Activation of natural killer cells in the pathogenesis of preeclampsia

Mingyue Du (✉ mingyue.du@foxmail.com)

Guangzhou First People's Hospital <https://orcid.org/0000-0003-4131-8237>

Weijia Wang

Department of Obstetric and Gynecology, Guangzhou First People's Hospital, School of Medicine, South China University of Technology

Lina Huang

Department of Obstetric and Gynecology, Guangzhou First People's Hospital, School of Medicine, South China University of Technology

Xiuting Guan

Department of Obstetric and Gynecology, Guangzhou Eighth People's Hospital, School of Medicine, South China University of Technology

Wen Lin

Department of Obstetric and Gynecology, Guangzhou First People's Hospital, School of Medicine, South China University of Technology

Jingxin Yao

Department of Obstetric and Gynecology, Guangzhou First People's Hospital, School of Medicine, South China University of Technology

Liping Li

Department of Obstetric and Gynecology, Guangzhou First People's Hospital, School of Medicine, South China University of Technology

Research

Keywords: preeclampsia, natural killer cells, vessel remodeling, extravillous trophoblast cells (EVTs), maternal-fetal interface

Posted Date: December 17th, 2019

DOI: <https://doi.org/10.21203/rs.2.19061/v1>

License: This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background: Preeclampsia (PE) is a serious pregnancy-specific systemic inflammatory disorder. The characteristic pathological abnormality in PE is impaired uterine spiral artery (SA) remodeling. Natural killer (NK) cells have been proposed to play an important role in uterine SA remodeling, particularly in early pregnancy. Still, the definitive role of NK cells in later phases of PE is largely unstudied. In the present study, we investigated the association between NK cells and PE in late human pregnancy.

Methods: Collected normal control (n = 18), late-onset (n = 28) and early-onset PE patients peripheral blood and decidua, isolated mononuclear cells, the ratio of NK cells and the level of intracellular cytokines production were evaluated using Flow cytometry. Co-culture first trimester cytotrophoblast cells with conditioned medium (CM) from decidual NK (dNK) cells, evaluated cytotrophoblast cells migration, invasion, NK cytotoxicity and soluble factors secreted by dNK cells. For multiple group comparisons, data were analyzed using one-way analysis of variance with Bonferroni post-testing when the variances were homogeneous or with Tamhane's T2 post-testing when the variances were not homogeneous. $P < 0.05$ was considered significant.

Results: We found that the numbers of both peripheral blood NK (pNK) cells and dNK cells and intracellular interferon (IFN)- γ , perforin and granzyme B production were significantly higher in PE compared with normal pregnancies at the time of delivery for both early-onset and late-onset disease. dNK cells from PE pregnancies not only killed primary first trimester trophoblast cells in vitro but also inhibited their migration and invasion when compared to normal controls. Using recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-8, IFN- γ and tumor necrosis factor (TNF)- α , and neutralizing Abs against these factors, we demonstrated that GM-CSF and IL-8 in the CM from dNK cells promoted, while IFN- γ and TNF- α inhibited trophoblast cell migration and invasion.

Conclusion: Our results suggest that activation of NK cells may play an important role in the pathogenesis of PE in late pregnancy. Elevated local levels of dNK cell-derived IFN- γ and TNF- α and decreased GM-CSF and IL-8 in preeclamptic pregnancies may directly mediate known reductions in trophoblast cell migration and invasion in deciduae from preeclamptic pregnancies.

Background

Preeclampsia (PE) is a serious pregnancy-specific disorder that affects approximately 3–8% of pregnancies and is defined by the development of maternal hypertension and proteinuria after 20 weeks of gestation [1]. PE is a systemic inflammatory disease that may lead to maternal multi-organ damage and fetal growth restriction [2]. The disorder is a leading cause of maternal and fetal morbidity and mortality, accounting for an estimated 14% of pregnancy-related maternal deaths and 15% of premature births worldwide [3, 4]. The effects of PE can last long beyond pregnancy for both mother and child. In fact, the long-term risks of cardiovascular, cerebrovascular, and renal diseases are increased in women with a history of PE [3–5].

Despite well-recognized public health significance and intensive investigation, the pathogenesis of PE is still poorly understood. Delivery of the placenta remains the only effective treatment for PE, indicating that the placenta plays an important role in disease pathogenesis. One key to successful human pregnancy is a well-described and dramatic remodeling of the uterine spiral arteries (SAs) [6]. In early pregnancy, progenitor cytotrophoblast cells at the tips of anchoring villi differentiate into extravillous trophoblast cells (EVTs) that invade the uterine SAs of the decidua and myometrium and replace the endothelial layer of these uterine vessels. This transforms uterine SAs from high-resistance vessels to flaccid, high-capacitance vessels [6]. Such changes allow the increased blood supply needed to sustain the growing fetus and expanding placenta throughout pregnancy. Characteristic pathological changes in PE include deficient EVT invasion and incomplete uterine SA remodeling.

Natural killer (NK) cells are a subset of lymphocytes of the innate immune system characterized by high cytolytic potential against MHC class I negative virus-infected and tumor-transformed cells [7, 8]. NK cells constitute the major immune cell type in the decidua during the first trimester of human pregnancy, accounting for approximately 70% of the local lymphocytes [9], although these decidual NK (dNK) cells appear to have less cytolytic potential and more cytokine and chemokine secretory activities than their peripheral counterparts [10]. The abundance of NK cells in the decidua has suggested that these cells may play an important role in pregnancy support and placental development. Decidual NK cells have been proposed to contribute directly to the initiation of uterine SA remodeling [11] by secreting numerous cytokines, angiogenic factors and enzymes, including matrix metalloproteinases, that disrupt vascular extracellular matrix connections [12–14]. In addition, a number of studies have shown that dNK cells have indirect effects on uterine vessel remodeling by modulating EVT growth, differentiation, migration and invasion [15, 16]. Moreover, dNK cells from pregnancies at high risk of PE are less able to induce EVT motility and fail to induce vascular cell apoptosis when compared to dNK cells isolated from low-risk pregnancies, providing functional evidence of altered dNK cell function in PE [17]. In contrast, it has also been reported that dNK cells are a source of decidual interferon (IFN)- γ during early human pregnancy and likely participate in the inhibition of EVT invasion [18, 19].

