Decidual Lymphatic Endothelial Cell-derived GM-CSF induces M1 Macrophage Polarization via the NF-κB Pathway in Severe Preeclampsia

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Directed by Professor Ja-Young Kwon

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ABSTRACT

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Objective: The impaired immune tolerance and excessive inflammatory response at the maternal-fetal interface led to the development of preeclampsia (PE). Recent evidence suggests that decidual macrophages may be polarized into the proinflammatory M1 phenotype in PE and mediate inflammatory responses. Decidual macrophages are in close contact with uterine immune cells during placentation, and decidual lymphatic endothelial cells (DLEC) have a high probability of crosstalk with resident macrophages. It is likely to affect the decidual macrophage polarization, in addition to aspirin and folic acid that can contribute to the immune tolerance in PE. This thesis aimed to investigate whether DLECs influence decidual macrophage polarization in normal and preeclamptic pregnancy, find candidate cytokine expressed in PE-DLECs modulating macrophage polarization, and evaluate the effect of aspirin and folate on the interaction between decidual LECs and resident macrophage differentiation in PE.

Material and methods: Primary DLECs were isolated from decidua obtained from 10 pregnant women with PE and 10 gestational age-matched controls. To analyze the effects of normal and PE-DLECs on macrophage polarization, phorbol-12-myristate-13-acetate (PMA)-stimulated THP-1 cells were cultured in a DLEC-conditioned medium (CM) or co-cultured with DLECs from patients

with PE and controls. Surface marker expression of M1 and M2 macrophages was detected by flow cytometry. Secreted cytokine expression levels from DLECs were determined by microarray. Granulocyte-macrophage colony-stimulating factor (GM-CSF) expression was measured by enzyme-linked immunosorbent assay, and transcripts for GM-CSF were detected by quantitative real-time reverse transcription polymerase chain reaction. Phosphorylation of extracellular signal-related kinase (ERK) and activation of nuclear factor-κB (NF-κB) were evaluated by western blotting and immunocytochemical analysis, respectively. Normal and PE-DLECs were exposed to four different concentrations of aspirin and folate during 24 hr, measuring protein and mRNA levels of GM-CSF.

Results: In a coculture and culture with DLEC-CM, the contribution of PE-DLECs to M1 polarization of macrophages was confirmed by M1-related marker expression using flow cytometry. GM-CSF secretion and expression levels were increased in PE-DLECs. The addition of rhGM-CSF to M0 macrophages with normal CM promoted M1 polarization, whereas knockdown of GM-CSF in PE-DLECs by shRNA significantly reduced M1 polarization. The results showed that GM-CSF expression from PE-DLECs is downstream of NF-κB activation using an NF-κB inhibitor (PDTC), and this NF-κB/GM-CSF signaling pathway can modulate the decidual M1 polarization. In addition, pretreatment of aspirin and folate significantly decreased the expression of GM-CSF of PE-DLECs at both protein and mRNA levels in a dose-dependent manner.

Conclusions: GM-CSF induces M1 macrophage polarization through NF-κB pathway in PE-DLECs. Since aspirin and folate modulate GM-CSF release, the present data suggest aspirin and folate may mitigate the progression of PE.

Keywords: pregnancy, lymphatic endothelial cell, macrophage polarization, preeclampsia

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I. INTRODUCTION

Pre-eclampsia (PE) is a human-specific syndrome with unknown etiology causing maternal-fetal morbidities and mortalities. This syndrome is a hypertensive disorder of pregnancy, which affects 5–10% of all pregnancies, and presents after 20 weeks of gestation [1,2]. Generally, PE develops after abnormal trophoblast invasion and a decreased spiral artery remodeling and defective placentation. However, its etiology is not fully understood. An increasing amount of data indicates maternal immune system alterations associated with the pathophysiology of PE [3,4].

Maternal-fetal immune tolerance, the unresponsiveness of the adaptive immune system to paternal alloantigens, is important in the maintenance of pregnancy to term [5,6]. Decidua, the maternal-fetal interface, contains a large amount of immune cells that maintain immune tolerance. Various decidual immune cells, including decidual NK cells, macrophages, T cells, and dendritic cells (DCs), are involved in processes of placentation, such as extracellular matrix remodeling, trophoblast invasion, and angiogenesis [7].

Among them, decidual macrophages are relatively abundant, comprising

10–20% of the human decidual leukocyte population in the first trimester, and are present throughout pregnancy [8]. Resident macrophages play a major role in acquiring immune tolerance during pregnancy through polarization to the pro-inflammatory M1 or immunomodulatory M2 phenotype. Several studies indicate that M2 polarized decidual macrophages contribute to fetal tolerance and are involved in several processes, including trophoblast invasion and vascular remodeling in normal pregnancy [9-12]. Conversely, M1 macrophages mainly distribute in maternal blood and decidua of preeclamptic pregnancy and mediate inflammatory responses [13,14]. Aberrantly activated decidual M1 macrophages and pro-inflammatory cytokine secretion could affect angiogenesis, tissue homeostasis, trophoblast invasion, spiral artery remodeling, and placental apoptosis [15]. However, the underlying molecular mechanisms of imbalanced decidual macrophage polarization in PE remain unclear.

Emerging evidence indicates that lymphatic vessels are important to regulate immune cell trafficking, antigen presentation, tolerance, and immunity, all of which may affect the acquisition of immune tolerance during pregnancy [16-19]. Lymphatic endothelial cells can modulate dendritic cell and macrophage trafficking through the expression of the chemokine-scavenging decoy receptor D6 and C-C chemokine ligand 2 [19]. Tissue-resident macrophages also regulate lymphangiogenesis during organ development and tumor progression [20,21]. Therefore, there is a possible interaction between macrophages and LECs even at the maternal-fetal interface. Previous studies have reported that the distribution of decidual lymphatic vessels and lymphangiogenic function of LECs were reduced in PE [22,23]. Although the relationship between lymphatics and macrophage differentiation has not been elucidated, the altered lymphatic environment in PE may affect the polarization of resident macrophages.

Many pharmacological interventions with anti-inflammatory and immunomodulatory properties are used or studied to prevent PE, among which aspirin and high-dose folate have been widely used clinically [24,25]. Aspirin

inhibits the production of prostaglandin and thromboxane by inactivating COX-1. Although the mechanism by which aspirin prevents PE is unclear, it is presumed to enhance trophoblast invasion, reduce placental infarction, and induce anti-inflammatory effects and endothelial stabilization [26]. Folate, which is essential for DNA synthesis, is important for cell-mediated and humoral immunity. Moreover, the suggested mechanism by which folate supplementation help prevent the development of PE is when an adequate supply of folate necessary for implantation, angiogenesis, trophoblast invasion, and endothelial-dependent vascular relaxation during pregnancy was acquired [27-29]. Due to limited studies on the effects of aspirin and folate on resident immune cell function and lymphangiogenesis in PE [26,27], studies on the immunological role of aspirin and folate at the maternal-fetal interface are needed.

Based on the research results to date, it can be hypothesized that lymphangiogenesis in the maternal-fetal interface during pregnancy influences the differentiation of resident macrophages, and the induction of proinflammatory changes in PE may be highly relevant. Aspirin and folate, which have immune-modulating properties, are also expected to have a positive effect on the interaction between decidual lymphatics and resident macrophages in PE. This study aimed (1) to investigate whether decidual lymphatic endothelial cells influence decidual macrophage polarization associated with immune tolerance in normal and preeclamptic pregnancy, (2) find candidate cytokine expressed in preeclamptic DLECs that could affect macrophage polarization, and (3) evaluate the effect of aspirin and folate on the interaction between decidual LECs and resident macrophage differentiation in PE.

