Received: 19 March 2022 | Revised: 1 August 2022 | Accepted: 8 August 2022

DOI: 10.1111/aji.13611

AJRI American Journal of Reproductive Immunology WILEY

ORIGINAL ARTICLE

Macrophage polarization in placenta accreta and macrophage-trophoblast interactions

Jia-hui Che[1] | Zi-meng Zheng[1,2] | Ming-qing Li[1,2,3] | Xiaoying Yao[1]

[1]Institute of Obstetrics and Gynecology, Obstetrics and Gynecology Hospital of Fudan University, Shanghai, China

[2]NHC Key Lab of Reproduction Regulation (Shanghai Institute for Biomedical and Pharmaceutical Technologies), Obstetrics and Gynecology Hospital of Fudan University, Fudan University, Shanghai, China

[3]Shanghai Key Laboratory of Female Reproductive Endocrine Related Diseases, Obstetrics and Gynecology Hospital of Fudan University, Fudan University, Shanghai, China

Correspondence
Xiaoying-Yao and Ming-Qing Li, Institute of Obstetrics and Gynecology, Obstetrics and Gynecology Hospital of Fudan University, Fudan University, No.1326, Pingliang Road, Shanghai 200080, China.
Email: 13916738653@139.com and mqli@fudan.edu.cn

Funding information
Program of Shanghai Municipal Health Commission, Grant/Award Number: 201540361; Shanghai Municipal Health Bureau

Abstract

Problem: Placenta accreta (PA) is defined by an abnormal invasion of placental trophoblasts into the myometrium, which can lead to serious postpartum complications. Macrophages play an important role in the regulation of trophoblast function. Both granulocyte colony-stimulating factor (G-CSF) and its receptor (granulocyte colony-stimulating factor receptor, G-CSFR) have effects on trophoblast invasion. However, the current understanding of G-CSF secretion, G-CSFR expression, abnormal polarization of decidual macrophages (dMϕ) in PA and the abnormal invasion of placental trophoblasts into the myometrium are limited.

Method of Study: The polarization of dMϕ in PA was analyzed by flow cytometry (FCM), and the expression of G-CSFR in placental trophoblasts in PA was evaluated by immunohistochemistry. In an in vitro co-culture model, we investigated the effects of HTR-8/SVneo trophoblasts cell line (HTR-8) on macrophage human monocyte cell line (THP-1) polarization and G-CSF secretion, and we also analyzed the effects of THP-1 cells, especially M2-like subtype, on primary trophoblasts and HTR-8 proliferation, invasion, and adhesion. FCM, transwell assays, adhesion assays, and proliferation assays were used in the above model.

Results: Compared with controls ($n = 9$), dMϕ showed significantly lower levels of M1 markers CD80 and CD86 and higher levels of the M2 markers CD163 and CD206, and G-CSFR expression of placental trophoblasts was increased in PA ($n = 5$). In vitro experiments showed that the trophoblast HTR-8 cell line induced polarization of THP-1 cells to an M2-like subtype and increased their secretion of G-CSF. Furthermore, IL-4/IL-13-induced M2-like THP-1 macrophages were able to increase the expression of G-CSFR, proliferation, invasion and adhesion of both primary trophoblasts and HTR-8 trophoblasts.

Conclusions: There is an altered immune imbalance at the maternal-fetal interface in PA, which further may lead to abnormal trophoblast function. G-CSF and its receptors may play important roles in abnormal polarization of macrophages and abnormal invasion of trophoblasts.

KEYWORDS
G-CSF, G-CSFR, macrophage polarization, placenta accreta, trophoblasts

1 | INTRODUCTION

Placenta accreta (PA) refers to a group of disorders in which placental trophoblasts aberrantly invade the myometrium to varying degrees,[1] and cause postpartum hemorrhage, shock, and even lead to hysterectomy. Its incidence is increasing, ranging between 0.01% and 1% of livebirths,[2] along with an increase in high-risk factors such as a history of previous cesarean section, multiple abortions, and a history of myomectomy. Such incidences seriously endanger the safety of the mother and the fetus.

Macrophages are the second-largest immune cell population at the maternal-fetal interface, accounting for 20%–30% of leukocytes,[3,4] and they maintain the local immune microenvironment by secreting cytokines that regulate trophoblast invasion and placental development.[5] Macrophage polarization is the process by which macrophages adopt distinct functional phenotypes by responding to specific microenvironmental stimuli and signals.[6,7] Although not proven in humans, the polarization pattern of decidual macrophages (dMϕs) varies with the gestational age. In the peri-implantation period, macrophages polarize into M1 phenotype.[8] When trophoblasts invade the uterine stroma, dMϕs begin to convert to a mixed M1/M2 polarization pattern, which is maintained until mid-pregnancy. After placental development is completed, dMϕ shift towards a predominantly M2-subtype phenotype to sustain maternal-fetal tolerance during pregnancy.[9] At term delivery, the number of M1 dMϕ increases to participate in the onset of term delivery.[10,11] The balance of macrophage polarization is important for various processes in normal pregnancy, such as trophoblast invasion, spiral artery remodeling and apoptotic cell phagocytosis.[12] Some studies have shown abnormal macrophage polarization is associated with multiple pathological pregnancies, such as preterm labor, gestational hypertensive disorders, and recurrent spontaneous abortion.[11] However, the abnormal polarization and possible mechanisms of macrophages in PA are largely unknown.

Some studies have shown that macrophages interact with trophoblasts through cytokines by the maternal-fetal interface.[3,13] Macrophages regulate the behavior of trophoblasts by secreting various soluble mediators. Simultaneously, various factors produced by trophoblasts can alter the polarization status of macrophages, thereby regulating the function of the macrophages.[3,14–16] Placental implantation depends on the balance between the aggressiveness of the chorionic tissue and the "protection" of the decidual cells. Once there is defective decidualization or a decidua injury, villi can significantly invade the myometrium and lead to placental implantation.[17,18]

Granulocyte colony-stimulating factor (G-CSF) belongs to the colony-stimulating factor (CSF) family and can be synthesized by macrophages, fibroblasts, and endothelial cells.[19] G-CSF can bind its receptors (granulocyte colony-stimulating factor receptor, G-CSFR),[20] and further affect trophoblast invasion,[21–23] but the specific mechanism needs further investigation. In this study, we investigated the polarization of decidual macrophages and the expression of G-CSFR in placental trophoblasts in full-term pregnant patients with placental implantation, observed the effect of abnormally polarized macrophages on trophoblast function, and further performed a preliminary investigation of the immune mechanism associated with PA.

2 | MATERIALS AND METHODS

2.1 | Human and tissue samples

Maternal decidua and placentae were obtained from pregnant women with term delivery by cesarean section. Participants were assigned into the PA ($n = 5$) or control ($n = 9$) group, according to whether PA occurred during caesarean section. The indications for elective cesarean section included: breech presentation ($n = 1$), previous cesarean delivery ($n = 7$), placenta previa ($n = 1$) and cephalopelvic disproportion ($n = 5$). The exclusion criteria were the following: pregnant women with complications and/or comorbidities of pregnancy (including hypertensive disorders of pregnancy, diabetes, thyroid disorders, chorioamnionitis, etc.), fetal growth restriction, intrauterine fetal death, or abnormalities other than PA found by placental pathology for various reasons. The placentae were fixed in 4% paraformaldehyde for paraffin-embedding in blocks and the deciduae were placed in the RPIM-1640 medium (Gibco, USA) to isolate human dMϕ within one hour after caesarean section. The deciduae were collected from the basal plate of the placenta and separated from placentae based on anatomical and histological features. Villous tissues ($n = 15$) were derived from elective termination of pregnancy in the first trimester (gestational age 6—8 weeks) for no medical reason in the first trimester. Villus tissues were stored in ice-cold Dulbecco's modified Eagle's medium (DMEM high D-glucose; Gibco) and transported to the laboratory within 30 min after surgery. This protocol of study was approved by the Ethical Committee of Obstetrics and Gynecology Hospital of Fudan University. All participants signed a written informed consent form.

2.2 | Cell culture and reagent

Then decidual tissues taken back to the lab were washed with phosphoric acid buffer saline (PBS) to remove blood clots and cut into small pieces. Minced decidual tissues were incubated in 30 ml of RPMI 1640 containing type IV collagenase (Worthington Biochemical Corporation, USA), hyaluronidase (Sigma, USA), and Dnase I (Roche, Germany) for 30 min at 37°C with shaking. After filtering with 100-, 200-, 400-mesh sieves, the filtrate was washed with PBS and the centrifuged at 1300 bpm for 5 min. The collected cells were analyzed by flow cytometry after discarding the supernatant.