Although a possible etiological role for NK cells in the pathogenesis of PE has long aroused the interest of scientists, their role in later phases of PE is largely unstudied. In addition, there are conflicting reports regarding the numbers of dNK cells in PE deciduae. Some reports show an increase in dNK cell number in PE, albeit with an altered phenotype [20, 21], whereas others report decreased dNK cell numbers in preeclamptic placental samples [22, 23]. Increased secretion of angiogenic factors by dNK cells from pregnancies with high uterine artery resistance has also been reported [24].

In short, we continue to search for the definitive role of NK cells in the pathogenesis of PE, particularly in late pregnancy. In the present study, we examined NK cell frequencies and function between normal and preeclamptic pregnancies. We report that in the third trimester, the numbers of both peripheral blood NK (pNK) cells and dNK cells, as well as intracellular IFN- γ , perforin and granzyme B production are significantly increased in PE compared with normal pregnancies. Functional analyses revealed that dNK cells from PE pregnancies at late gestation kill primary first trimester trophoblast cells in vitro. These dNK

cells also inhibit primary trophoblast cell invasion and migration, and the effects may be due to increases in dNK cell IFN- γ and tumor necrosis factor (TNF)- α , and decreases in granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-8. We hypothesize that activation of NK cells may contribute to the pathogenesis of PE in late human pregnancy.

Methods

Collection of human tissues

This study was approved by the Medical Ethics Committee of Guangzhou First People's Hospital, Guangzhou, China. Informed written consent was obtained from each participant prior to participation. Placental tissues (8–10 weeks; $n = 20$) from women with normal pregnancies undergoing elective terminations of pregnancy for nonmedical reasons were obtained. In addition, we collected third trimester peripheral blood and decidual tissue samples from 42 women with early-onset or late-onset PE (14 and 28, respectively) and 18 healthy pregnant women at the time of delivery. All participants delivered by caesarean section without prior labor. Clinical characteristics of these participants are shown in Table 1. PE was defined by elevated blood pressures (BPs) ($BP \geq 140$ mmHg systolic or ≥ 90 mmHg diastolic on 2 occasions at least 4 hours apart) that occurred after 20 weeks of gestation in a woman with previously normal BP accompanied by proteinuria (excretion of 300 mg or more of protein in a 24-hour urine collection, or a urine protein/creatinine ratio of at least 0.3) [2]. PE with severe features was diagnosed when any of the following findings was present: $BP \geq 160$ mmHg systolic or ≥ 110 mmHg diastolic, thrombocytopenia, impaired liver function, progressive renal insufficiency, pulmonary edema, and/or new-onset cerebral or visual disturbances [2]. The occurrence of PE at less than 34 weeks of gestation was defined as early-onset PE [2]. Pregnant women with multifetal gestation, chronic hypertension, chronic hypertension with superimposed PE, gestational hypertension, primary diseases of other organs, endocrine diseases, autoimmune diseases and acute systemic and genital tract infection were excluded. The decidual basalis was collected immediately after placental delivery using direct wiping with gauze. First trimester placental samples and third trimester decidual samples were obtained to isolate cytotrophoblast cells and decidual mononuclear cells, respectively. Peripheral blood was obtained just before delivery by venipuncture using heparin as an anticoagulant to isolate peripheral blood mononuclear cells (PBMCs). All samples were processed immediately following collection.

Table 1
Clinical characteristics of normal and preeclamptic pregnancies

Clinical parameters	Normal group (n = 18)	Late-onset PE group (n = 28)	Early-onset PE group (n = 14)	P value
Age (years)	33.5 ± 5.7	33.8 ± 5.2	34.5 ± 6.5	0.889
Height (cm)	160.9 ± 5.3	159.4 ± 3.8	158.7 ± 4.5	0.344
Weight (Kg)	67.5 ± 8.9	67.9 ± 11.3	66.7 ± 7.7	0.952
Gravidity	2.3 ± 1.3	2.3 ± 1.1	2.5 ± 1.3	0.838
Parity	1.6 ± 0.7	1.6 ± 0.7	1.7 ± 0.8	0.968
Systolic BP (mmHg)	124.9 ± 8.3	157.6 ± 13.8	166.8 ± 14.9	0.000
Diastolic BP (mmHg)	78.9 ± 4.2	101.7 ± 11.0	108.5 ± 8.5	0.000
Gestational age (weeks)	38.7 ± 0.9	37.7 ± 1.3	33.1 ± 3.3	0.000
Fetal weight (g)	3338.6 ± 242.7	2942.6 ± 265.7	2017.7 ± 428.4	0.000
Data are presented as means ± SDs. PE: preeclampsia; BP: blood pressure.				

Isolation of PBMCs and decidual mononuclear cells

Peripheral blood was obtained and PBMCs were isolated over a Ficoll-Paque Premium (GE Healthcare, Pittsburgh, PA) by centrifugation at 400 g for 30 min at 20 °C and re-suspended in 50 µl phosphate-buffered saline (PBS) for flow cytometry. Decidual tissues were collected, washed in PBS, and cut into small pieces. Minced decidual tissue was digested in PBS containing 1 mg/ml Dispase II (Roche, Basel, Switzerland) and 0.1 mg/ml DNase I (Roche) at 37 °C for 30 min. Released cells were separated from undigested tissue pieces by filtering through a 40 µm strainer (Thermo Fisher Scientific, Waltham, MA, USA). Decidual mononuclear cells were enriched via centrifugation over the Ficoll-Paque Premium at 400 g for 30 min at 20 °C.

