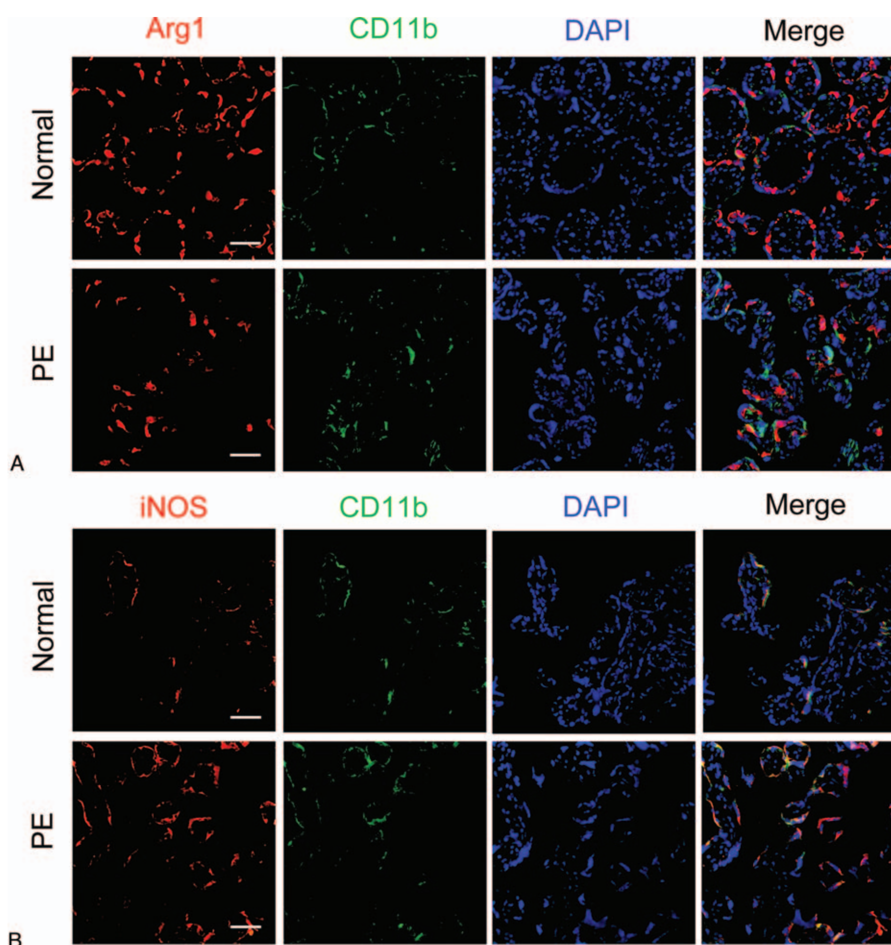

Figure 3. Expression of M1 and M2 macrophages in human placental tissues from control (normal pregnant) women and women with PE. Representative images of colocalization of CD11b (green) with iNOS (red, M1) (A) or Arg1 (red, M2) (B) in the placentas of control and PE groups. Nuclei were counterstained with DAPI (blue). Scale bar: 50 μm.

unable to comment whether the observed increased proinflammatory factors and M1 monocytes/macrophages in women with PE is the cause or the consequence of the condition. Longitudinal study may be needed to explore this question.

In conclusion, our study identified that peripheral blood monocytes, placental macrophages and pro-inflammatory factors are collectively associated with the excessive inflammatory response in women with PE. Although we could not determine whether inflammation is the cause or effect of PE, our findings may provide the notion that PE is an inflammatory process and proper suppression of excessive inflammatory response definitely inhibits the development of PE.

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Author contributions

Conceptualization: Yu Ma.
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