The villous tissues were Isolated from the trophoblast according to the procedure described above.[24,25] The obtained villous tissues were mixed by 0.25% (wt/vol) tryspin and 0.02% (wt/vol) DNase type I (Sigma) for 5 min at 37°C with gentle agitation, followed by four cycles of 10-min digestion. Filter the trypsin-treated cell suspension through sterile gauze (pore diameter sizes 100, 300, and 400 mesh) and centrifuge the filtered suspension at 400 × g for 10 min. After discarding

the supernatant, the cell pellet was suspended in DMEM containing high D-glucose and carefully layered over discontinuous Percoll gradients (50%–20% in 10% steps), And centrifuge at 800 × *g* for 20 min. Cells pelleted at a density between 1.048 and 1.062 were collected and washed with DMEM supplemented with 20% (vol/vol) heat-inactivated FBS. This method resulted in a 92% purity in trophoblast cells.

All cell lines came from Laboratory for Reproductive Immunology, Hospital of Obstetrics and Gynecology, Fudan University Shanghai Medical College. The trophoblast cell line, HTR-8/SVneo (HTR-8) was cultured and grown in DMEM-F12 medium (Gibco, USA) with 10% fetal bovine serum (FBS) (Gibco, USA), and the human monocyte cell line (THP-1), was in RPMI-1640 medium with 10% FBS, at 37°C in the presence of 5% CO_2. The THP-1 cells were induced to M0 macrophages with Phorbol 12-myristate 13-acetate (PMA; Sigma, USA) (100 ng/ml) for 24 h. Afterwards, to obtain M1 or M2 macrophages, M0 macrophages were treated with lipopolysaccharides (LPS; Sigma, USA) (100 ng/ml)/interferon-γ (IFN-γ; PeproTech, USA) (20 ng/ml) or interleukin-4 (IL-4; PeproTech, USA) (20 ng/ml)/Interleukin-13(IL-13; PeproTech, USA) (20 ng/ml) for 48 h.[26] To Direct co-culture different subtypes of THP-1 cells with HTR-8, 4 × 10^5 THP-1 cells were firstly put in 6-well plates in RPMI 1640 medium supplemented with 10% FBS. After induced with PMA and cytokines mentioned above, different subtypes of THP-1 cells were culture with HTR-8 or primary trophoblasts at a 1:1 ratio.

2.3 | Flow cytometry (FCM)

To evaluate the expression of cell surface and intracellular markers, flow cytometric analysis was used. The following antibodies were used for the FCM analysis: phycoerythrin (PE)- and Cy7-conjugated anti-human CD206 (BioLegend, USA), allophycocyanin (APC)-conjugated anti-human CD80 (BioLegend, USA), fluorescein isothiocyanate (FITC)-conjugated anti-human CD14 (BioLegend, USA), PE-anti-human CD86 (BioLegend, USA), Brilliant Violet 421-conjugated antihuman CD163 (BioLegend, USA), APC-anti-human CD45 (BioLegend, USA), Brilliant Violet 605-conjugated antihuman Ki-67 (BioLegend, USA), Alexae-Fluor® 488-conjugated anti-human matrix metalloprotein-9(MMP-9, Abacm, USA), APC-anti-human CD61 (integrin β3, ITGB3, BioLegend, USA), PE-anti-human CD54 (Intercellular adhesion molecule-1, ICAM-1, BioLegend, USA), PerCP/Cyanine5.5 anti-human CD114 (G-CSFR, BioLegend, USA), PE-anti-human HLA-G (BioLegend, USA), (PE)- and Cy7-conjugated anti-human HLA-G (BioLegend, USA). Cells were collected by digesting with trypsin, washed with PBS twice and then stained with antibodies for 30 min at 4°C. After incubation, cells were washed twice and resuspended in 100 µl PBS. For intracellular staining, cells were fixed and permeabilized using the Fix/Perm kit (BioLegend, USA). FCM was performed on a Beckman-Coulter CyAn ADP cytometer and Data was analyzed using Flowjo software (Tree Star, USA). All experiments were performed in triplicate.

2.4 | Transwell invasion assay

According to previous procedure,[27–29] Transwell (8 µm, Corning, USA) migration assay was performed to estimate the invasion ability of the HTR-8. Matrigel (BD Biosciences, USA) was diluted with serum-free RPMI-1640 medium at a ratio of 1:8. 50 µl of the mixture was smeared onto the upper chamber and incubated overnight at 4°C. 3×10^4 HTR-8 were suspended in 50 µl RPMI-1640 medium without serum and seeded on the upper chambers, while the lower chambers were seeded 1 × 10^5 THP-1-M1or M2, added 200µl RPMI-1640 medium with 10% FBS. After co-culture for 48 h at 37°C, the cells were fixed with 4% paraformaldehyde and stained with 1% crystal violet (Sigma, USA). The invading cell numbers were counted in five random fields under ×200 magnification. All experiments were performed in triplicate.

2.5 | Immunohistochemistry (IHC)

The placenta tissues of all participants were extracted, and the expression of G-CSFR was detected by IHC. IHC was completed as described above.[30] Anti-G-CSFR (Bioss, China) antibody was 1:150 diluted. IHC staining intensity and percentage of positive cells were assessed according to the following criteria. Staining intensity: 1 = weak staining, 2 = moderate staining, 3 = strong staining. Percentage of stained cells: 0 = 0%–5%, 1 = 6%–25%, 2 = 26%–50%, 3 = 51%–75%, 4 = 75%–100%. The final score is obtained by multiplying the staining intensity and percent scores. All scores were independently performed by two persons, and five visual fields under optical microscope (200×) were randomly selected for evaluation.

2.6 | Adhesion assay

Non-contact co-culture transwell system (6 walls, 0.4µm, Corning, USA) was used to facilitate the acquisition of HTR-8 directly after co-culture. 1.0 × 10^5 THP-1 cells were seeded in upper inserts and induced to THP-1-M1/M2 as described previously.[27] After induction, the upper chamber was replaced with new 800µl culture medium and 2 × 10^5 HTR-8 were seeded in the lower chamber and co-cultured for 48 h. After co-culture, HTR-8 were digested with trypsin. Then HTR-8 were seeded in 24-well plates, in which 5000 cells per well were suspended with 500 µl of culture medium. The culture medium and unadhered cells were aspirated at selected time (30 min, 1 h and 2 h) and then observed under a microscope. The adhesive cell numbers were counted in five random fields under ×100 magnification. All experiments were performed in triplicate.

2.7 | Enzyme-linked immunosorbent assay (ELISA)

To evaluate the concentration of G-CSF in the supernatant of co-culture HTR-8 with THP-1-M0 for 24h and 48h, ELISA kits

TABLE 1 Comparison of clinical characteristics between PA and control group of participants

	PA ($n = 5$)	CON ($n = 9$)	p
Age	30.17 ± 5.04	33.90 ± 3.38	0.096
Gravidity	2.50 ± 2.43	1.70 ± 0.95	0.361
Parity	0.33 ± 0.52	0.40 ± 0.52	0.806
Termination time (week)	37.68 ± 0.96	38.53 ± 1.50	0.238
Newborn weight (g)	3353.33 ± 338.72	3133.00 ± 434.18	0.348
Postpartum hemorrhage (ml)	566.67 ± 350.24	235.00 ± 74.72	0.011*

Data are represented as the mean ± SD; Statistical significance (*t*-test): * $p < 0.05$; PA, placenta accreta; CON, control.

(Absin, China) were used following the manufacturer's instructions. All experiments were performed in triplicate.

2.8 | Cell proliferation assay

Cell proliferation assays were performed with the Cell Counting Kit-8 (CCK-8; Dojindo, Japan) assay according to the manufacturer's recommendations. After non-contact co-culture, HTR-8 were seeded into 96-well plates at a density of 5×10^4 cells with 100 μl medium per well, and CCK-8 solution was added (10 μl/well). The plates were incubated at 37°C for 2 h. The absorbance value was measured at 450 nm using an ELISA plate reader (Biotek, USA). All groups were evaluated with a minimum of three separate wells per experiment. All experiments were performed in triplicate.

2.9 | Statistics analysis

All analyses were carried out using SPSS 25 (SPSS Statistics, USA) and GraphPad Prism 8 software (GraphPad, USA). A t-test was used to analyze continuous variables that were normally distributed, and the results were expressed as means ± SD or SEM. A non-parametric Mann-Whitney *U* test was used to analyze continuous variables that were not normally distributed, and the results were presented as the median and interquartile range. $P < 0.05$ was considered statistically significant.