II. MATERIALS AND METHODS

1. Study population and sample collection

Deliveries at 33–38 weeks of gestation were selected at the Severance Hospital between January 2019 and February 2021. Enrolled pregnant women were divided into the normal and PE groups. Severe PE was diagnosed in accordance with the American College of Obstetricians and Gynecologists criteria [30], as the presence of hypertension associated with the following: thrombocytopenia (platelet count < 100,000/μL); impaired liver function indicated by abnormally elevated blood concentrations of liver enzymes (twice the normal concentration); severe persistent right upper quadrant or epigastric pain unresponsive to medication and not accounted for by alternative diagnosis, or both; progressive renal insufficiency (serum creatinine concentration > 1.1 mg/dL or a doubling of the serum creatinine concentration in the absence of other renal diseases); new-onset cerebral or visual disturbance; and pulmonary edema. Hypertension was defined as blood pressure > 160/110 mmHg on two occasions at least 4 hr apart while the patient is on bed rest. Exclusion criteria were as follows: clinical or pathological diagnosis of chorioamnionitis, maternal autoimmune disease, or multiple pregnancies. Gestational age-matched controls, who had delivered for other indications, were not diagnosed with PE or chronic hypertension. Fetal chorioamniotic membranes containing decidual tissue were obtained at the time of cesarean delivery after fetal expulsion from preeclampsia (n=30) and normal pregnancy (n=30). This study was approved by the Institutional Review Board (IRB) of Severance Hospital (4-2016-0450), and written informed consent was obtained from all patients.

2. Cell isolation and cultivation

A. Primary human decidual lymphatic endothelial cell (DLEC) isolation

Decidual tissues were aseptically scraped from fetal chorioamniotic membranes with sterile forceps and scissors and minced into 1–2 mm pieces, followed by enzyme digestion with warming 0.25% collagenase II during 2 hr at 37℃. Digested cells were passed through a 100 μM cell strainer once. Primary DLECs will be cultured on fibronectin-coated culture dishes using the EBM medium. Cells were grown at 37℃ in a humidified atmosphere containing 5% CO_2. After 24 hr, unbounded cells were washed, and new endothelial cell media were added. Cell supernatant was collected and spun down to remove any cellular debris. Cell-free supernatant will be aliquoted and stored at -80℃ until use.

B. THP-1 differentiation

THP-1 cells (Korean Cell Line Bank) were cultured in an RPMI-1640 medium (GE Healthcare Life Sciences, Marlborough, MA, USA) supplemented with 10% FBS, 10 mM HEPES (Sigma-Aldrich, St. Louis, MO, USA), 1 mM sodium pyruvate (Sigma-Aldrich), 0.05 mM β-mercaptoethanol (Sigma-Aldrich), and 1% penicillin-streptomycin (Sigma-Aldrich) in a humidified incubator (37℃/5% CO_2). THP-1 cells were differentiated in six-well plates ($5x10^5$ cells/well). For preparation of M0 THP-1 macrophage, 100 nM phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) was added to THP-1 cells for 72 hr. Macrophages were obtained in M1 macrophages by incubation with 20 ng/mL of interferon γ (IFN-γ; Rocky Hill, NJ, USA) and 20 ng/mL of lipopolysaccharides (LPS; Sigma-Aldrich) for 48 hr. M2 macrophage differentiation was obtained by incubation with 20 ng/mL of interleukin 4 (IL-4; Peprotech) and 20 ng/mL of interleukin 13 (IL-13; Peprotech) for 48 hr.

3. Conditioned medium and coculture assays

A. Co-culture of THP-1 cells with DLECs

In the M0 macrophage-DLEC coculture model, 2.5×10^5 THP-1 cells in RPMI containing 10% FBS were cultured and incubated with 100 nM PMA for 72 hr. After the replacement of the medium, 5×10^5 DLECs were added to six-well plates (M0:DLEC ratio, 1:2) and incubated for 48 hr. After 48 hr, cells were washed with PBS, added with cell dissociation buffer, and gently scraped.

B. DLEC conditioned medium preparation

DLEC-conditioned medium (CM) was prepared from second and third passage DLECs. Briefly, 5×10^5 DLECs were cultured in an EBM medium supplemented with 10% FBS for 2 days. The media from these plates was collected and centrifuged at 1500 rpm for 5 min. Supernatants were then transferred to fresh tubes and mixed with an equal volume of fresh medium with the same supplements to form CM.

4. Flow cytometry

Fluorescence-activated cell sorting (FACS) was used to determine the expression of THP-1 macrophage cell surface marker proteins. After the polarization of macrophages for 48 hr, cells were washed with PBS, added with cell dissociation buffer, and gently scraped. Cells were then stained with fluorochrome-tagged monoclonal antibodies (all from BD Biosciences, Bedford, MA, USA) against surface HLA-DR (PE-Cy5) and CD80 (FITC) to characterize the M1 phenotype, and against CD209 (PerCP-Cy5.5) and CD163 (FITC) to characterize the M2 phenotype [31]. Upon labeling, cells were washed twice and fixed with 1% paraformaldehyde for 10 min at 4℃. Cells were analyzed with a FACS LSR II SORP system (BD Biosciences), recording at least 10,000 events for each sample. Data were analyzed using the FacsDiva software (BD Biosciences).

5. Microarray analysis

Ten micrograms of total RNA from DLECs were hybridized to the HG-U133A 2.0 microarray (54675 human genes; Affymetrix, Santa Clara, CA). The standard protocol used for sample preparation and microarray processing is available from Affymetrix. Expression data were analyzed using Microarray Suite version 5.0 (Affymetrix) and GenPlex v2.4 software (ISTECH, Inc., Seoul, Korea).

6. Determination of cytokine production by enzyme-linked immunosorbent assay (ELISA)

Normal and PE-DLECs were seeded in 96-well plates at a density of 5×10^5 cells/well to analyze the GM-CSF production. The cell culture supernatant was collected at 6, 12, and 24 hr of culture. GM-CSF production was determined using the human GM-CSF Quantikine ELISA kit (R&D systems, Minneapolis, MN, USA) in each supernatant, according to the manufacturer's instructions.

7. GM-CSF shRNA transfection

To silence the expression of GM-CSF in PE-DLEC, cells were cultured in six-well plates and transfected with shRNA Lentiviral Particles (Santa Cruz Biotechnology, Santa Cruz, CA, USA), following the manufacturer's instructions. The expression of GM-CSF mRNA was analyzed by quantitative RT-PCR 48 hr after transfection.

8. Cell treatment

After 72 hr of culture of THP-1 derived M0 macrophage in RPMI 1640 media, M0 were treated with normal-DLEC conditioned media with or without 25 or 50 ng/mL recombinant human GM-CSF (Sigma-Aldrich) to evaluate the effect of treatment with GM-CSF in normal DLEC on macrophage polarization. After 48 hr, cells were harvested and analyzed by flow cytometry using specific

markers of M1/M2 macrophages.

To investigate the role of nuclear factor-κB (NF-κB) and extracellular signal-related kinase (ERK) pathway in the production of GM-CSF in DLECs, normal and PE-DLECs were pretreated with 100 μM pyrrolidine dithiocarbamate (PTDC; NF-κB inhibitor, Sigma-Aldrich) and 5 μM U0126 (ERK pathway inhibitor, Sigma-Aldrich) for 1 hr, and were then cultured for 24 hr at 37℃ in a 5% CO_2 incubator. Cell supernatants and lysates were harvested for analysis.

To determine the effect of aspirin and folate concentration on DLEC, cells (2×10^5 cells/well) were plated with various concentrations of aspirin (0, 3, 30, 300 μM) or folate (0, 20, 200, 2000 ng/mL) in 96-well plates, and cultured for 24 hr at 37℃ in a 5% CO_2 incubator. Aspirin and folate were purchased from Sigma-Aldrich. All cells were exposed to the treatment for 24 hr, at which point cells and supernatants were harvested for analysis unless otherwise specified.