Isolation of dNK cells

Decidual mononuclear cells were re-suspended. Non-NK cells were removed by negative selection using a NK Cell Biotin-Ab Cocktail (Miltenyi Biotec, Bergisch Gladbach, Germany) and a NK Cell MicroBead Cocktail (Miltenyi Biotec) according to the manufacturer's protocol. CD56⁺ NK cells were then purified with microbeads conjugated to anti-human CD56 Ab (Miltenyi Biotec). The purity of isolated CD56⁺ dNK cells was more than 95% as determined by flow cytometry (data not shown).

Preparation of conditioned medium (CM) from dNK cells

Isolated dNK cells (1 × 10⁶/ml) were cultured in complete RPMI 1640 medium containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine (all from Life

Technologies, Grand Island, NY, USA) at 37 °C. After 48 hours, the cell culture supernatants were collected as dNK cell CM. The CM was aliquoted and stored at -80 °C before use.

Multiplex immunoassays

CM from dNK cells were collected and stored at -80 °C for batched cytokine determination. Levels of GM-CSF, IFN- γ , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10 and TNF- α were assessed simultaneously using the Human Ultrasensitive Cytokine Magnetic 10-Plex Panel (R&D Systems, Minneapolis, MN, USA) and the Luminex™ detection MAGPIX System (R&D Systems). All assays were conducted according to the manufacturer's instructions.

Isolation of human cytotrophoblast cells

Cytotrophoblast cells from human first trimester [25] and term placentae [26] have been successfully isolated. Here, we isolated cytotrophoblast cells from human first trimester placentae using similar methods with minor modifications. Briefly, villous tissues were dissected free of membranes, rinsed and minced in PBS. The villous samples were digested in a digestion enzyme medium containing 1 mg/ml Dispase II and 0.1 mg/ml DNase I at 37 °C for 30 minutes. Released cells were then purified on a discontinuous Percoll gradient (GE Healthcare) and centrifuged at 730 g without braking at 4 °C for 20 min. The layer between the 45% and 35% Percoll aliquots containing cytotrophoblast cells (density 1.050–1.060 g/ml) were collected. First trimester cytotrophoblast cells were further immunopurified by eliminating CD45RB positive cells of myeloid origin using a PE-conjugated anti-CD45RB Ab (Miltenyi Biotec) and anti-PE-microbeads (Miltenyi Biotec), and depleting fibroblasts using anti-fibroblast microbeads (Miltenyi Biotec) according to the manufacturer's instructions. The purity of isolated cytotrophoblast cells was consistently more than 95% as determined by flow cytometry for cytokeratin-7 positivity.

Matrigel invasion assay

Cytotrophoblast cell invasion was evaluated in a Matrigel-coated transwell system with 8 μ m pore size polyethylene terephthalate membranes (BD Biosciences, San Jose, CA, USA) as previously described [25] with minor modifications. In brief, 2×10^5 cytotrophoblast cells in 200 μ l of Dulbecco modified Eagle medium (DMEM) (Life Technologies) containing 10% FBS, 100 U/ml penicillin, 100 μ g/ml streptomycin and 2 mM L-glutamine were seeded in the upper chamber of a 24-well plate. A volume of 600 μ l of CM from dNK cells from normal pregnancies or preeclamptic pregnancies with anti-GM-CSF Ab (10 μ g/ml), anti-IL-8 Ab (10 μ g/ml), anti-TNF- α Ab (10 μ g/ml), anti-IFN- γ Ab (10 μ g/ml), or an isotype control Ab (10 μ g/ml) was placed in the lower chamber. Medium alone with the isotype Ab in the lower chamber served as a negative control. Media supplemented with the isotype Ab and 10 ng/ml recombinant human GM-CSF, IL-8, IFN- γ or TNF- α in the lower chamber served as positive controls. Cells were incubated at 37 °C for 24 h. The membranes were then stained using crystal violet (Sigma-Aldrich, St. Louis, MO, USA) and mounted onto glass slides. Stained cells were counted at a magnification of 200 $\times$ under a Leica DMIL microscope (Leica Microsystems, Wetzlar, Germany). The invasion index was defined as the

number of invading cells in the experimental group divided by that of the negative control group. The assay was carried out in triplicate and repeated 6 times independently. Recombinant human cytokines, neutralizing Abs and the isotype Ab were all purchased from R&D Systems.

Migration assay

We evaluated the migration of cytotrophoblast cells using an 8 µm membrane insert system (Corning, NY, USA). Isolated cytotrophoblast cells (2×10^5) were seeded in the upper chamber of a 24-well plate. A volume of 600 µl of CM from dNK cells from normal or preeclamptic pregnancies with anti-GM-CSF Ab (10 µg/ml), anti-IL-8 Ab (10 µg/ml), anti-TNF-α Ab (10 µg/ml), anti-IFN-γ Ab (10 µg/ml), or an isotype control Ab (10 µg/ml) was added to the lower chamber. Basal culture medium with the isotype Ab in the lower chamber served as a negative control. Media supplemented with the isotype Ab and 10 ng/ml GM-CSF, IL-8, IFN-γ or TNF-α in the lower chamber served as positive controls. After 24 hours, cells in the lower chamber were collected and labelled with fluorescence-conjugated anti-cytokeratin-7 Ab (BD Biosciences). The numbers of cytokeratin-7⁺ cells were calculated using flow cytometry. Results were expressed as fold change of the numbers of cytokeratin-7⁺ cells relative to those isolated from basal culture medium controls. The assay was carried out in triplicate and repeated 6 times independently.