3 | RESULTS

3.1 | Clinical characteristics of participants

Basic participants' characteristics of PA and control groups were shown in Table 1. Compared to patients without PA, patients with PA had more postpartum bleeding (566.67 ± 350.24 vs. 235.00 ± 74.72, $p < 0.05$). There were no significant differences in age, termination time, gravidity, parity and newborn weight. And the indications for elective cesarean section between PA and control group of participants were shown in Table 2.

3.2 | Polarization of dMϕ and expression of placental trophoblast G-CSFR in normal pregnancy and PA

dMϕ phenotypic markers were analyzed via flow cytometry. Compared with control group, there were significantly lower levels of M1 markers CD80 and CD86 and higher levels of the M2 markers CD163 and CD206 in PA group (Figure 1A,B), suggesting that macrophages in deciduae of patients with PA are more polarized to M2 subtype.

Immunohistochemistry analysis showed that expression of G-CSFR in placental trophoblasts of PA group was higher than that of control group (Figure 1C), with a strong staining. The staining score of the placenta accreta group was 8.28 ± 2.07, while the control group was 3.08 ± 2.10 (Figure 1D). Therefore, we observed there was a statistically significant difference between the two groups ($p < 0.05$).

3.3 | HTR-8 cell induces M2 polarization of THP-1 macrophages and increases G-CSF secretion

Compared with the control group, the phenotype of THP-1-M0, after co-culture with HTR-8 cells for 48h, showed significantly lower levels of M1 markers CD80 and CD86 and higher levels of the M2 markers CD163 and CD206 (Figure 2A,B). Besides, the concentration of G-CSF in direct co-culture model of THP-1-M0 and HTR-8 cells increased significantly, at both 24 and 48 h (Figure 2C).

3.4 | IL-4/IL-13-induced M2-like THP-1 macrophages increase expression of G-CSFR and promote invasion, proliferation, and adhesion of HTR-8 trophoblast cells and primary trophoblasts

Flow cytometry showed that co-culture with IL-4/IL-13-induced M2-like THP-1 macrophages (THP-1-M2) could significantly increase the G-CSFR level in HTR-8 cells and primary trophoblasts (Figure 3A and B).

CCK8 assays and the expression of Ki-67 were performed to determine the effects of THP-1-M2 cells on trophoblast proliferation. As shown in Figure 3D and E, the expression of Ki-67 markedly increased

TABLE 2 Indications for elective cesarean section between PA and control group of participants

	PA ($N = 5$)	CON ($N = 9$)
Breech presentation	/	1(11.1)
Previous cesarean delivery	2(40.0)	5(55.6)
Placenta previa	1(20.0)	/
Cephalopelvic disproportion	2(40.0)	3(33.3)

Data are represented as the *n* (%); PA, placenta accreta; CON, control.

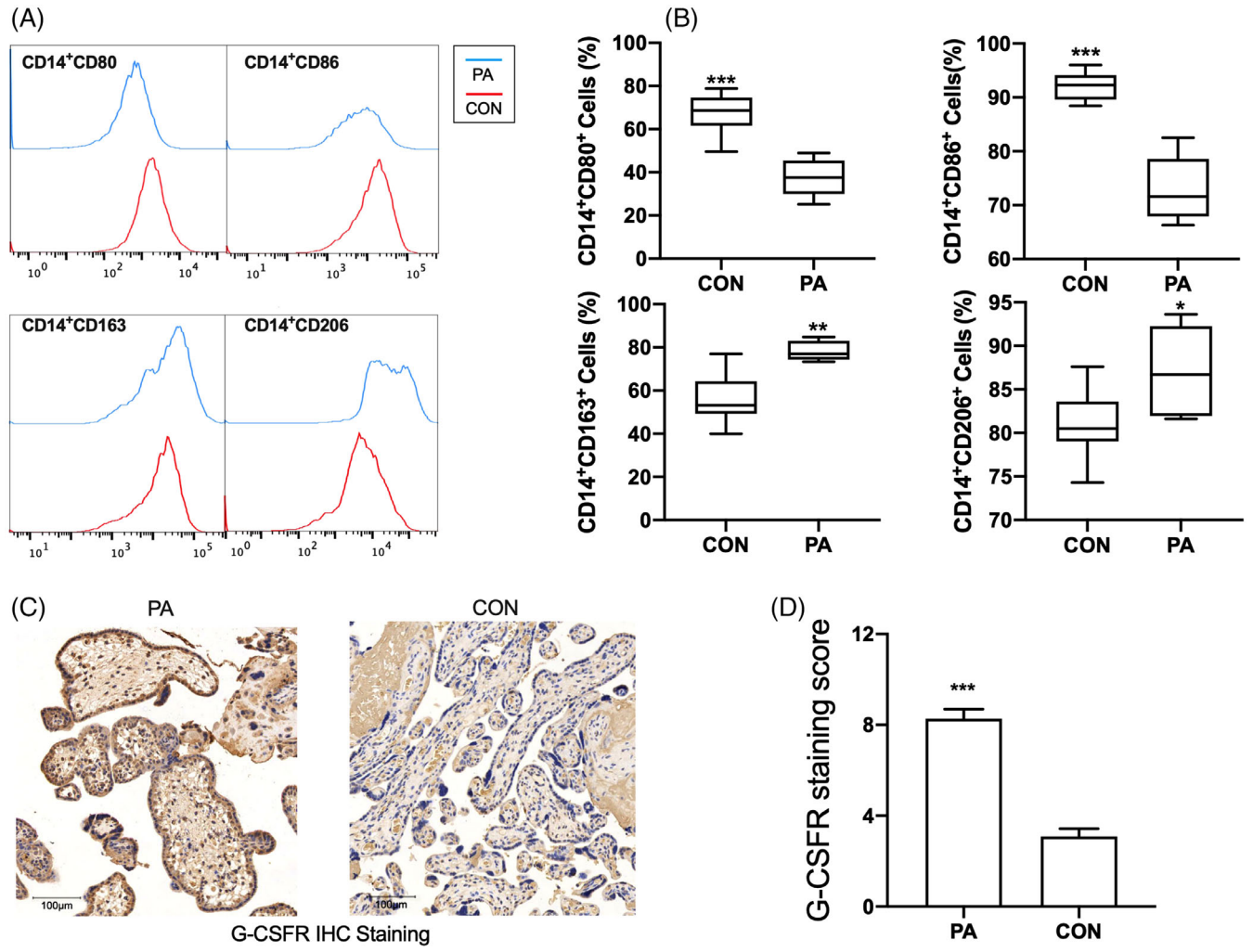

FIGURE 1 Decidual macrophages polarize towards the M2 subtype in placenta accreta patients and G-CSFR expression is increased in placental trophoblasts. A and B, The expression of CD80, CD86, CD163 and CD206 in decidual macrophages in control and PA group by FCM. The data are expressed as the median, interquartile range, and range (minimum and maximum). C, Expression of G-CSFR in placental trophoblasts in control and PA group by IFC, scale, 100μm. D, Staining score of G-CSFR in placental trophoblasts in control and PA group by IFC. The data are expressed as the mean ± SEM. Statistical significance (*t* test or Mann-Whitney *U* test): * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. PA, placenta accreta; CON, control.

in the HTR-8 cells and primary trophoblasts after direct co-culture with THP-1-M2 cells. The CCK8 analysis also showed that THP-1-M2 cells induced the proliferation of HTR-8 cells compared with LPS/IFN-γ-induced M1-like THP-1 macrophages (THP-1-M1) group or HTR-8 cell alone (Figure 3C).