9. Real-Time Reverse-Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was isolated from the DLECs derived from normal and PE using a Trizol reagent (Invitrogen, Carlsbad, CA, USA). Power SYBR Green RNA-to-CTTM 1-Step kit (Applied Biosystems, Foster City, CA, USA) and StepOnePlusTM (Applied Biosystems) were used to measure the mRNA expression of human genes, according to the manufacturer's instructions. PCR conditions were as follows: 48℃ for 30 min and 95℃ for 10 min, followed by 40 cycles of 95℃ for 15 s and 55℃ for 1 min. The results were based on the cycle threshold (Ct) values. Differences between the Ct values for the experimental and reference (*GAPDH*) genes were calculated, and the results are expressed as the ratio of each RNA level to the calibrated sample. The primer sequences were as follows: CSF2, 5′-ATGGGGAAGGTGAAGGTCG-3′ (sense) and 5′-GGGGTCATTGATGGCAACAATA-3′ (antisense); GAPDH,

5′-AGCCACTACAAGCAGCAC-3′(sense) and 5′-GATGACAAGCAGAAA GTCCT-3′ (antisense).

10. Immunohistochemistry

Nuclear translocation of the p65 subunit of NF-κB was examined by an immunocytochemical method, as previously described [32]. Briefly, treated cells were fixed with 2% paraformaldehyde and permeabilized with 0.2% Triton X-100. After washing in phosphate-buffered saline, the slides were blocked with 3% bovine serum albumin for 1 hr, and the cells were incubated with goat polyclonal anti-p65 antibody (Santa Cruz Biotechnology) (1:100). After 2 hr at 4℃, the cells were washed and incubated with anti-goat IgG-rhodamine (Santa Cruz Biotechnology) (1:100) for 1 hr. Nuclei were stained with DAPI (Santa Cruz Biotechnology). The cells were then mounted with a mounting medium and observed under a fluorescence microscope (Olympus, Tokyo, Japan).

11. Western blotting

DLEC cell lysates were fractionated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. The blocked membranes were incubated with the appropriate antibody, and immunoreactive bands were visualized using a chemiluminescent reagent, as recommended by Amersham Biosciences, Inc. (GE Healthcare, Chicago, IL, USA).

12. Statistical analysis

All experiments were repeated at least three times. Data are presented as means ± standard error (S.E.), and statistical comparisons between groups were performed by one-way ANOVA, followed by Student's t test.

III. RESULTS

1. Establishment of the THP-1 differentiation protocol for polarization

Based on the literature, THP-1 cells were induced with 100 nM PMA for 72 hr to differentiate into M0 macrophages. M0 macrophages were then treated with LPS (20 ng/mL)/IFN-γ (20 ng/mL) for 48 hr to induce M1 macrophages and IL-4 (20 ng/mL)/IL-13 (20 ng/mL) for 48 hr to induce M2 macrophage polarization [33].

Phase-contrast microscopic images showed that there were significant morphological differences among M0, M1, and M2 macrophages. M0 macrophages were small round-shaped cells, while M1 macrophages displayed a spindle-like shape with pseudopods. Most M2 macrophages adopted a round shape with fewer pseudopods as compared with the M1 macrophages. The immunofluorescent images showed that M1 macrophage-specific markers, CD80 and HLA-DR, could be found in M1 macrophages. M2 specific markers, CD163 and CD209, could be found in M2 macrophages. **(Figure 1A)** Flow cytometric analysis confirmed that 65.2% of M1 polarized macrophages were double positive for HLA-DR and CD80. A 60.5% of M2 polarized macrophages were double positive for CD163 and CD209 **(Figure 1B).** These results indicated that the THP-1 differentiation protocol is suitable for these experiments.

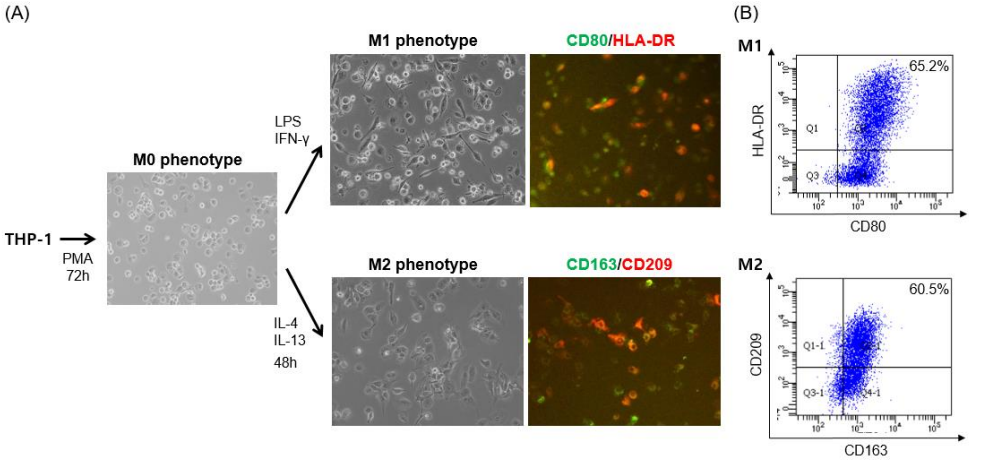

Figure 1. Optimization of the differentiation protocol for polarization of THP-1 monocytes into M0/M1/M2 macrophages by analyzing cell surface markers

(A) THP-1 differentiation protocol with phase contrast and fluorescent microscopy of THP-1 cells. After PMA stimulation of THP-1 cells (M0) for 72 hr, macrophages with M1 and M2 phenotypes were acquired from THP-1 cells, following the treatment with LPS/IFN-γ and IL-4/IL-13 for 48 hr, respectively. (B) Representative flow cytometry plots of M1 ($HLA\text{-}DR^{+}CD80^{+}$) and M2 ($CD163^{+}CD209^{+}$) macrophages. M1 macrophages showed high expression of HLA-DR and CD80. CD163 and CD209 were highly expressed on M2 macrophages.

2. PE-DLECs induce M1 macrophage polarization

To investigate the roles of DLECs in the polarization of resident macrophages, M0 differentiated THP-1 cells were cultured in DLEC conditioned medium or co-cultured with DLECs from normal and preeclamptic pregnancy.

To establish the impact of DLEC-produced soluble factors on resident macrophages in PE, CM was collected from normal and PE-DLECs, as described in the Materials and Methods section. The media was centrifuged to eliminate any remaining cell debris and frozen at −70°C until used. In all cases, CM was diluted with 50% fresh media before adding it to the M0 macrophages. M0 differentiated THP-1 cells were cultured in the normal and PE-DLEC CM for 48 hr. After harvesting, polarization markers were analyzed using HLA-DR, CD80, CD163, and CD209 **(Figure 2A)**. Flow cytometric results showed that the treatment with PE-DLEC CM increased the percentage of HLA-DR^{+}CD80^{+} (M1 phenotype) cells in total macrophages than normal. No significant differences were observed in CD163 and CD209 expression. The ratio of M1/M2 was significantly higher in the PE-DLEC CM than in the normal DLEC CM. **(Figure 2B)**

To examine the direct effects of PE-DLECs on the polarization of resident macrophage, M0 macrophages were cocultured with normal and PE-DLECs. Co-cultures (DLEC:M0, 2:1) were analyzed by flow cytometry for 24 hr to further confirm that PE-DLECs induced a M1 polarization **(Figure 2C)**. The results represented that a significant increase of HLA-DR and CD80 was detected on macrophages cocultured with PE-DLECs. No significant differences in the percentages of CD163^{+}CD209^{+} cells were detected between normal and PE-DLEC cocultures. The ratio of M1/M2 was also significantly higher in the PE-DLEC coculture group than in the normal DLEC coculture group. **(Figure 2D)**

These results indicated that the M1 polarization of resident macrophages might be facilitated by chemokines or cytokines secreted from PE-DLECs.