NK cytotoxicity assay

Cytotoxicity of NK cells was assessed by lysis of cytotrophoblast cells using an In Vitro Lactic Dehydrogenase Based Toxicology Assay Kit (Sigma-Aldrich) according to the manufacturer's instructions. Different concentrations of dNK cells (2.0×10^5 , 1.0×10^5 , 0.5×10^5 and 0.2×10^5) from normal, late-onset PE and early-onset PE pregnancies were co-cultured with cytotrophoblast cells (0.1×10^5) in a flat-bottomed 96-well culture plate (Corning) at 37°C for 4 h. Cultures without cytotrophoblast cells served as controls. All of the experiments were set up as triplicate cultures and repeated 6 times independently. Absorbance of each well was measured at a wavelength of 490 nm using a GloMax® Navigator Microplate Luminometer (Promega, Madison, WI, USA). The background absorbance of the plate was measured at 690 nm. NK cell cytotoxicity was calculated using the following formula: Percent cytotoxicity = $100 \times (\text{Experimental absorbance} - \text{Background absorbance}) / (\text{Control absorbance} - \text{Background absorbance})$.

Flow cytometry

Freshly isolated PBMCs or decidual mononuclear cells were stained with fixable viability dye eFluor 520 (eBioscience, San Diego, CA, USA) to quantify dead cells. For cell surface staining, cells were incubated with fluorescence-conjugated Abs against CD3, CD56 and CD16 for 30 min at 4 °C. After washing twice with PBS, cells were then washed and fixed in a fixation buffer. For intracellular cytokine analysis, PBMCs or decidual mononuclear cells (10^6 cells/ml) were incubated with 50 ng/ml phorbol myristate acetate (PMA) (Sigma-Aldrich), 500 ng/ml ionomycin (Sigma-Aldrich) and 5 µg/ml brefeldin A (Sigma-Aldrich) at 37 °C in 5% CO₂ for 4 h. Cells were collected and stained for surface markers. Subsequently these cells were fixed in a fixation buffer, washed and re-suspended in a permeabilization wash buffer. Cells were

then stained with fluorescence-conjugated Abs against IFN- γ , perforin and granzyme B for 30 min at 4 °C. Isotype controls were established using matched fluorescence-labeled isotype control Abs and equivalent immunostaining conditions. Immunostained cells were analyzed on a FACSCanto flow cytometer (BD Biosciences) using FACSDiva software (BD Biosciences). Post-acquisition FACS data were analyzed using FlowJo software (Tree Star, Ashland, OR, USA). The percentages of CD3⁺CD56⁺ NK cells among lymphocytes and NK cell intracellular production of IFN- γ , perforin and granzyme B were calculated. Fluorescence-conjugated Abs, matched fluorescence-labeled isotype control Abs, the permeabilization wash buffer and the fixation buffer were all purchased from BioLegend (San Diego, CA, USA).

Statistical analyses

All statistical analyses were performed using SPSS 23.0 software (IBM, Armonk, NY, USA). For multiple group comparisons, data were analyzed using one-way analysis of variance with Bonferroni post-testing when the variances were homogeneous or with Tamhane's T2 post-testing when the variances were not homogeneous. Results were expressed as means $\pm$ SDs. A P-value of < 0.05 was considered significant.

Results

Association between PE and dNK cell frequencies and intracellular IFN- γ , perforin and granzyme B production during late pregnancy

We determined the frequencies of dNK cells and levels of intracellular IFN- γ , perforin and granzyme B production using flow cytometry in women with PE in the third trimester. As shown in Figure 1A-H, the frequencies of dNK cells and the levels of dNK cell intracellular IFN- γ , perforin and granzyme B were all significantly higher in late-onset PE when compared to normal pregnancies ($P < 0.01$ for all comparisons), but significantly lower than those in specimens from women with early-onset PE ($P < 0.01$ for all comparisons).

Association between PE and pNK cell frequencies and intracellular IFN- γ , perforin and granzyme B production during late pregnancy

To complement our comparisons of dNK cell numbers in early and late onset PE and control pregnancies, we used flow cytometry to assess and compare the number of maternal pNK cells in women with these conditions. The number of pNK cells in late-onset PE patients was significantly higher than that in normal pregnancies ($P < 0.01$) and significantly lower than that in early-onset PE ($P < 0.01$, Figure 2A and E). Similar results were noted for the levels of pNK cell intracellular IFN- γ , perforin and granzyme B ($P < 0.01$ for all comparisons, Figure 2B-D, F-H).

Immune activation of dNK cells in PE during late pregnancy

Since dNK cells play an important role in regulating trophoblast invasion and migration [15, 16], we analyzed the effects of dNK cells on these processes. As shown in Figure 3A and B, while CM from dNK cells from women with late-onset PE significantly decreased the invasion and migration of first trimester

trophoblast cells when compared with normal pregnancies ($P < 0.01$ for both comparisons), the effect was even more robust when using CM from early-onset PE dNK cells ($P < 0.01$ for both comparisons). Additionally, dNK cell anti-trophoblast cytotoxicity was highest for dNK cells isolated from women with early-onset PE, lower for those isolated from late-onset PE subjects, and lowest when the source was the deciduae of unaffected control pregnancies ($P < 0.01$ for all comparisons, Figure 3C).

Levels of soluble factors in dNK cell CM

Since CM from dNK cells isolated from normal and preeclamptic pregnancies demonstrated different effects on trophoblast cell migration and invasion, we hypothesized that these effects may be related to differences in the secretion of biologically active factors and that such changes might be detected by comparing dNK cell CM of normal and preeclamptic pregnancies. We simultaneously analyzed 10 candidate soluble factors in dNK cell CM using multiplex immunoassays. As demonstrated in Figure 4, levels of GM-CSF and IL-8 in dNK cell CM from women with late-onset PE were significantly lower than those in samples from normal pregnancies ($P < 0.01$ for both comparisons), but significantly higher than those in samples from early-onset PE ($P < 0.01$ for both comparisons). In contrast, concentrations of IFN- γ and TNF- α in dNK cell CM from late-onset PE were significantly higher than those in specimens from normal pregnancies ($P < 0.01$ for both comparisons), but significantly lower than those in specimens from early-onset PE ($P < 0.01$ for both comparisons). Levels of IL-1 β , IL-2, IL-4, IL-5, IL-6 and IL-10 were low in all samples and no significant differences were detected among normal and preeclamptic pregnancies.