To examine whether THP-1-M2 cells could directly promote adhesion of HTR-8 cell, adhesion assays were performed. It was apparent from Figure 4C that the adhesion ability of trophoblast HTR-8 increased after co-cultured with THP-1-M2 cells. The expression of ICAM1 and ITGB3 in the HTR-8 cells and primary trophoblasts,

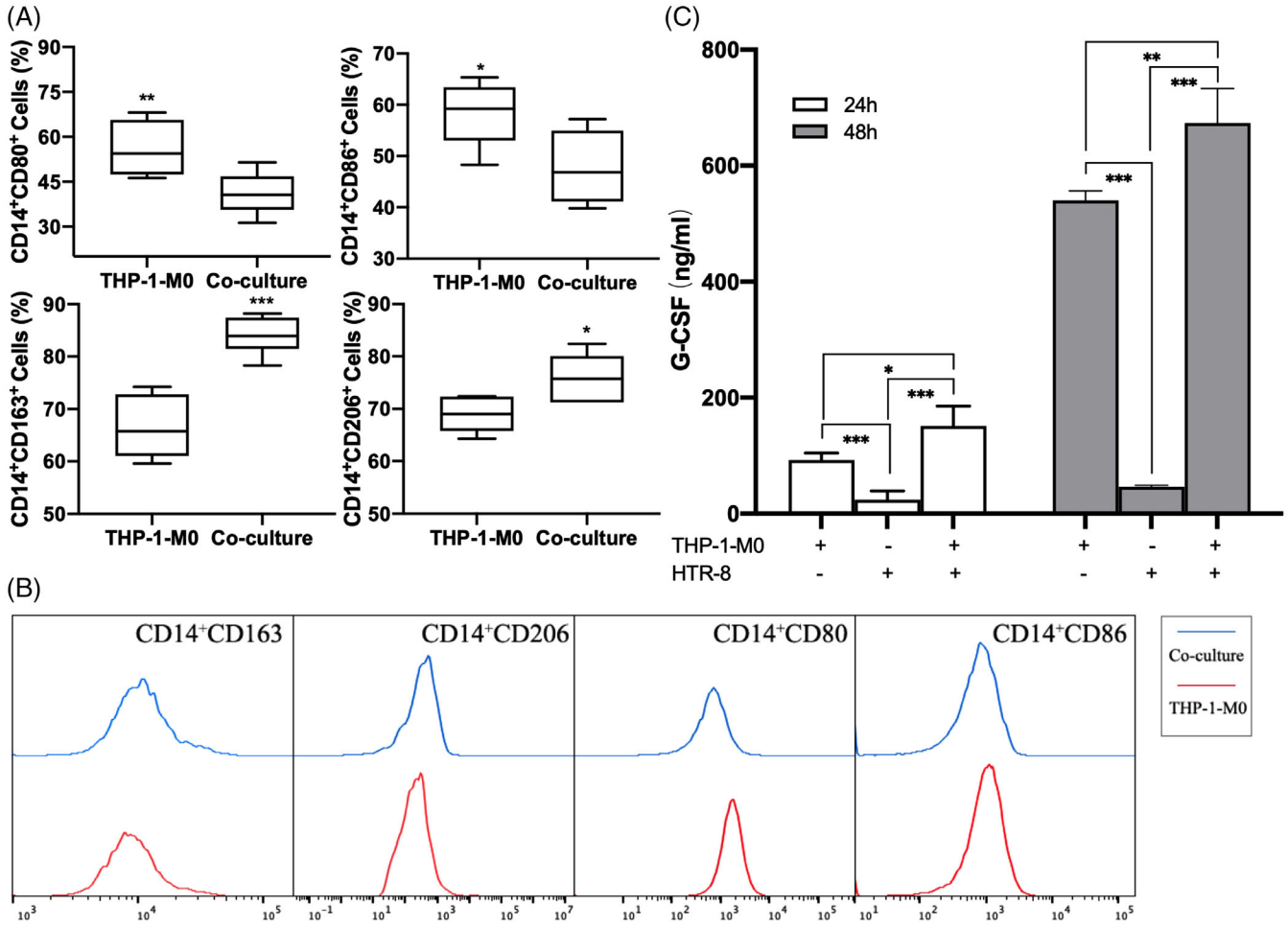

FIGURE 2 THP-1 cells polarize towards the M2 subtype and increase G-CSF secretion after co-culture with HTR-8 trophoblast cells. A and B, Changes in the expression of CD80, CD86, CD163 and CD206 before and after co-culture with HTR-8 in THP-1-M0 by FCM. The data are expressed as the median, interquartile range, and range (minimum and maximum). C, Secretion of G-CSF (24 and 48 h) in THP-1-M0 alone, HTR-8 alone and THP-1-M0-co-cultured HTR-8 by ELISA. The data are expressed as the mean ± SD. Statistical significance (*t* test or Mann-Whitney *U* test): * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. G-CSF, colony-stimulating factor.

two transmembrane glycoproteins associated with adhesion capacity, extremely increased (Figure 4A, B, D and E).

To confirm whether THP-1-M2 cells directly induced the invasion of HTR-8, transwell invasion assays were performed. The results showed that HTR-8 co-cultured with THP-1-M2 cells exhibited a more invasion cell count (Figure 5C,D). Moreover, THP-1-M2 cells increase the MMP-9 expression in HTR-8 cells and primary trophoblasts (Figure 5A,B). The results suggested that THP-1-M2 cells could distinctly promote the invasion of HTR-8 cells and primary trophoblasts.

4 | DISCUSSION

Macrophages, especially M2 subtype macrophages, may play a key role in PA. dMϕ contribute to trophoblast remodeling and invasion of spiral arteries.[31,32] Jonathan et al. found high macrophage infiltration at the uteroplacental interface in PA, and a similar pattern of immune cell infiltration in the decidua and placenta in patients with PA.[17] Zhou et al. showed that uterine injuries can lead to a decrease in the number of macrophages and an increase in the proportion of M2 subtype macrophages in the decidua and placenta of mice during the third trimester of pregnancy,[33] indicating that M2 macrophages may be one of the key factors in uterine injury leading to PA. During pregnancy, when trophoblasts attach to the endometrial endometrium and invade the uterine stroma, the proportion of M2 subtype dMϕ begins to rise to promote maternal immune tolerance to fetuses and trophoblast invasion.[9,34] M1 dMϕ predominates over the M2 subset again during the period of parturition. These accumulated M1 macrophages contribute to the uterus contraction, baby expulsion, placenta ejection and uterine involution.[35] Therefore, excessive polarization of macrophages toward the M2 subtype may lead to abnormal invasion of trophoblasts into the myometrium in the third trimester.

A CSF family includes macrophage CSF (M-CSF), granulocyte macrophage CSF (GM-CSF), and G-CSF.[36] Miyama et al. have confirmed that dMϕ are the source of G-CSF,[23] and G-CSF can