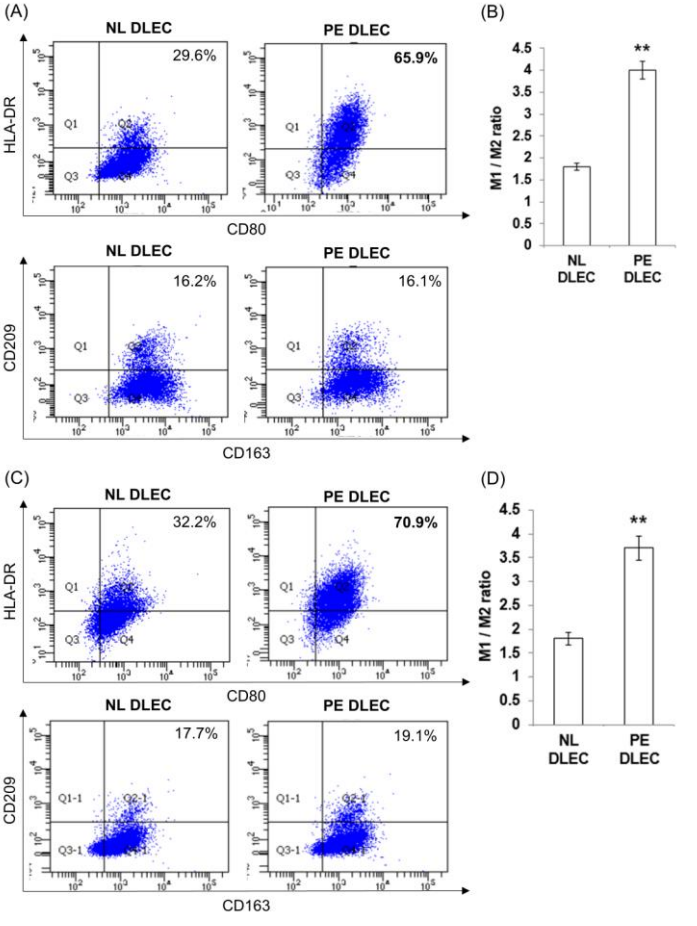

Figure 2. PE-DLECs induces M1 macrophage polarization

(A) Representative flow cytometry plots of M1 (HLA-DR^{+}CD80^{+}) and M2 (CD163^{+}CD209^{+}) macrophages cultured in normal and PE-DLEC CM. (B) The ratio of M1/M2 was markedly higher in PE-DLEC CM than normal DLEC CM. (C) Representative flow cytometry plots of M1 (HLA-DR^{+}CD80^{+}) and M2 (CD163^{+}CD209^{+}) macrophages cocultured with normal and PE-DLECs. M0 macrophages were predominantly polarized towards the M1 macrophage after coculture with PE-DLECs. (D) The M1/M2 ratio was higher in coculture with PE-DLECs. The values are presented as the mean ± SEM from at least three independent experiments. ** P <0.01.

3. GM-CSF production increased in PE-DLEC

To explore the mechanism under which PE-DLECs induce the polarization of macrophages toward the M1 phenotype, a cytokine microarray was performed to screen the PE-DLEC secreting factors associated with macrophage differentiation. Interestingly, microarray data reveals that PE-DLEC expressed significantly increased levels of GM-CSF gene in M1 polarization of macrophage **(Figure 3).**

As shown in **Figure 4**, the ELISA assay showed that the values of GM-CSF concentration in the supernatant of normal and PE-DLECs were increased in a time-dependent manner. In addition, a significant increase of GM-CSF was observed in PE-DLECs compared to normal DLECs. Therefore, these findings indicated that PE-DLECs could secret GM-CSF and possibly play a role in inducing the polarization of M1 macrophage.

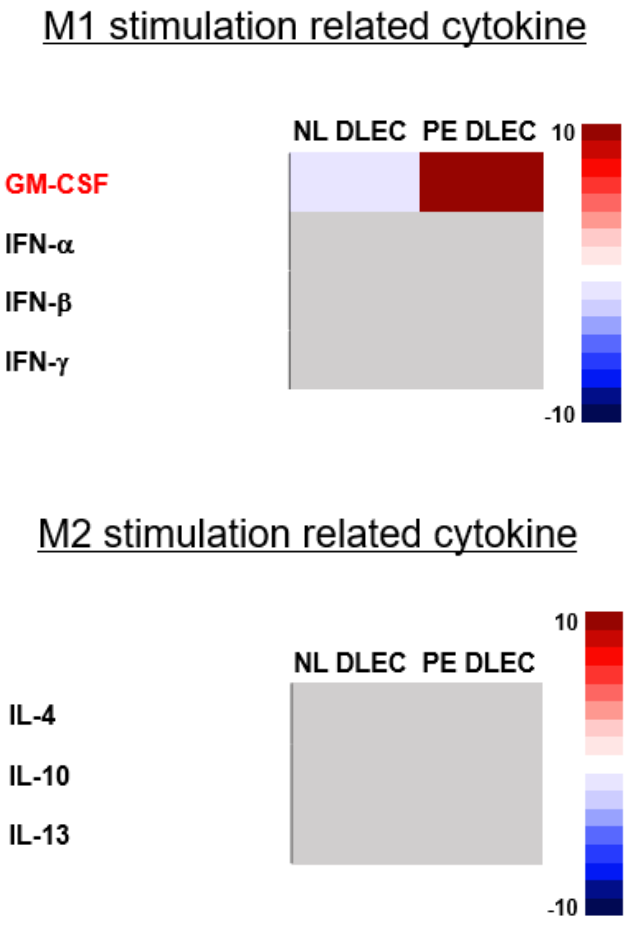

Figure 3. The aberrant upregulation of GM-CSF in PE-DLECs

Heatmap diagram summarizing cytokine expression in normal and PE-DLECs. Relative gene expression was depicted according to the color scale. The result showed that the expression of GM-CSF was significantly upregulated in PE-DLEC.

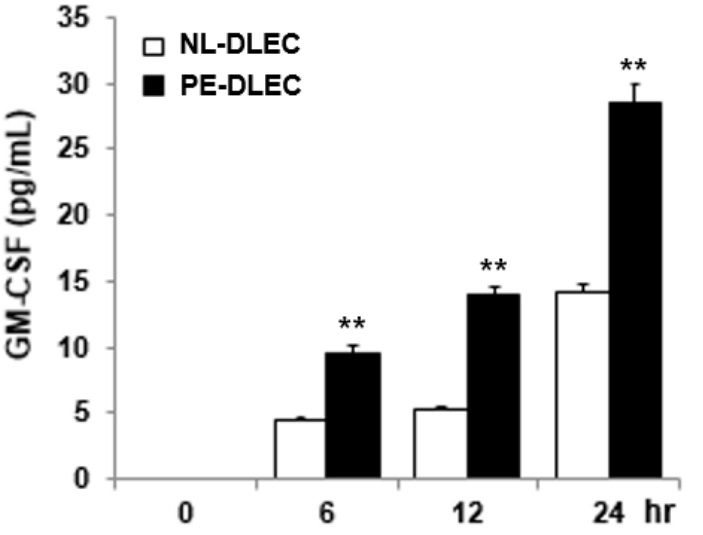

Figure 4. GM-CSF secretion increases in PE-DLECs

GM-CSF concentration in the supernatants of normal and PE-DLECs was determined by ELISA. The results showed that GM-CSF concentration in the PE-DLEC supernatants was significantly increased in a time-dependent manner. The results were based on three repeated experiments. The values are presented as the mean ± SEM. ** $P < 0.01$.