Effects of soluble factors secreted by dNK cells on trophoblast cell migration and invasion

Having detected differences in the levels of GM-CSF, IL-8, IFN- γ and TNF- α in dNK cell CM among normal and early- and late-onset PE, we next examined the functional effects of these factors on trophoblast cell migration and invasion. Since dNK cells from normal pregnancies secreted elevated levels of GM-CSF and IL-8, we concentrated on these two factors when assessing the effects of adding neutralizing Abs to dNK cell CM from normal pregnancies on trophoblast cell function. Stimulation with GM-CSF or IL-8 was used as positive controls and the addition of isotype control Ab to unconditioned media was included as negative controls. As shown in Figure 5A, dNK cell CM from normal pregnancies significantly increased trophoblast cell migration and invasion compared with the medium control group ($P < 0.01$ for both comparisons). Normal dNK cell CM supplemented with neutralizing Abs against GM-CSF or IL-8 significantly decreased trophoblast cell migration and invasion compared to dNK cell CM from normal pregnancies ($P < 0.01$ for each comparison). Addition of IL-8 or GM-CSF caused expected increases in both trophoblast cell migration and invasion when compared to unconditioned medium controls ($P < 0.01$ for each comparison).

Decidual NK cells from late-onset PE produced moderate amounts of GM-CSF, IL-8, IFN- γ and TNF- α , so we analyzed roles of these factors in trophoblast cell migration and invasion using neutralizing Abs. As shown in Figure 5B, dNK cell CM from late-onset PE significantly decreased trophoblast cell migration and invasion when compared to unconditioned medium controls ($P < 0.01$ for both comparisons).

Supplementation of CM with neutralizing Abs against GM-CSF or IL-8 significantly decreased trophoblast cell migration and invasion compared with dNK cell CM from late-onset PE ($P < 0.01$ for each comparison). However, supplementation with neutralizing Abs against IFN- γ or TNF- α significantly increased trophoblast cell migration and invasion compared with dNK cell CM from late-onset PE ($P < 0.01$ for each comparison).

In similar experiments, since dNK cells from early-onset PE secreted a considerable amount of IFN- γ and TNF- α , we examined the effects of these factors on trophoblast cell migration and invasion using recombinant human IFN- γ and TNF- α , and neutralizing Abs against IFN- γ and TNF- α . As shown in Figure 5C, dNK cell CM from early-onset PE significantly decreased trophoblast cell migration and invasion when compared to unconditioned medium controls ($P < 0.01$ for both comparisons). Supplementation with neutralizing Abs against IFN- γ or TNF- α significantly increased trophoblast cell migration and invasion when compared with dNK cell CM from early-onset PE ($P < 0.01$ for each comparison). The addition of recombinant IFN- γ or TNF- α significantly decreased trophoblast cell migration and invasion when compared to unconditioned medium controls ($P < 0.01$ for each comparison).

Discussion

Although great progress has been made during the past decade toward our understanding of the pathogenesis of PE, several formidable investigative challenges leave much about its etiology and pathophysiology unclear [27]. First, while the disease is associated with inadequate EVT invasion and insufficient uterine SA remodeling beginning in early pregnancy, significantly delayed symptom onset in the late second or early third trimester [28] makes etiologic studies in humans daunting. Further, even if one could definitively identify in early pregnancy those women who would ultimately develop signs and symptoms of PE, access to relevant placental or decidual tissues for single or multiple time point investigations is essentially impossible. Finally, PE is considered to be a disease specific to humans. Animal models in which the disease spontaneously occurs are lacking [29] and animal models utilizing induction techniques do not typically demonstrate all disease manifestations [30].

A two-stage model for PE pathogenesis has been proposed [31, 32]. The first stage involves reduced EVT invasion and inadequate uterine vessel remodeling in the deciduae. These structural abnormalities are, in turn linked to insufficient and hyperactive uteroplacental circulation that causes overall placental hypoxia with interspersed periods of intermittent hyperoxia [31]. The resulting hypoxic and oxidative damage promotes a second stage of release of a series of proinflammatory factors and vasoactive factors from the placenta into maternal circulatory system that cause the clinical syndrome in late pregnancy [33, 34]. In the present study, we investigated the role of NK cells in the pathogenesis of the second stage of PE when the maternal inflammatory responses cause the majority of disease signs and symptoms.

We divided women with PE into early-onset and late-onset PE groups, a subclassification that often correlates with disease severity [35]. Using flow cytometry, we found that the numbers of NK cells in the

peripheral blood of women with both early-onset and late-onset PE were significantly higher than those in normal control pregnancies and that the extent of these changes was highest in early-onset PE.

We also assessed NK cells within the deciduae of samples obtained at the time of delivery in our control and PE subjects. These cells were present in normal pregnancies at term. Their functional role at this stage of gestation is not known and will be the topic in future investigations. Like our results for pNK cells, the percentages of NK cells in the decidual basalis of preeclamptic pregnancies was significantly higher than those in normal pregnancies, and the effect was most marked in women with early-onset PE. This supports prior immunohistochemical analyses of frozen decidual tissues [20] showing that CD56⁺dNK cell numbers were higher in women with PE when compared with normal term control pregnancies. Similarly, Wilczyński et al. [21] used flow cytometry to demonstrate that preeclamptic patients had a higher percentage of CD3⁻CD56⁺CD16⁺ NK cells in the deciduae compared with age-matched term control pregnancies.