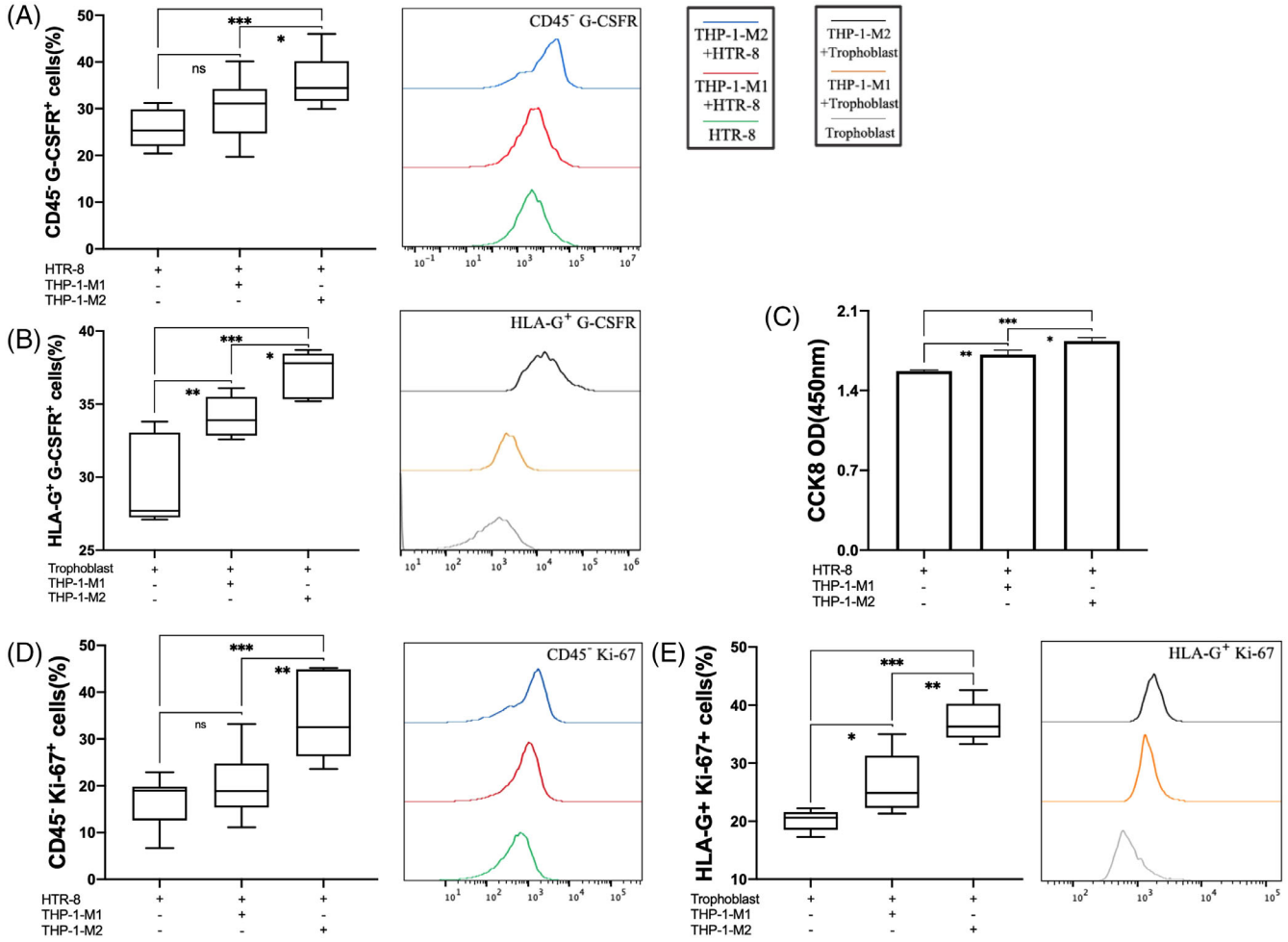

FIGURE 3 THP-1-M2 cells can increase the expression of G-CSFR in HTR-8 trophoblast cells and primary trophoblasts, and can increase the proliferation of HTR-8 and primary trophoblasts. A and D, Expression of G-CSFR and Ki-67 in HTR-8 alone, THP-1-M1/M2-co-cultured HTR-8 by FCM. The data are expressed as the median, interquartile range, and range (minimum and maximum). B and E, Expression of G-CSFR and Ki-67 in primary trophoblasts alone, THP-1-M1/M2-co-cultured primary trophoblasts by FCM. The data are expressed as the median, interquartile range, and range (minimum and maximum). C, Cell proliferation in HTR-8 alone, THP-1-M1 or M2-co-cultured HTR-8 with CCK-8 assays. The data are expressed as the mean± SD. Statistical significance (*t* test or Mann-Whitney *U* test): * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. G-CSFR, granulocyte colony-stimulating factor receptor; CCK-8, Cell Counting Kit-8.

promote the differentiation of macrophages to an anti-inflammatory phenotype.[37,38] It has been shown that G-CSF increases the expression of adhesion receptors and induce migration of endothelial cells.[39,40] Several studies have shown that G-CSF affects embryo implantation, trophoblast migration, angiogenesis, VEGF secretion, endometrial thickness and blood supply.[21,22,41,42] Neoangiogenesis is one of the pathological features of PA, and G-CSF can promote endothelial progenitor cell mobilization and angiogenesis by stimulating VEGF secretion.[43] Treatment with anti-VEGF (Bevacizumab) with anti-G-CSF mAb reduced tumor growth and angiogenesis more effectively than that after anti-VEGF monotherapy.[44] Thus, an increase in the number of blood vessels and the abnormal invasion of trophoblasts in PA may be related to the abnormal secretion of G-CSF. On the other hand, G-CSFR can promote the growth of trophoblasts.[23] In this study, we found that G-CSFR expression increased in placental trophoblasts in patients with PA in late pregnancy, but the mechanism needs to be further studied. Several studies have shown that trophoblast-derived M-CSF was involved in the differentiation and regulation of dMϕ to facilitate the induction of maternal-fetal immune tolerance.[16,27,45] In addition, trophoblasts can produce GM-CSF in early pregnancy and participate in regulating the growth, proliferation and differentiation of primary trophectoderm.[46–48] However, future studies are needed to clarify the regulatory roles of M-CSF and GM-CSF in the interaction between macrophages and trophoblasts in the third trimester of pregnancy.

Excessive invasion of trophoblasts is an important sign of PA. MMP-9 was significantly upregulated in implanted placenta samples,[49] and in vitro experiments also demonstrated that the MMP family positively regulates trophoblast invasion.[49] Integrins are heterodimeric cell surface glycoproteins associated with the cell-matrix or cell adhesion.

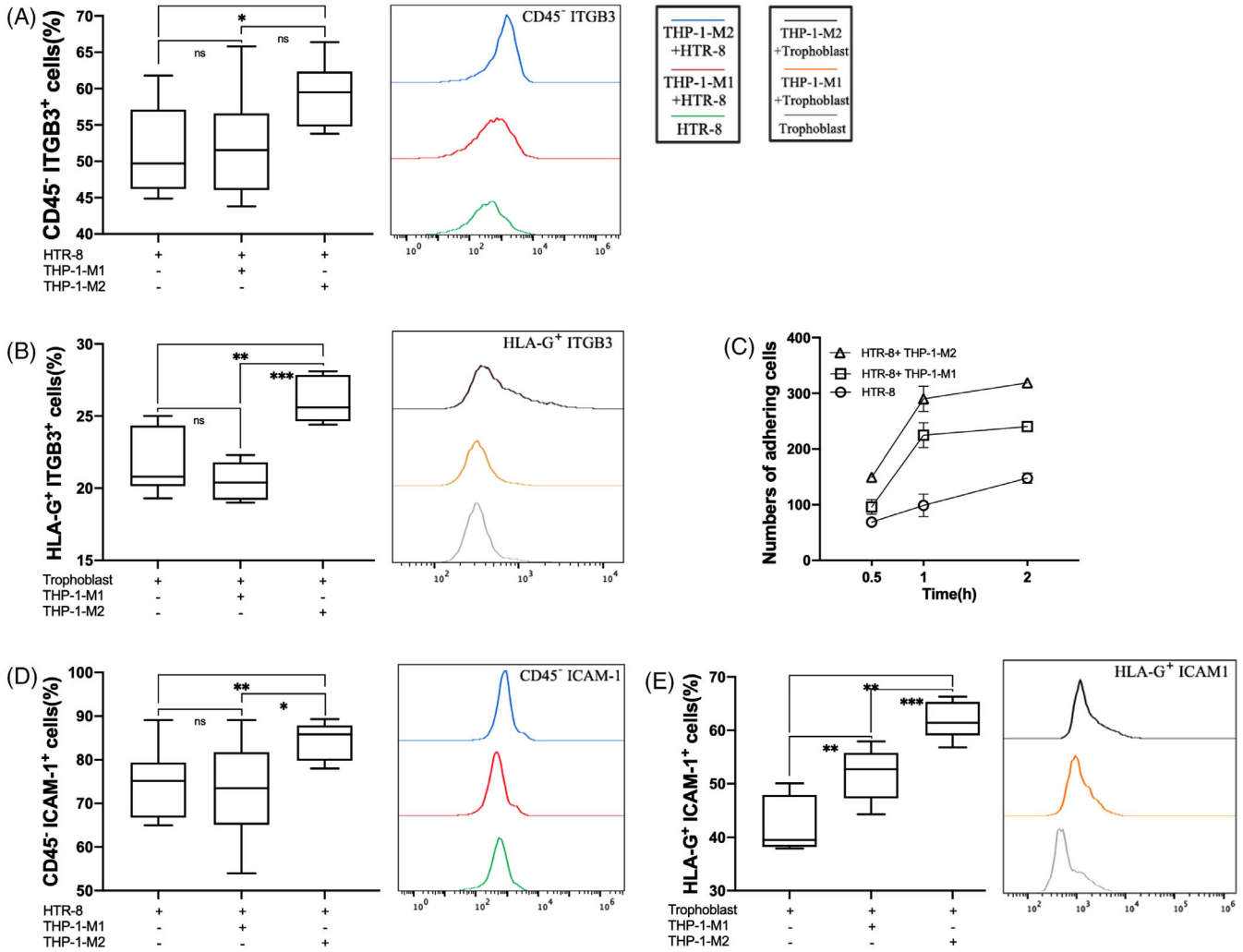

FIGURE 4 THP-1-M2 cells can increase the adhesion of HTR-8 cells and primary trophoblasts. A and D, Expression of ITGB3 and ICAM-1 in HTR-8 alone, THP-1-M1/M2-co-cultured HTR-8 by FCM. The data are expressed as the median, interquartile range, and range (minimum and maximum). B and E, Expression of ITGB3 and ICAM-1 in primary trophoblasts, THP-1-M1/M2-co-cultured primary trophoblasts by FCM. The data are expressed as the median, interquartile range, and range (minimum and maximum). C, Cell adhesion in HTR-8 alone, THP-1-M1 or M2-co-cultured HTR-8 in adhesion assay, the number of adhering cells was statistically different among the groups at each time period. The data are expressed as the mean± SD. Statistical significance (*t* test or Mann-Whitney *U* test): * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. ITGB3, integrin $\beta 3$; ICAM-1, Intercellular adhesion molecule-1.