4. PE-DLEC derived GM-CSF may play a role in polarization of macrophage to the M1 phenotype

To confirm the role of GM-CSF by PE-DLECs in the M1 macrophage polarization, M0 macrophages were cultured with normal DLEC CM containing rhGM-CSF (0, 25, 50 ng/mL), and macrophages phenotype markers were analyzed using flow cytometry. The result showed that the expression of M1 macrophages markers were significantly increased in macrophages cultured in NL-DLEC CM with rhGM-CSF (44.4% vs. 32.2%). The percentage of M1 macrophages also increased with the concentration of rhGM-CSF in a dose-dependent manner. **(Figure 5A)**. In contrast, no significant difference in M2 polarization was observed between normal DLEC CM with or without rhGM-CSF. rhGM-CSF treatment with normal DLEC CM significantly increased the ratio of M1/M2 in M0 macrophages compared to normal DLEC CM alone **(Figure 5B).**

To further validate that GM-CSF regulates M1 macrophage polarization, GM-CSF expression in PE-DLECs was silenced by shRNA transfection, and shGM-CSF efficiently repressed GM-CSF in PE-DLECs by qRT-PCR **(Figure 6C)**. The result showed that the expression of M1 macrophage phenotype markers was significantly decreased when M0 macrophages cultured with CM from PE-DLECs with GM-CSF knockdown as compared to PE-DLECs (43.0% vs. 58.0%) **(Figure 6A).** PE-DLEC with GM-CSF knockdown significantly reduced the M1/M2 ratio in M0 macrophages compared to PE-DLEC CM alone **(Figure 6B).** These results confirmed that PE-DLECs induce M1 macrophage polarization by secreting GM-CSF.

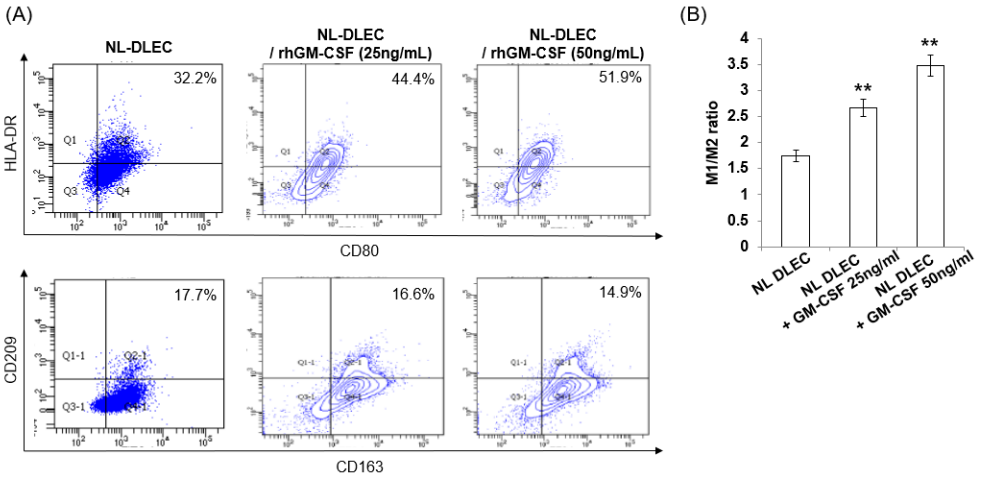

Figure 5. Effect of GM-CSF treatment on macrophage polarization in normal DLECs

(A) Representative flow cytometry plots of M1 (HLA-DR^{+}CD80^{+}) and M2 (CD163^{+}CD209^{+}) macrophages cultured in NL-DLECs CM with or without rhGM-CSF at 25 or 50 ng/mL. The rhGM-CSF treatment upregulated the expression of M1 macrophage phenotype markers. (B) Ratio of M1/M2 macrophages was calculated. rhGM-CSF treatment with normal DLEC CM increased the M1/M2 ratio in a dose-dependent manner. The results were based on three repeated experiments. All data are expressed as the mean ± SEM. ** P <0.01

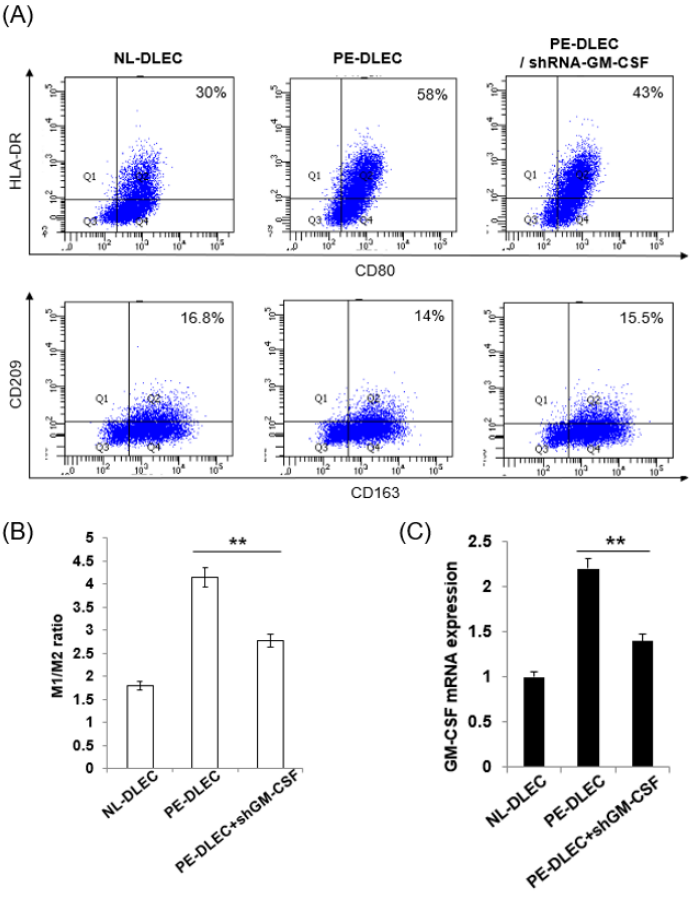

Figure 6. Effect of GM-CSF knockdown on macrophage polarization in PE-DLECs

(A) Representative flow cytometry plots of M1 (HLA-DR^{+}CD80^{+}) and M2 (CD163^{+}CD209^{+}) macrophages cultured in CM from NL-DLECs or PE-DLECs with or without GM-CSF knockdown. Knockdown of GM-CSF in PE-DLECs downregulated the expression of M1 macrophage phenotype markers. (B) Ratio of M1/M2 macrophages was calculated. Knockdown of GM-CSF in PE-DLECs decreased the M1/M2 ratio compared to PE-DLEC. (C) Quantification of real-time PCR analysis of GM-CSF mRNA levels in normal DLECs, PE-DLECs, and shGM-CSF PE-DLECs. The results were based on three repeated experiments. The values are presented as the mean ± SEM. ** P <0.01.

5. NF-κB dependent GM-CSF production in PE-DLEC

Previous studies have shown that NF-κB and ERK are key transcriptional regulators of GM-CSF gene expression [34]. However, studies on GM-CSF expression and related signaling pathways in preeclamptic DLEC remain unelucidated. Thus, it is supposed that NF-κB or ERK pathway may regulate the expression of GM-CSF in PE-DLECs. To clarify the mechanism of GM-CSF secretion in PE-DLECs, the effects of NF-κB and ERK inhibitors on GM-CSF secretion were observed in PE-DLECs. Normal and PE-DLECs were pretreated with or without 100 μM PDTC (NF-κB inhibitor) and 5 μM U0126 (ERK inhibitor) for 1 hr, and cultured for 24 hr. GM-CSF concentration was analyzed by ELISA. PDTC treatment markedly inhibited the release of GM-CSF from normal and PE-DLECs. In contrast, the U0126 treatment slightly increased the release of GM-CSF from normal and PE-DLECS, although not statistically significant. **(Figure 7A).** To confirm this inhibitory effect, NF-κB activity was analyzed using immunocytochemical localization of p65. Normal and PE-DLECs were treated with PDTC or U0126 for 1 hr, followed by immunofluorescence using an antibody for p65. p65 was translocated from cytoplasm to the nucleus in normal and PE-DLECs without pretreatment. In the control group, the localization of NF-κB p65 is mostly cytoplasmic. The nuclear translocation of the p65 subunit of NF-κB was significantly increased in PE-DLECs. **(Figure 7B).** The NF-κB nuclear translocation, which was also quantified based on the fluorescent intensity of the immunostainings, was induced in PE-DLEC and inhibited by PDTC **(Figure 7C)**. ERK activity was measured by western blotting with antibody against the phosphorylated form of ERK**.** As shown in **Figure 7D**, U0126 completely inhibited ERK activation in normal and PE-DLECs, whereas GM-CSF expression was slightly increased in both normal and PE-DLECs treated with U0126 **(Figure 7A)**.