In contrast, using immunohistochemical analyses of frozen tissues, Williams et al. [22] reported a reduction in dNK cells in placental bed biopsies from women with PE compared with control third trimester deciduae. Further, using immunofluorescence and frozen tissues, Lockwood et al. [23] reported that deciduae from women with PE displayed significantly lower dNK cell numbers than term controls. Likewise, Rieger et al. [36] have shown that the number of decidual CD56⁺CD16⁺ NK cells was lower in deciduae from women with PE than that in gestational age-matched preterm deliveries caused by preterm labour, multiple gestation, fetal distress, intrauterine fetal growth restriction, fetal abnormalities, placenta praevia or uterine rupture. The reasons for the discrepancies between our results and these latter findings are not immediately evident, but may result from differences in study design, subject selection, specimen origin (e.g. decidua basalis vs. placental bed biopsies), reagent specificity, sample size and/or analytic methods.

We next turned to more functional analyses and reported that the production of intracellular IFN- γ , perforin and granzyme B by both pNK and dNK cells is higher in pregnancies affected by PE when compared to controls and that the effect is most marked in women with early-onset PE. This suggests that both systemic NK cells and NK cells at the maternal-fetal interface are hyperactivated in PE and the degree of activation correlates positively with disease activity. More detailed mechanisms underlying NK cell activation in PE is currently under investigation. One hypothesis is that dNK cells may become activated and dysfunctional under the stress of pathological placental hypoxia [37]. To this point, a conversion of tolerogenic dNK cells to a largely cytolytic phenotype [38] was reported in affected rats exposed to reduced uterine perfusion pressure to model human PE. This shift was associated with higher mean arterial pressures, fetal intrauterine growth restriction and increased inflammation, changes that were reversed by the depletion of NK cells [38].

In the present study, activated dNK cells from subjects experiencing PE not only killed first trimester trophoblast cells at a higher level than those from unaffected women, but also inhibited trophoblast cell invasion and migration to a greater extent. All dNK samples were subjected to stimulation with primary

cytotrophoblast cells isolated from the first trimester placentae instead of with cytotrophoblast cells of the same donors near term because most of the first trimester cytotrophoblast cells will differentiate into EVT_s, while the third trimester cytotrophoblast cells will essentially universally differentiate into syncytiotrophoblast cells in vitro [39]. To our knowledge, this is the first report to include the frequencies and the function of both dNK cells and pNK cells isolated in late pregnancy from women experiencing PE.

We next selected 10 candidate soluble factors in dNK CM that might modulate trophoblast function and further dissected their production levels and effects on trophoblast cell migration and invasion by disease status. Decidual NK cells from normal pregnancies produced high levels of GM-CSF and IL-8, while dNK cells from preeclamptic pregnancies, especially early-onset PE, produced abundant IFN- γ and TNF- α . We then demonstrated that GM-CSF and IL-8 in dNK CM promoted, while IFN- γ and TNF- α inhibited trophoblast cell migration and invasion using recombinant human GM-CSF, IL-8, IFN- γ and TNF- α , and neutralizing Abs against these factors. To our knowledge, this is the first report to describe the regulation of trophoblast cell function by effector factors produced by dNK cells in late pregnancy and the first to link these findings to PE.

One limitation of our study is that we selected for study only 10 candidate soluble factors in dNK cell CM. Other factors produced by dNK cells may have effects on trophoblast cell function of equal or greater importance than those studied here and these effects will have gone undetected. As with many studies on PE at delivery, there was a gestational age difference in the time of sample collection among disease and control groups. Unfortunately, choice of an appropriate gestational age-matched normal control population is difficult as premature deliveries are essentially never normal.

Conclusions

Activation of both dNK cells and pNK cells may contribute to the pathogenesis of symptomatic PE in late pregnancy. Inhibition of trophoblast cell migration and invasion by dNK CM from preeclamptic pregnancies may be due to elevated production of IFN- γ and TNF- α and decreased production of GM-CSF and IL-8 by dNK cells in affected pregnancies.

Declaration

Ethics approval and consent to participate

The study was approved by the Medical Ethics Committee of Guangzhou First People's Hospital, School of Medicine, South China University of Technology. Written informed consent was obtained from the study participants prior to their enrollment.

Consent for publication

Not applicable

Availability of data and materials

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests

Funding

This work was supported by funding from the National Natural Science Foundation of China (81571510 and 81871217) to L.L.

Authors' contributions

L.L. conceived and designed the study, directed the project and wrote the manuscript. M.D., W.W. and L.H. collected samples, performed experiments and analyzed data. X.G., W.L. and J.Y. performed experiments and analyzed data.