Trophoblasts express ITGB3, a vitronectin receptor that mediates trophoblast migration and invasion.[50] The results of a study by Chen *et al.* showed that in preeclampsia, affecting the expression of FOXO1 can enhance the expression of ITGB3 in trophoblasts, thereby regulating cell adhesion and migration.[51] ICAM-1, a member of the immunoglobulin superfamily of proteins, mediates cell-to-cell adhesion and plays an important role in trophoblast migration.[52] Immunohistochemical studies have shown that ICAM-1 is highly expressed in adherent placenta.[53] In this study, we observed the increase in the adhesion ability of trophoblasts after co-culture with M2 macrophages, indicating upregulation of cell adhesion-related genes at the maternal-fetal interface in PA. Ki-67 is a marker of proliferation.[54] Multiple studies have found overexpression of Ki-67 in the placenta of patients with diabetes, maternal anemia, and hypertensive disorders of pregnancy.[55–57] However, whether the proliferation ability of trophoblasts in patients with PA is enhanced needs to be verified by conducting studies with animal models.

Our study showed that dMϕ in patients with PA exhibited M2 polarization, and G-CSFR is highly expressed in placental trophoblasts. In vitro studies have shown that, HTR-8 cells can induce the polarization of THP-1cells to the M2 subtype and increase the secretion of G-CSF by THP-1cells. Moreover, THP-1-M2 cells can increase the expression of G-CSFR in trophoblasts and enhance their ability to adhere, proliferate, and invade. The above findings suggest that there could be a regulatory mechanism for the interactions between macrophages and trophoblasts during PA (Figure 6). Abnormal trophoblasts might induce macrophage polarization to the M2 subtype and secrete cytokines such as G-CSF. The cytokines secreted by the M2 subtype macrophages

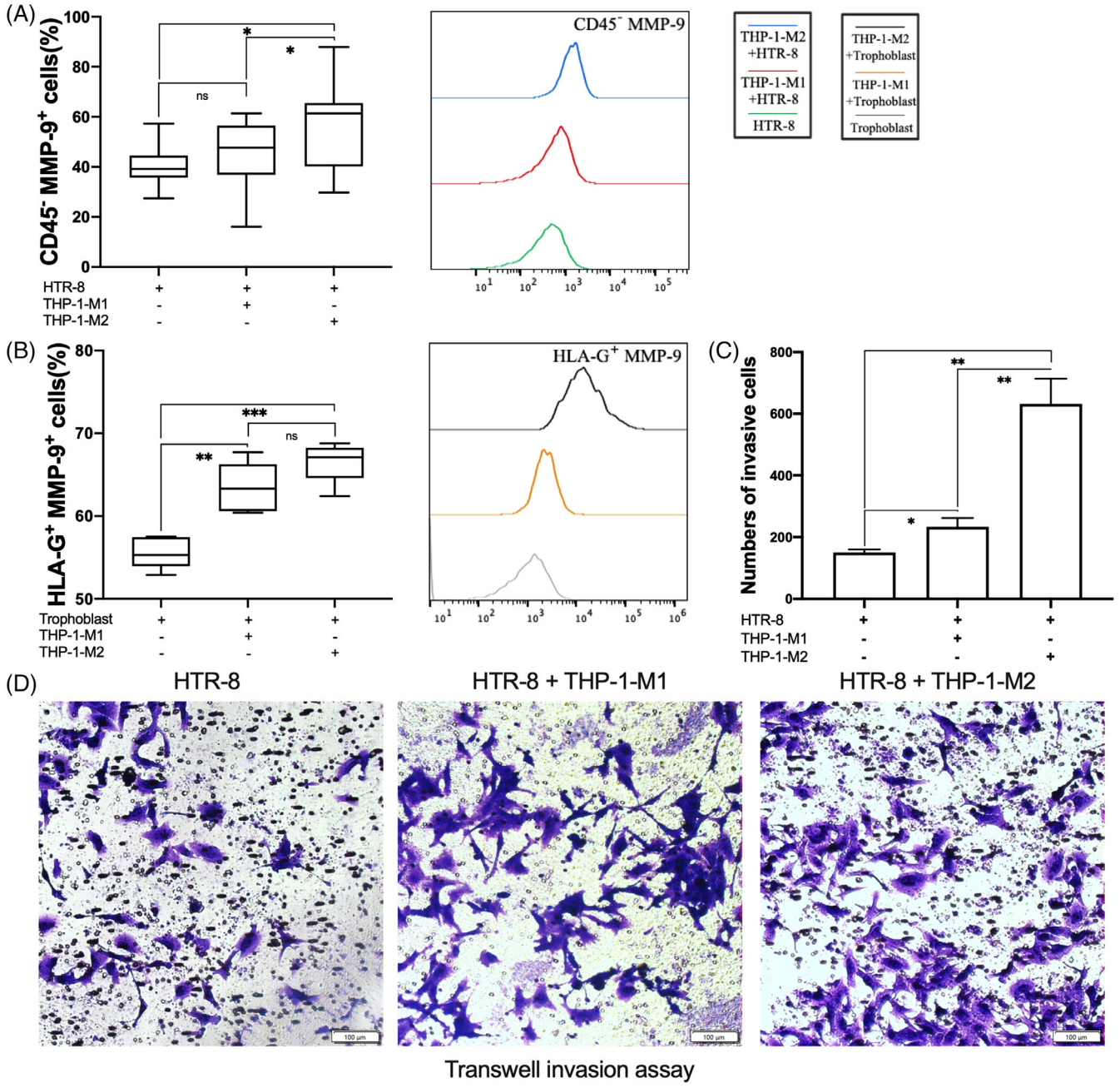

FIGURE 5 THP-1-M2 cells can increase the invasion of HTR-8 trophoblast cells and primary trophoblasts. A, Expression of MMP-9 in HTR-8 alone, THP-1-M1/M2-co-cultured HTR-8 by FCM. The data are expressed as the median, interquartile range, and range (minimum and maximum). B, Expression of MMP-9 in trophoblasts alone, THP-1-M1/M2-co-cultured primary trophoblasts by FCM. The data are expressed as the median, interquartile range, and range (minimum and maximum). C and D, Cell invasion in HTR-8 alone, THP-1-M1 or M2-co-cultured HTR-8 were determined in transwell invasion assays, magnification, ×200. The data are expressed as the mean± SD. Statistical significance (*t* test or Mann-Whitney *U* test): * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. MMP-9, human matrix metalloprotein-9.

may alter the expression of membrane proteins, such as G-CSFR in trophoblasts, and enhance their ability to adhere, proliferate, and invade. This may, in turn, promote invasion of trophoblasts into the myometrium, eventually causing PA. However, there are certain limitations in this study. For example, because of the altered properties of cell lines compared to primary cells, some of the findings involving cell lines cannot be translated to human cells. Additionally, these results should be verified by term placenta trophoblast cells and in vivo models. The sequence and specific mechanism of abnormal polarization of decidual macrophages and abnormal function of placental trophoblasts at the initial stage of PA are not known. Moreover, whether abnormal PA starts during the implantation window, or after the formation of the placenta is undetermined. These aspects need to be further studied.

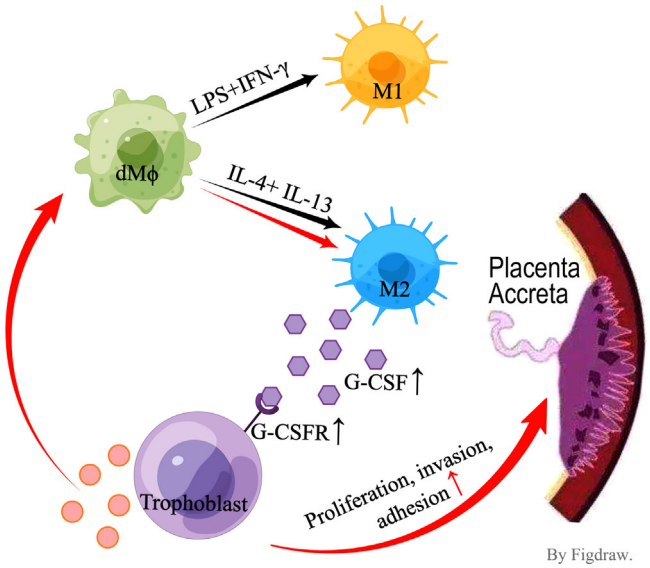

FIGURE 6 Regulatory mechanism for the interactions between macrophages and trophoblasts during PA. Abnormal trophoblasts induce macrophage polarization to the M2 subtype and secretion of cytokines such as G-CSF during pregnancy. Cytokines secreted by M2 macrophages up-regulate the expression of membrane proteins such as G-CSFR in trophoblasts, and further enhance their ability to adhere, proliferate, and invade, and promote trophoblast invasion into the myometrium, ultimately leading to PA.