These results indicated that the NF-κB pathway regulates the expression of GM-CSF in PE-DLECs.

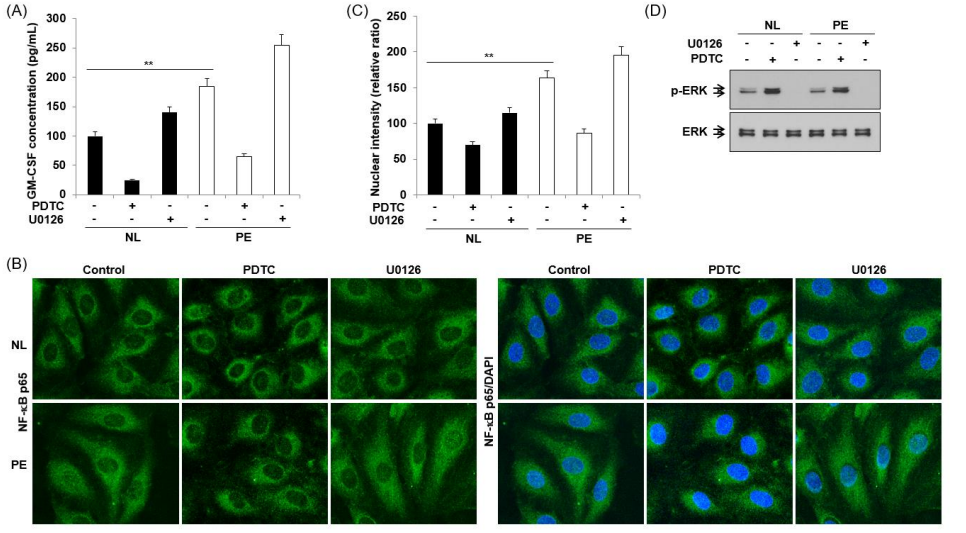

Figure 7. Inhibition of NF-κB decreases the production of GM-CSF in PE-DLECs

Normal and PE-DLECs were pretreated with or without 100 μM PTDC (NF-κB inhibitor) and 5 μM U0126 (ERK inhibitor) for 1 hr, and cultured for 24 hr. (A) Concentration of GM-CSF in supernatants were determined by ELISA. GM-CSF secretion was increased in PE-DLECs. In contrast, PDTC treatment inhibited the production of GM-CSF. (B) Immunocytochemical analysis of p65 localization. Green: p65 staining; blue: cell nuclei. (C) Fluorescence intensity of the NF-κB immunostaining in cell nuclei and cytoplasm. Results showed that p65 nuclear translocation was promoted in PE-DLECs, whereas PDTC treatment inhibited this process. (D) Representative western blotting image of phosphorylated ERK. The results were based on three repeated experiments. The values are presented as the mean ± SEM. ** $P < 0.01$.

6. Aspirin and folate reduce GM-CSF mRNA expression and release in PE DLEC

Aspirin and folate may play a protective role against PE through beneficial effects on immune modulation and vasculogenesis [35,36]. Generally, there is little information on the contributing role of aspirin and folate on the polarization of proinflammatory M1 phenotype in PE. Further studies are necessary to determine the effects of aspirin and folate on the production of GM-CSF and the polarization of resident macrophages in PE.

To determine the effect of aspirin and folate treatment on the GM-CSF expression in normal and PE-DLECs, normal and PE-DLECs were cultured in 60mm plates with various concentrations of aspirin (0, 3, 30, 300 μM) or folate (0, 20, 200, 2000 ng/mL) for 24 hr, at which point cells and supernatants were harvested for analysis. GM-CSF concentration in supernatants of DLECs was analyzed by ELISA, and GM-CSF transcription in DLECs was analyzed by qRT-PCR.

GM-CSF mRNA expression was increased in PE-DLECs than in normal DLECs. In normal DLECs, aspirin and folate treatment did not change GM-CSF mRNA expression. Interestingly, GM-CSF mRNA expression was decreased in PE-DLECs with aspirin or folate treatment. **(Figure 8**). GM-CSF concentration in the supernatants from PE-DLECs was also significantly increased than that in normal DLECs, and was reduced in a dose-dependent manner by treatment with aspirin (3-300 μM) (**Figure 9A**). GM-CSF release in aspirin-treated normal DLECs was decreased compared to normal DLECs without aspirin treatment, although not statistically significant. Folate treatment also reduced GM-CSF mRNA expression in PE-DLECs **(Figure 8**). Folate decreased GM-CSF secretion from PE-DLEC in a concentration-dependent manner **(Figure 9B).**

As a result, we confirmed that aspirin and folate have positive effects on reducing GM-CSF secretion of PE-DLEC.

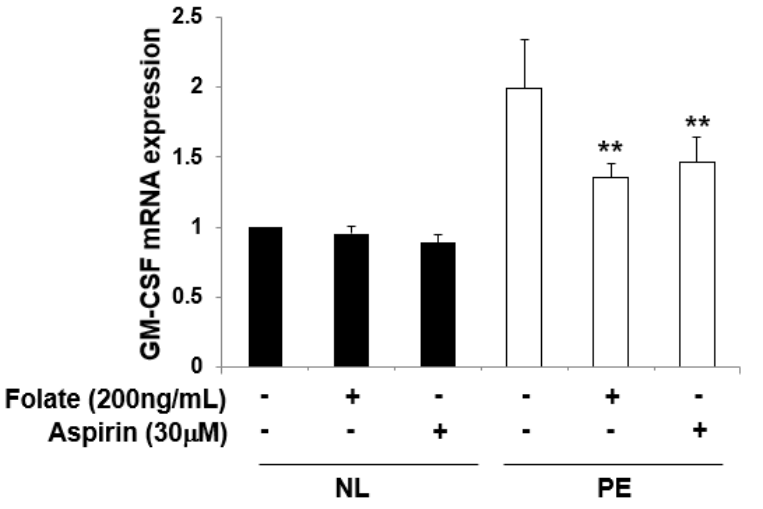

Figure 8. Effects of aspirin and folate on GM-CSF gene transcription in normal and PE-DLECs

Normal and PE-DLECs were treated with aspirin (30 μM) or folate (200 ng/mL) for 24 hr. The mRNA expression of GM-CSF was analyzed by qRT-PCR. GM-CSF mRNA expression was decreased in PE-DLECs with aspirin or folate treatment. The results are representative of three independent experiments and expressed as the mean ± SEM. ** $P < 0.01$.

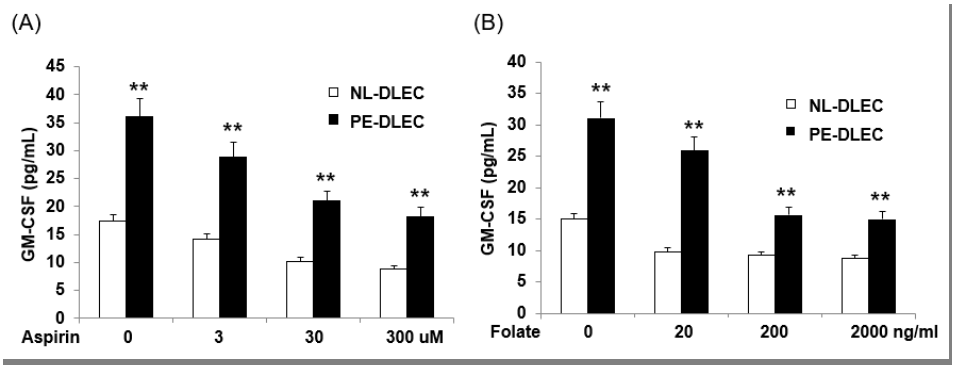

Figure 9. Effect of aspirin and folate on GM-CSF secretion from normal and PE-DLECs

(A) Concentration of GM-CSF released by normal and PE-DLECs with aspirin treatment for 24 hr (0-300uM). (B) Concentration of GM-CSF released by normal and PE-DLECs after folate treatment for 24 hr (0-2000 ng/mL). GM-CSF concentration in the supernatants of DLECs was analyzed by ELISA. Aspirin and folate treatment reduced GM-CSF secretion from PE-DLECs in a concentration-dependent manner than the control. The results are representative of three independent experiments and expressed as the mean ± SEM. ** P <0.01.