Acknowledgement

Not applicable

Abbreviations

PE: Preeclampsia

SA: spiral artery

dNK: decidual natural killer

pNK: peripheral blood NK

IFN- γ : interferon- γ

EVT: extravillous trophoblast cell

GM-CSF: colony-stimulating factor

IL-8: interleukin-8

TNF: tumor necrosis factor

CM: conditioned medium

BPs: blood pressures

PBMCs: peripheral blood mononuclear cells

PBS: phosphate-buffered saline

References

1. Yeh CC, Chao KC, Huang SJ. Innate immunity, decidual Cells, and preeclampsia. *Reprod Sci* 2013; 20: 339-353.
2. American College of Obstetricians and Gynecologists; Task Force on Hypertension in Pregnancy. Hypertension in pregnancy. Report of the American College of Obstetricians and Gynecologists' Task Force on Hypertension in Pregnancy. *Obstet Gynecol* 2013; 122: 1122-1131.
3. Ilekis JV, Reddy UM, Roberts JM. Preeclampsia - a pressing problem: an executive summary of a National Institute of Child Health and Human Development workshop. *Reprod Sci* 2007; 14: 508-523.
4. Young BC, Levine RJ, Karumanchi SA. Pathogenesis of preeclampsia. *Annu Rev Pathol* 2010; 5: 173-192.
5. Bello N, Rendon ISH, Arany Z. The relationship between pre-eclampsia and peripartum cardiomyopathy: a systematic review and meta-analysis. *J Am Coll Cardiol* 2013; 62: 1715-1723.
6. Moffett A, Colucci F. Uterine NK cells: active regulators at the maternal-fetal interface. *J Clin Invest* 2014; 124: 1872-1879.
7. Caligiuri MA. Human natural killer cells. *Blood* 2008; 112: 461-469.
8. Vivier E, Tomasello E, Baratin M, Walzer T, Ugolini S. Functions of natural killer cells. *Nat Immunol* 2008; 9: 503-510.
9. Zhang J, Tian Z. UNK cells: their role in tissue re-modelling and preeclampsia. *Semin Immunopathol* 2007; 29: 123-133.
10. Erlebacher A. Immunology of the maternal-fetal interface. *Annu Rev Immunol* 2013; 31: 387-411.
11. Lash GE, Schiessl B, Kirkley M, Innes BA, Cooper A, Searle RF, Robson SC, Bulmer JN. Expression of angiogenic growth factors by uterine natural killer cells during early pregnancy. *J Leukoc Biol* 2006; 80: 572-580.
12. Wallace AE, Fraser R, Cartwright JE. Extravillous trophoblast and decidual natural killer cells: a remodelling partnership. *Hum Reprod Update* 2012; 18: 458-471.
13. Kalkunte SS, Mselle TF, Norris WE, Wira CR, Sentman CL, Sharma S. Vascular endothelial growth factor C facilitates immune tolerance and endovascular activity of human uterine NK cells at the maternal-fetal interface. *J Immunol* 2009; 182: 4085-4092.
14. Anacker J, Segerer SE, Hagemann C, Feix S, Kapp M, Bausch R, et al. Human decidua and invasive trophoblasts are rich sources of nearly all human matrix metalloproteinases. *Mol Hum Reprod* 2011; 17: 637-652.
15. Wallace AE, Host AJ, Whitley GS, Cartwright JE. Decidual natural killer cell interactions with trophoblasts are impaired in pregnancies at increased risk of preeclampsia. *Am J Pathol* 2013; 183:

1853-1861.

16. Lash GE, Otun HA, Innes BA, Percival K, Searle RF, Robson SC, et al. Regulation of extravillous trophoblast invasion by uterine natural killer cells is dependent on gestational age. *Hum Reprod* 2010; 25: 1137-1145.
17. Fraser R, Whitley GS, Johnstone AP, Host AJ, Sebire NJ, Thilaganathan B, et al. Impaired decidual natural killer cell regulation of vascular remodelling in early human pregnancies with high uterine artery resistance. *J Pathol* 2012; 228: 322-332.
18. Lash GE, Otun HA, Innes BA, Kirkley M, De Oliveira L, Searle RF, et al. Interferon- γ inhibits extravillous trophoblast cell invasion by a mechanism that involves both changes in apoptosis and protease levels. *FASEB J* 2006; 20: 2512-2518.
19. Hu Y, Dutz JP, MacCalman CD, Yong P, Tan R, von Dadelszen P. Decidual NK cells alter in vitro first trimester extravillous cytotrophoblast migration: a role for IFN- γ . *J Immunol* 2006; 177: 8522-8530.
20. Bachmayer N, Rafik Hamad R, Liszka L, Bremme K, Sverremark-Ekström E. Aberrant uterine natural killer (NK)-cell expression and altered placental and serum levels of the NK-cell promoting cytokine interleukin-12 in pre-eclampsia. *Am J Reprod Immunol* 2006; 56: 292-301.
21. Wilczyński JR, Tchórzewski H, Banasik M, Głowacka E, Wieczorek A, Lewkowicz P, et al. Lymphocyte subset distribution and cytokine secretion in third trimester decidua in normal pregnancy and preeclampsia. *Eur J Obstet Gynecol Reprod Biol* 2003; 109: 8-15.
22. Williams PJ, Bulmer JN, Searle RF, Innes BA, Robson SC. Altered decidual leucocyte populations in the placental bed in pre-eclampsia and foetal growth restriction: a comparison with late normal pregnancy. *Reproduction* 2009; 138: 177-184.
23. Lockwood CJ, Huang SJ, Chen CP, Huang Y, Xu J, Faramarzi S, et al. Decidual cell regulation of natural killer cell-recruiting chemokines: implications for the pathogenesis and prediction of preeclampsia. *Am J Pathol* 2013; 183: 841-856.
24. Wallace AE, Fraser R, Gurung S, Goulwara SS, Whitley GS, Johnstone AP, et al. Increased angiogenic factor secretion by decidual natural killer cells from pregnancies with high uterine artery resistance. *Hum Reprod* 2014; 29: 652-660.
25. Li L, Tu J, Jiang Y, Zhou J, Yabe S, Schust DJ. Effects of lipopolysaccharide on human first trimester villous cytotrophoblast cell function in vitro. *Biol Reprod* 2016; 94: 33.
26. Li L, Schust DJ. Isolation, purification and in vitro differentiation of cytotrophoblast cells from human term placenta. *Reprod Biol Endocrinol* 2015; 13: 71.
27. Rabaglino MB, Post Uiterweer ED, Jeyabalan A, Hogge WA, Conrad KP. Bioinformatics approach reveals evidence for impaired endometrial maturation before and during early pregnancy in women who developed preeclampsia. *Hypertension* 2015; 65: 421-429.
28. Sibai B, Dekker G, Kupferminc M. Pre-eclampsia. *Lancet* 2005; 365: 785-799.
29. Granger JP, George EM, Roberts JM. Animal model for investigating pathophysiological mechanisms of preeclampsia. In: Taylor RN, Roberts JM, Cunningham FG, Lindheimer MD (ed.) *Chesley's hypertensive disorders in pregnancy*. Amsterdam: Elsevier-Academic Press, 2014: 209-220.