5 | CONCLUSION

Our current study has revealed abnormal polarization of dMϕ and abnormal expression of G-CSFR in trophoblasts in PA. In addition, HTR-8 cells can induce polarization of THP-1 cells to the M2 subtype and increase G-CSF secretion by THP-1 cells, and THP-1-M2 cells have an important role in increasing the expression of G-CSFR and the adhesion, proliferation, invasion ability of HTR-8 cells. Therefore, immune imbalance may contribute to abnormal trophoblast function at the maternal-fetal interface in PA.

ACKNOWLEDGMENTS

We would like to thank Prof. Jiang-Feng Ye from Institute for Molecular and Cell Biology, Agency for Science, Technology and Research, Singapore for editing this manuscript, and Laboratory for Reproductive Immunology, Hospital of Obstetrics and Gynecology, Fudan University Shanghai Medical College for the gift of HTR-8/SVneo and THP-1 cells. This study was supported by the Program of Shanghai Municipal Health Commission (No. 201540361).

CONFLICT OF INTEREST

The author reports no conflicts of interest in this work.

DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available within the article.

ORCID

Jia-hui Che https://orcid.org/0000-0001-9249-3102
Ming-qing Li https://orcid.org/0000-0002-9276-0722

REFERENCES

1. Afshar Y, Dong J, Zhao P, et al. Circulating trophoblast cell clusters for early detection of placenta accreta spectrum disorders. *Nat Commun*. 2021;12(1):4408. https://doi.org/10.1038/s41467-021-24627-2
2. Jauniaux E, Bunce C, Grønbeck L, Langhoff-Roos J. Prevalence and main outcomes of placenta accreta spectrum: a systematic review and meta-analysis. *Am J Obstet Gynecol*. 2019;221(3):208-218. https://doi.org/10.1016/j.ajog.2019.01.233
3. Bulmer JN, Williams PJ, Lash GE. Immune cells in the placental bed. *Int J Dev Biol*. 2010;54(2-3):281-294. https://doi.org/10.1387/ijdb.082763jb
4. Williams PJ, Searle RF, Robson SC, Innes BA, Bulmer JN. Decidual leucocyte populations in early to late gestation normal human pregnancy. *J Reprod Immunol*. 2009;82(1):24-31. https://doi.org/10.1016/j.jri.2009.08.001
5. Wang XH, Liu W, Fan DX, et al. IL-33 restricts invasion and adhesion of trophoblast cell line JEG3 by downregulation of integrin $\alpha 4\beta 1$ and CD62L. *Mol Med Rep*. 2017;16(4):3887-3893. https://doi.org/10.3892/mmr.2017.7085
6. Murray PJ. Macrophage Polarization. *Annu Rev Physiol*. 2017;79:541-566. https://doi.org/10.1146/annurev-physiol-022516-034339
7. Sica A, Mantovani A. Macrophage plasticity and polarization: in vivo veritas. *J Clin Invest*. 2012;122(3):787-795. https://doi.org/10.1172/jci59643
8. Jaiswal MK, Mallers TM, Larsen B, et al. V-ATPase upregulation during early pregnancy: a possible link to establishment of an inflammatory response during preimplantation period of pregnancy. *Reproduction*. 2012;143(5):713-725. https://doi.org/10.1530/rep-12-0036
9. Svensson J, Jenmalm MC, Matussek A, Geffers R, Berg G, Ernerudh J. Macrophages at the fetal-maternal interface express markers of alternative activation and are induced by M-CSF and IL-10. *J Immunol*. 2011;187(7):3671-3682. https://doi.org/10.4049/jimmunol.1100130
10. Xu Y, Romero R, Miller D, et al. An M1-like macrophage polarization in decidual tissue during spontaneous preterm labor that is attenuated by rosiglitazone treatment. *J Immunol*. 2016;196(6):2476-2491. https://doi.org/10.4049/jimmunol.1502055
11. Yao Y, Xu XH, Jin L. Macrophage polarization in physiological and pathological pregnancy. *Front Immunol*. 2019;10:792. https://doi.org/10.3389/fimmu.2019.00792
12. Jóźwik M, Okungbowa OE, Lipska A, et al. Surface antigen expression on peripheral blood monocytes in women with gynecologic malignancies. *BMC Cancer*. 2015;15:129. https://doi.org/10.1186/s12885-015-1136-x
13. Houser BL. Decidual macrophages and their roles at the maternal-fetal interface. *Yale J Biol Med*. 2012;85(1):105-118.
14. Tian FJ, Qin CM, Li XC, et al. Decreased stathmin-1 expression inhibits trophoblast proliferation and invasion and is associated with recurrent miscarriage. *Am J Pathol*. 2015;185(10):2709-2721. https://doi.org/10.1016/j.ajpath.2015.06.010
15. Ding J, Yin T, Yan N, Cheng Y, Yang J. FasL on decidual macrophages mediates trophoblast apoptosis: a potential cause of recurrent miscarriage. *Int J Mol Med*. 2019;43(6):2376-2386. https://doi.org/10.3892/ijmm.2019.4146
16. Svensson-Arvelund J, Mehta RB, Lindau R, et al. The human fetal placenta promotes tolerance against the semiallogeneic fetus by inducing regulatory T cells and homeostatic M2 macrophages. *J Immunol*. 2015;194(4):1534-1544. https://doi.org/10.4049/jimmunol.1401536
17. Hecht JL, Karumanchi SA, Shainker SA. Immune cell infiltrate at the utero-placental interface is altered in placenta accreta spectrum