IV. DISCUSSION

Macrophages are found in the female reproductive system and are important in the maintenance of tissue homeostasis through the clearance of dead cells and the remodeling and repair tissue after inflammation [37]. The number of CD14-positive uterine macrophages immediately increases after fertilization, and 20–30% of all decidual leukocytes are macrophages in the first trimester of pregnancy [38]. Decidual macrophages can induce apoptosis of damaged cells generated during placentation and remove apoptotic cells before releasing their intracellular components, which are antigenically foreign to the maternal immunity and may initiate immunological reactions for the semi-allogenic fetus. Decidual macrophages show immunosuppressive characteristics induced by different cells at the maternal-fetal interface, including trophoblasts, decidual stromal cells, and other immune cells through various cytokines and immune checkpoints [39, 40]. Decidual macrophages are also key components in inducing immunosuppressive phenotype of decidual NK and T cells and support biological functions of trophoblasts and decidual stromal cells, which contributes to the maintenance of immune tolerance and successful pregnancy [41].

Emerging evidence suggests that adequate macrophage polarization at the maternal-fetal interface is important in the maintenance of pregnancy. During the first trimester, proinflammatory M1 macrophages are mainly distributed to inhibit infection and restore uterine epithelium. During the second and third trimester, anti-inflammatory M2 macrophages are predominantly distributed to maintain immune tolerance [11,15,42]. However, imbalanced decidual macrophage polarization can induce poor placentation, impaired trophoblast function, and lead to adverse pregnancy outcomes, including PE, intrauterine growth restriction, and miscarriage [12]. Previous studies reported that monocytes from PE women express significantly higher levels of the M1 markers, TLR4 and

CD64, while the expression of the M2 markers, CD163 and CD206, were significantly lower than the normal-term pregnant women [43-45]. Schonekeren, et al. showed that decreased numbers of M2 macrophages are present in the decidua of PE women [14]. Moreover, recent data showed that the counts of $CD14^{+}CD11c^{+}CD163^{-}$ M1 phenotype monocytes in the PE group were significantly increased, which correlated with the increased level of pro-inflammatory factors (IL-1, IL-6, and MCP-1) [46]. The shift in the balance of M2/M1 macrophages towards M1 is also explained by high levels of pro-inflammatory cytokines and low levels of anti-inflammatory cytokines within the preeclamptic placenta [47,48]. During PE, increased numbers of decidual macrophages are observed, which may be of the M1 phenotype and produce pro-inflammatory cytokines, including IFN-γ, TNF-a, and IL6 [9].

Although various factors may induce the regulation of M1 and M2 polarization during pregnancy, the molecular mechanisms of macrophage recruitment and polarization in the decidua are not fully understood. To date, a number of researchers have conducted studies on the interaction between trophoblasts and macrophages, which are mainly found in the uterus. Wang et al. reported that trophoblast-derived CXCL16 induced the polarization of M2 macrophage and the inactivation of NK cells at the maternal–fetal interface [49]. Zhang et al. reported that trophoblast-secreted soluble-PD-L1 modulates M2 macrophage polarization [50]. The crosstalk between trophoblasts and decidual immune cells via cell–cell direct interaction and soluble factors, such as chemokines and cytokines, contributes to the unique immunotolerant microenvironment [51].

However, no studies have been conducted on the effects of decidual lymphatic vessels on the macrophages, which move macrophages into the uterus and play a key role in immune tolerance. Lymphatic vasculature is essential to maintain immune tolerance through their roles in adequate antigen presentation, trafficking and migration, modulation of T cells, and induction of regulatory T cells [16-19]. Lymphatic endothelial cells have a high possibility of crosstalk with

resident macrophages, similar to trophoblast. In some reports, it is also known that M1 macrophages are differentiated into lymphatic endothelial cells by vascular endothelial growth factor (VEGF)-C and participate in lymphangiogenesis [52]. However, there is a few research on the effect of lymphatic endothelial cells on the differentiation of macrophages.

This study showed that DLECs from PE induce pro-inflammatory polarization of macrophages, suggesting that DLECs influence the polarization of resident macrophages. In particular, it is shown that PE-DLEC secretes granulocyte-macrophage colony-stimulating factor (GM-CSF) excessively, polarizing decidua macrophages to M1. GM-CSF is a cytokine that was initially described by its ability to induce colonies of granulocytes and macrophages from myeloid progenitor cells [53]. GM-CSF is known as a cytokine related to M1 differentiation, and its expression is regulated mainly through various signal transduction pathways such as NF-κB, ERK, MAPK, and STAT [54,55]. Until recently, as studies on the pathogenesis of PE were actively conducted, studies on the increase of GM-CSF in the placenta, serum, and decidual cells have been reported [56,57]. In this study, when the NF-κB inhibitor was administered, the secretion of GM-CSF from PE-DLEC was decreased, confirming that GM-CSF was regulated by the NF-κB pathway in DLEC. Within the last decade, scientists have demonstrated that the trophoblast/uterine NK cell/macrophage crosstalk is crucial for implantation and spiral arteries remodeling during normal pregnancy. In particular, NF-κB regulates this process through modification of cytokine expression, as well as cell phenotype and function. NF-κB also mediates production of proinflammatory cytokines by the uterine epithelium, which activates decidual macrophages, and regulates M1/M2 polarization of macrophages [58]. Based on these findings, this research demonstrated that PE-DLECs secrets GM-CSF through NF-κB pathway to induce proinflammatory macrophage polarization. These results experimentally suggest that crosstalk between LECs and macrophages can modulate immune

reaction, making it immunogenic or tolerogenic.

To date, PE is a disease that has not been overcome, but with the discovery of several immune mechanisms, researchers are looking for ways to suppress the incidence and exacerbation of the disease. Studies have been conducted using various substances to prevent PE, but there were no significant results except for low-dose aspirin [30]. Aspirin is believed to inhibit the generation of thromboxane A2 (TXA2) by platelets, increasing the prostacyclin/TXA2 ratio and reducing platelet aggregation, thereby inhibiting the development of PE [59], but this has not been clearly elucidated. Yin et al. reported that the anti-inflammatory properties of aspirin and salicylate are mediated in part by their specific inhibition of IKK-β, thereby preventing activation by NF-κB of genes involved in the pathogenesis of the inflammatory response [60]. Previous studies also reported that aspirin inhibited LPS-induced proinflammatory macrophage activation and lymphatic vessel remodeling via inhibiting IκK/IκB/NF-κB pathways [61]. Aspirin suppressed lymphatic vessel remodeling by inhibiting NF-κB/VCAM-1 pathway in human dermal LECs [26]. Considering these facts, aspirin is thought to be involved in the regulation of GM-CSF production by inhibiting the NF-κB pathway of PE-DLEC.

In addition to aspirin, epidemiological studies of the association between folate supplementation and the incidence of PE have shown a potential preventive effect, although findings have been inconsistent [62,63]. Based on the fact that folate is required for nucleotide synthesis, cellular methylation therefore modifies DNA synthesis, cell proliferation, and gene regulation [64]. Several proposed mechanisms of action have been proposed to explain that folate reduces the risk of developing PE. Briefly, folate is thought to help adequate placentation, to induce lower serum homocysteine levels, and to increase systemic endothelial function, reducing pregnancy complications such as PE [65]. Folate diminished the expression of proinflammatory cytokines of M1 macrophage and that is associated with NF-κB pathway in macrophage [27,66].