30. Aubuchon M, Schulz LC, Schust DJ. Preeclampsia: animal models for a human cure. *Proc Natl Acad Sci USA* 2011; 108: 1197-1198.
31. Laresgoiti-Servitje E. A leading role for the immune system in the pathophysiology of preeclampsia. *J Leukoc Biol* 2013; 94: 247-257.
32. Sargent IL, Borzychowski AM, Redman CW. Immunoregulation in normal pregnancy and pre-eclampsia: an overview. *Reprod Biomed Online* 2006; 13: 680-686.
33. Furuya M, Kurasawa K, Nagahama K, Kawachi K, Nozawa A, Takahashi T, et al. Disrupted balance of angiogenic and antiangiogenic signalings in preeclampsia. *J Pregnancy* 2011; 2011: 123717.
34. Redman CW, Sargent IL. Immunology of pre-eclampsia. *Am J Reprod Immunol* 2010; 63: 534-543.
35. Miko E, Meggyes M, Bogar B, Schmitz N, Barakonyi A, Varnagy A, et al. Involvement of Galectin-9/TIM-3 pathway in the systemic inflammatory response in early-onset preeclampsia. *PLoS One* 2013; 8: e71811.
36. Rieger L, Segerer S, Bernar T, Kapp M, Majic M, Morr AK, et al. Specific subsets of immune cells in human decidua differ between normal pregnancy and preeclampsia—a prospective observational study. *Reprod Biol Endocrinol* 2009; 7: 132.
37. Sava RI, March KL, Pepine CJ. Hypertension in pregnancy: Taking cues from pathophysiology for clinical practice. *Clin Cardiol* 2018; 41: 220-227.
38. Elfarrar J, Amaral LM, McCalmon M, Scott JD, Cunningham MW Jr, Gnam A, et al. Natural killer cells mediate pathophysiology in response to reduced uterine perfusion pressure. *Clin Sci (Lond)* 2017; 131: 2753-2762.
39. Cervar-Zivkovic M, Stern C. Trophoblast isolation and culture. In: Kay HH, Nelson DM, Wang Y (ed.) *The placenta: from development to disease*. England: Wiley-Blackwell, 2011: 155-162.

Figures

Figure 2

The frequencies of decidual natural killer (dNK) cells and dNK cell intracellular interferon (IFN)- γ , perforin and granzyme B production in women with preeclampsia (PE) at the time of delivery. Representative experiments and graphical summaries of the percentages of dNK cells (A and E), and dNK cell intracellular IFN- γ (B and F), perforin (C and G) and granzyme B (D and H) levels in normal control (n = 18), late-onset PE (n = 28) and early-onset PE (n = 14) samples are shown. Deciduae were obtained at the time of cesarean section delivery without preceding labor. Red: isotype control. Blue: normal pregnancies. Orange: late-onset PE. Green: early-onset PE. $P < 0.01$, by 2-sided one-way ANOVA. Data represent the means $\pm$ SDs.

Figure 4

The frequencies of peripheral blood natural killer (pNK) cells and pNK cell intracellular interferon (IFN)- γ , perforin and granzyme B production in women with preeclampsia (PE) at the time of delivery. Representative experiments and graphical summaries of the percentages of pNK cells (A and E), and pNK cell intracellular IFN- γ (B and F), perforin (C and G) and granzyme B (D and H) levels in normal control (n = 18), late-onset PE (n = 28) and early-onset PE (n = 14) samples are shown. Blood was obtained at the time of cesarean section delivery without preceding labor. Red: isotype control. Blue: normal pregnancies. Orange: late-onset PE. Green: early-onset PE. P < 0.01, by 2-sided one-way ANOVA. Data represent the means $\pm$ SDs.

Figure 6

Functional activation of dNK cells in preeclampsia (PE). Effects of dNK cells from normal, late-onset PE and early-onset PE pregnancies on the invasion (A) and migration (B) of first trimester trophoblast cells. (C) Lysis of first trimester trophoblast cells by dNK cells from normal, late-onset PE and early-onset PE pregnancies. *P < 0.01, by 2-sided one-way ANOVA. Data represent the means $\pm$ SDs of six independent experiments. E:T ratio: the ratio of effector cells (dNK cells) to target cells (trophoblast cells).

Figure 7

Comparison of the levels of soluble factors in the conditioned medium (CM) from decidual natural killer (dNK) cells between normal and preeclamptic pregnancies. Levels of colony-stimulating factor (GM-CSF), interferon (IFN)- γ , interleukin (IL)-1 β , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10 and tumor necrosis factor (TNF)- α in dNK cell CM from normal controls (n = 18), late-onset (n = 28) and early-onset preeclampsia (PE) (n = 14)

were assessed simultaneously using multiplex immunoassays. * $P < 0.01$, by 2-sided one-way ANOVA. Data represent the means $\pm$ SDs.

Figure 9

Effects of soluble factors secreted by decidual natural killer (dNK) cells on trophoblast cell migration and invasion. We examined the effects of soluble factors in the conditioned medium (CM) of dNK cells from (A) normal controls, (B) late-onset and (C) early-onset preeclampsia (PE) on cytotrophoblast cell migration and invasion using recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-8, interferon (IFN)- γ and tumor necrosis factor (TNF)- α , and neutralizing Abs against these factors. * $P < 0.01$ and $\blacktriangle P < 0.05$, by 2-sided one-way ANOVA. Data represent the means $\pm$ SDs of six independent experiments.