disorders. *Arch Gynecol Obstet*. 2020;301(2):499-507. https://doi.org/10.1007/s00404-020-05453-1
18. Illsley NP, DaSilva-Arnold SC, Zamudio S, Alvarez M, Al-Khan A. Trophoblast invasion: lessons from abnormally invasive placenta (placenta accreta). *Placenta*. 2020;102:61-66. https://doi.org/10.1016/j.placenta.2020.01.004
19. Würfel W. Treatment with granulocyte colony-stimulating factor in patients with repetitive implantation failures and/or recurrent spontaneous abortions. *J Reprod Immunol*. 2015;108:123-135. https://doi.org/10.1016/j.jri.2015.01.010
20. Fukunaga R, Ishizaka-Ikeda E, Seto Y, Nagata S. Expression cloning of a receptor for murine granulocyte colony-stimulating factor. *Cell*. 1990;61(2):341-350. https://doi.org/10.1016/0092-8674(90)90814-u
21. Furmento VA, Marino J, Blank VC, Roguin LP. The granulocyte colony-stimulating factor (G-CSF) upregulates metalloproteinase-2 and VEGF through PI3K/Akt and Erk1/2 activation in human trophoblast Swan 71 cells. *Placenta*. 2014;35(11):937-946. https://doi.org/10.1016/j.placenta.2014.09.003
22. Marino VJ, Roguin LP. The granulocyte colony stimulating factor (G-CSF) activates Jak/STAT and MAPK pathways in a trophoblastic cell line. *J Cell Biochem*. 2008;103(5):1512-1523. https://doi.org/10.1002/jcb.21542
23. Miyama M, Umesaki N, Kawabata M. Identification of the granulocyte colony-stimulating factor (G-CSF) producing cell population in human decidua and its biological action on trophoblast cell. *Osaka City Med J*. 1998;44(1):85-96.
24. Li MQ, Hou XF, Shao J, Tang CL, Li DJ. The DSCs-expressed CD82 controls the invasiveness of trophoblast cells via integrin-beta1/MAPK/MAPK3/1 signaling pathway in human first-trimester pregnancy. *Biol Reprod*. 2010;82(5):968-979. https://doi.org/10.1095/biolreprod.109.080739
25. Meng YH, Zhou WJ, Jin LP, et al. RANKL-mediated harmonious dialogue between fetus and mother guarantees smooth gestation by inducing decidual M2 macrophage polarization. *Cell Death Dis*. 2017;8(10):e3105. https://doi.org/10.1038/cddis.2017.505
26. Genin M, Clement F, Fattaccioli A, Raes M, Michiels C. M1 and M2 macrophages derived from THP-1 cells differentially modulate the response of cancer cells to etoposide. *BMC Cancer*. 2015;15:577. https://doi.org/10.1186/s12885-015-1546-9
27. Yang HL, Lai ZZ, Shi JW, et al. A defective lysophosphatidic acid-autophagy axis increases miscarriage risk by restricting decidual macrophage residence. *Autophagy*. 2022:1-22. https://doi.org/10.1080/15548627.2022.2039000
28. Fan DX, Li MQ, Zhou WJ, Huang HL, Yang HL, Xu CJ. A defective CXCL16/CXCR6 axis increases the risk of pregnancy loss via the abnormal crosstalk between decidual $\gamma\delta$ T cells and trophoblasts. *Reprod Dev Med*. 2021;3:129-139. https://doi.org/10.4103/2096-2924.324878
29. Shi JW, Yang HL, Lai ZZ, et al. Aspirin enhances the protective effect of progesterone on trophoblast cell from oxidative stress and apoptosis. *Reprod Dev Med*. 2021;1:1-8. https://doi.org/10.4103/2096-2924.313686
30. Byun DS, Ahmed N, Nasser S, et al. Intestinal epithelial-specific PTEN inactivation results in tumor formation. *Am J Physiol Gastrointest Liver Physiol*. 2011;301(5):G856-G864. https://doi.org/10.1152/ajpgi.00178.2011
31. Gu Y, He Y, Zhang X, et al. Deficiency of monoclonal non-specific suppressor factor beta (MNSFB) promotes pregnancy loss in mice. *Mol Reprod Dev*. 2015;82(6):475-488. https://doi.org/10.1002/mrd.22495
32. Smith SD, Dunk CE, Aplin JD, Harris LK, Jones RL. Evidence for immune cell involvement in decidual spiral arteriole remodeling in early human pregnancy. *Am J Pathol*. 2009;174(5):1959-1971. https://doi.org/10.2353/ajpath.2009.080995
33. Zhou J, Chen H, Xu X, et al. Uterine damage induces placenta accreta and immune imbalance at the maternal-fetal interface in the mouse. *Placenta*. 2022;119:8-16. https://doi.org/10.1016/j.placenta.2022.01.002
34. Gustafsson C, Mjösberg J, Matussek A, et al. Gene expression profiling of human decidual macrophages: evidence for immunosuppressive phenotype. *PLoS One*. 2008;3(4):e2078. https://doi.org/10.1371/journal.pone.0002078
35. Zhang YH, He M, Wang Y, Liao AH. Modulators of the Balance between M1 and M2 macrophages during pregnancy. *Front Immunol*. 2017;8:120. https://doi.org/10.3389/fimmu.2017.00120
36. Rahmati M, Petitbarat M, Dubanchet S, Bensussan A, Chaouat G, Ledee N. Colony stimulating factors 1, 2, 3 and early pregnancy steps: from bench to bedside. *J Reprod Immunol*. 2015;109:1-6. https://doi.org/10.1016/j.jri.2015.01.005
37. Hollmén M, Karaman S, Schwager S, et al. G-CSF regulates macrophage phenotype and associates with poor overall survival in human triple-negative breast cancer. *Oncoimmunology*. 2016;5(3):e1115177. https://doi.org/10.1080/2162402x.2015.1115177
38. Wen Q, Kong Y, Zhao HY, et al. G-CSF-induced macrophage polarization and mobilization may prevent acute graft-versus-host disease after allogeneic hematopoietic stem cell transplantation. *Bone Marrow Transplant*. 2019;54(9):1419-1433. https://doi.org/10.1038/s41409-019-0449-9
39. Bussolino F, Ziche M, Wang JM, et al. In vitro and in vivo activation of endothelial cells by colony-stimulating factors. *J Clin Invest*. 1991;87(3):986-995. https://doi.org/10.1172/jci115107
40. Fusté B, Mazzara R, Escolar G, Merino A, Ordinas A, Díaz-Ricart M. Granulocyte colony-stimulating factor increases expression of adhesion receptors on endothelial cells through activation of p38 MAPK. *Haematologica*. 2004;89(5):578-585.
41. Gleicher N, Vidali A, Barad DH. Successful treatment of unresponsive thin endometrium. *Fertil Steril*. 2011;95(6):2123. e13-7. https://doi.org/10.1016/j.fertnstert.2011.01.143
42. Scarpellini F, Klinger FG, Rossi G, Sbracia M. Immunohistochemical study on the expression of G-CSF, G-CSFR, VEGF, VEGFR-1, Foxp3 in first trimester trophoblast of recurrent pregnancy loss in pregnancies treated with G-CSF and controls. *Int J Mol Sci*. 2019;21(1). https://doi.org/10.3390/ijms21010285
43. Ohki Y, Heissig B, Sato Y, et al. Granulocyte colony-stimulating factor promotes neovascularization by releasing vascular endothelial growth factor from neutrophils. *Faseb J*. 2005;19(14):2005-2007. https://doi.org/10.1096/fj.04-3496fje
44. Shojaei F, Wu X, Qu X, et al. G-CSF-initiated myeloid cell mobilization and angiogenesis mediate tumor refractoriness to anti-VEGF therapy in mouse models. *Proc Natl Acad Sci USA*. 2009;106(16):6742-6747. https://doi.org/10.1073/pnas.0902280106
45. Zhao L, Shao Q, Zhang Y, et al. Human monocytes undergo functional re-programming during differentiation to dendritic cell mediated by human extravillous trophoblasts. *Sci Rep*. 2016;6:20409. https://doi.org/10.1038/srep20409
46. Mo L, Hong S, Li Y, et al. Sevoflurane inhibited inflammatory response induced by TNF-α in human trophoblastic cells through p38MAPK signaling pathway. *J Recept Signal Transduct Res*. 2020;40(3):218-223. https://doi.org/10.1080/10799893.2020.1726951
47. Jeong W, Kim J, Bazer FW, Song G. Proliferation-stimulating effect of colony stimulating factor 2 on porcine trophectoderm cells is mediated by activation of phosphatidylinositol 3-kinase and extracellular signal-regulated kinase 1/2 mitogen-activated protein kinase. *PLoS One*. 2014;9(2):e88731. https://doi.org/10.1371/journal.pone.0088731
48. Dobbs KB, Khan FA, Sakatani M, et al. Regulation of pluripotency of inner cell mass and growth and differentiation of trophectoderm of the bovine embryo by colony stimulating factor 2. *Biol Reprod*. 2013;89(6):141. https://doi.org/10.1095/biolreprod.113.113183

49. Kocarslan S, Incebıyık A, Guldur ME, Ekinci T, Ozardali HI. What is the role of matrix metalloproteinase-2 in placenta percreta? *J Obstet Gynaecol Res*. 2015;41(7):1018-1022. https://doi.org/10.1111/jog.12667
50. Zhou Y, Fisher SJ, Janatpour M, et al. Human cytotrophoblasts adopt a vascular phenotype as they differentiate. A strategy for successful endovascular invasion? *J Clin Invest*. 1997;99(9):2139-2151. https://doi.org/10.1172/jci119387
51. Chen CP, Chen CY, Wu YH, Chen CY. Oxidative stress reduces trophoblast FOXO1 and integrin β3 expression that inhibits cell motility. *Free Radic Biol Med*. 2018;124:189-198. https://doi.org/10.1016/j.freeradbiomed.2018.06.006
52. Gearing AJ, Newman W. Circulating adhesion molecules in disease. *Immunol Today*. 1993;14(10):506-512. https://doi.org/10.1016/0167-5699(93)90267-o
53. Korkmazer E, Nizam R, Arslan E, Akkurt Ö. Relationship between intercellular adhesion molecule-1 and morbidly adherent placenta. *J Perinat Med*. 2018;47(1):45-49. https://doi.org/10.1515/jpm-2018-0086
54. Kaya B, Nayki U, Nayki C, et al. Proliferation of trophoblasts and Ki67 expression in preeclampsia. *Arch Gynecol Obstet*. 2015;291(5):1041-1046. https://doi.org/10.1007/s00404-014-3538-4
55. Suresh UR, Hale RJ, Fox H, Buckley CH. Use of proliferation cell nuclear antigen immunoreactivity for distinguishing hydropic abortions from partial hydatidiform moles. *J Clin Pathol*. 1993;46(1):48-50. https://doi.org/10.1136/jcp.46.1.48
56. Tomas SZ, Prusac IK, Roje D, Tadin I. Trophoblast apoptosis in placentas from pregnancies complicated by preeclampsia. *Gynecol Obstet Invest*. 2011;71(4):250-255. https://doi.org/10.1159/000320289
57. Prusac IK, Zekic Tomas S, Roje D. Apoptosis, proliferation and Fas ligand expression in placental trophoblast from pregnancies complicated by HELLP syndrome or pre-eclampsia. *Acta Obstet Gynecol Scand*. 2011;90(10):1157-1163. https://doi.org/10.1111/j.1600-0412.2011.01152.x

How to cite this article: Che J-H, Zheng Z-M, Li M-Q, Yao X. Macrophage polarization in placenta accreta and macrophage-trophoblast interactions. *Am J Reprod Immunol*. 2022;e13611. https://doi.org/10.1111/aji.13611