Also, folate inhibited endothelial cell function through cSrc/ERK2/NF-κB/p53 pathway mediated by folic acid receptor [67]. Although some studies have been conducted on the association of folate with macrophages and vascular endothelial cells, no studies have been conducted on LECs. In this study, similar to aspirin, folate inhibited GM-CSF secretion in PE-DLEC, which may be related to the NF-κB pathway. Therefore, further studies on the effect of folate on the NF-κB pathway related to GM-CSF expression are needed.

This thesis is the first study to investigate the effect of DLECs on macrophage polarization. It is difficult to obtain sufficient samples suitable for experiments in PE, and thus, studies on human DLECs during pregnancy are very limited so far. In addition, as a strength of this study, various concentrations were set in consideration of the maternal serum concentration of pregnant women during the pretreatment of aspirin and folate. In fact, when measuring the serum acetylsalicylic acid concentration of pregnant women who took aspirin for more than 4 weeks, the concentration of serum acetylsalicylic acid was measured at 2,417-3,214 ng/ml, up to a maximal concentration of 4,210 ng/mL [68,69]. The median concentration of aspirin was set to 30 μM (5,500 ng/mL), which is similar to the serum concentration level when taking a low dose of 100 mg aspirin. In addition, according to a previous report, there is a phenomenon that the cell viability of LECs decreases when aspirin exceeds 300 μM is administered [26]; therefore, the maximum concentration was set not to exceed 300 μM. According to the serum concentration set by the WHO, folate experimental treatments were selected to represent normal physiological concentration (20 ng/mL), elevated physiological concentration (200 ng/mL) and supraphysiological concentration (2,000 ng/mL) [70].

As a limitation of this study, relevant stimuli or factors affecting the activation of NF-κB have not been studied. Since NF-κB plays an important role in the interaction of various cells in decidua, a stimulus that induces activation of GM-CSF expression in decidua cells in contact with DLECs can be generated.

To date, studies on macrophage polarization and immune tolerance have been mainly focused on the relationship with trophoblast, uterine NK cells, and decidual stromal cells. Further studies on the interaction between DLEC and surrounding trophoblast, decidual stromal cells and several immune cells are important.

Furthermore, it is considered that additional studies are needed on the phosphorylation of signaling molecules and various stimuli activating NF-κB pathway. Contrary to inhibition of NF-κB, when ERK associated with GM-CSF expression in PE-DLEC was inhibited, a pattern of inversely increasing GM-CSF production was observed in this study. Previous study has shown that ERK has the potential to inhibit the canonical NF-κB pathway involving IKK-mediated IκBα phosphorylation in endothelial cells [32]. Considering this unexpected result, the possibility that the main mechanism regulating the expression of GM-CSF in PE-DLEC is the ERK pathway rather than the NF-κB cannot be excluded. Further studies are needed examining the reciprocal effects between NF-κB and ERK pathways in PE-DLECs.

V. CONCLUSION

GM-CSF induces proinflammatory M1 macrophage polarization through NF-κB pathway in PE-DLECs. Since aspirin and folate modulate GM-CSF release, the present data suggest aspirin and folate may mitigate the disease progression of preeclamptic pregnancy.

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ABSTRACT(IN KOREAN)

중증 자간전증의 탈락막 림프관 내피세포에서 NF-κB 기전을 통한 GM-CSF 의 M1 대식세포 분극화 유도

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목적: 모체-태아 접촉면에서 손상된 면역 관용과 그로 인한 과도한 염증 반응은 자간전증을 발생시키는 원인이다. 최근까지 많은 연구에서 탈락막 대식세포가 자간전증에서 주로 염증성의 M1 표현형으로 분극화되며, 이는 염증 반응을 매개한다고 제시하고 있다. 탈락막 대식세포는 태반 형성 과정에서 자궁 주변의 면역 세포들과 밀접하게 접촉하므로, 탈락막 림프관 내피세포도 상주 대식세포와 상호 작용할 가능성이 높다. 더불어, 자간전증에서 아스피린과 엽산이 면역 관용을 유지하는데 도움이 될 수 있으므로, 탈락막 내피세포의 분극화 과정에도 영향을 미칠 수 있다. 이에, 본 논문에서는 탈락막 내피세포가 정상과 자간전증에서 탈락막 대식세포의 분극화에 미치는 영향을 조사하고, 대식세포 분극화에 영향을 미칠 가능성이 있는 자간전증의 탈락막 림프관 내피세포에서 유래된 싸이토카인을 찾고, 아스피린과 엽산이 자간전증 탈락막 림프관 내피세포와 대식세포 분화의 상호작용에 미치는 영향을 평가하고자 한다.
대상과 방법: 10명의 자간전증 산모와 10명의 정상 산모군에서 획득한 탈락막에서 림프관 내피세포를 분리하였다. 정상과 자간전증 탈락막 림프관 내피세포가 대식세포 분극화에 미치는 영향을 분석하기 위하여, PMA 처리한 THP-1 세포를 정상 및 자간전증 탈락막 림프관 내피세포 또는 획득한 상층액를 이용하여 배양하였다. M1 및 M2 표현형은 유세포 분석을 시행하여 분석하였으며, 분비된

싸이토카인의 발현은 마이크로어레이 분석을 통해 확인하였다. GM-CSF 단백의 양과 발현량은 효소결합면역흡착 어세이(ELISA)와 역전사 중합효소 연쇄반응(RT-PCR)을 통해 분석하였다. ERK의 인산화와 NF-κB의 활성화는 웨스턴 블랏과 면역화학 염색으로 분석하여 평가하였다. 그리고 정상과 자간전증 탈락막 림프관 내피세포를 여러 농도의 아스피린과 엽산에 24시간 노출시킨 후 GM-CSF 단백의 양과 mRNA 발현을 분석하였다.

결과 : 자간전증 산모에서 획득한 탈락막 림프관 내피세포와 그 상층액을 이용하여 대식세포를 분화시킨 후 유세포 분석을 시행한 결과, 대식세포는 주로 M1 표현형으로 주로 분화되었다. 자간전증 탈락막 림프관 내피세포에서 GM-CSF 의 분비와 발현양이 증가되어 있었다. 정상 탈락막 림프관 내피세포에서 획득한 상층액에 rhGM-CSF 를 추가한 경우 M1 표현형의 대식세포 분극화가 촉진되며, 반면에 전자간증 탈락막 림프관 내피세포를 shRNA를 이용하여 GM-CSF 발현을 억제한 경우 M1 표현형의 대식세포 분극화가 감소되었다. NF-κB 억제제인 PDTC 를 전자간증 탈락막 림프관 내피세포에 처리한 경우, NF-κB 기전이 억제되어 GM-CSF 발현이 감소됨을 확인할 수 있었고, 이 결과 NF-κB/GM-CSF 신호 경로가 탈락막의 M1 대식세포 분극화를 조율할 수 있을 것으로 보인다. 더불어, 아스피린과 엽산을 정상과 자간전증 탈락막 림프관 내피세포에 농도별로 전처리한 결과, GM-CSF의 전사와 분비가 용량 의존적으로 유의하게 감소하였다.

결론 : 자간전증의 림프관 내피세포에서 NF-κB 경로를 통해 유래된 GM-CSF 는 M1 표현형의 대식세포 분극화를 유도한다. 아스피린과 엽산은 자간전증 탈락막 림프관 내피세포에서 GM-CSF 의 분비를 조절할 수 있으므로, 자간전증의 진행을 완화시킬 수 있는 효과를 기대할 수 있다.

핵심되는 말 : 임신, 림프관 내피 세포, 대식세포 분극화, 자간전증