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Immune involvement in recurrent pregnancy loss

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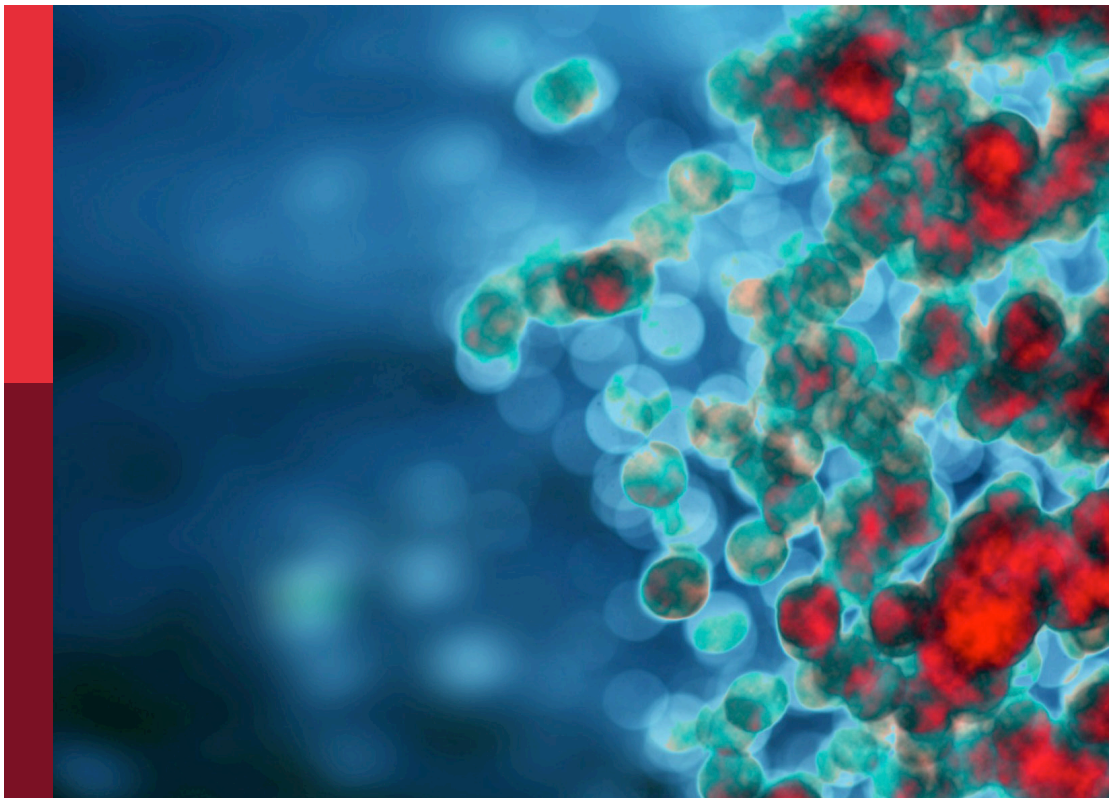

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Immune involvement in recurrent pregnancy loss

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Editorial: Immune involvement in recurrent pregnancy loss

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Editorial on the Research Topic
Immune involvement in recurrent pregnancy loss

Recurrent pregnancy loss (RPL) is defined as two or more consecutive pregnancy losses before the 20th week of gestation, occurring in 1%–3% of reproductive age women. RPL is a devastating condition, significantly negatively impacting the quality of life of affected couples. Unexplained RPL (URPL) is a heterogeneous condition affecting approximately 50% of RPL cases, with one contributing factor thought to be a disruption in maternal immune tolerance. Various immune effectors and molecules in the uterine microenvironment establish and maintain specific maternal tolerance toward the semi-allogeneic fetus during pregnancy. Immune cells including innate lymphoid cells (ILCs), myeloid cells, T cells and B cells have been found to contribute to maintaining this maternal immunological tolerance during pregnancy. ILCs have been found to be the most abundant immune cells in the pregnant uterus, with many studies describing the relationship between RPL and either T cells or natural killer (NK) cells in peripheral blood and the endometrium/decidua. Despite progress in uncovering the roles of uterine NK and regulatory T cells in pregnancy, the immune heterogeneity in patients with URPL remains inadequately understood.

In this Research Topic, we aimed to collect manuscripts that would contribute to our understanding of the disruption of maternal immune tolerance during pregnancy leading to URPL.

This Research Topic contains 9 manuscripts covering both original articles and reviews. The collected articles highlight the heterogeneity associated with URPL, and recurrent implantation failure (RIF) which shares some features with RPL, and the need to further stratify these women to allow for personalized treatment options. In addition, it highlights some of the novel approaches being taken to identify aetiologies associated with these conditions. Zhang et al. used a novel integrated bioinformatics approach combining both antiphospholipid syndrome and RIF datasets to determine commonly altered genes, all of which were associated with the immune system. Their report suggests the possible identification of four candidate genes that could be considered for the diagnosis of RIF with antiphospholipid syndrome. However, the use of bioinformatics in RPL research was questioned due to the large heterogeneity of the population, often small study sizes and challenges in a standard definition of RPL and relevant risk factors, taking lessons from cancer research as a 'gold standard' (Betti et al.).

Autoantibodies are a major risk factor for reproductive failure; not just antiphospholipid antibodies, but those against other targets as well. Nørgaard-Pedersen et al. described the potential risks associated with thyroid-peroxidase and anti-nuclear antibodies in addition to anticardiolipin antibodies, β2 glycoprotein antibodies and lupus anticoagulant in conjunction with HLA-DR typing. Three studies further showcased the dysregulation of different immune cells with the endometrium/decidua in women with RPL, uNK cells, low-density granulocytes and macrophages, indicating changes in cell surface receptors as well as numbers being associated with this condition (Ye et al.; Woon et al.; Sang et al.). However, association does not necessarily point to causality and thus we still do not fully understand their contribution to the aetiology of URPL, or how they may be targeted therapeutically. Review articles covered the roles of uNK cells and trophoblast (Wei et al.), macrophages (Zhao et al.) and matrix metalloproteinases (Jing et al.) in both the non-pregnant endometrium as well as the early pregnant decidua. Wei et al. discussed the contributions of uterine NK cells to the essential remodeling of the spiral artery, while Zhao et al. elaborated on possible connections between recurrent spontaneous miscarriage and the phenotypes and functions of decidual macrophages.

We hope this Research Topic will be useful for researchers and clinicians alike to help inform patients as well as further studies. Developing stratifying criteria for the different sub-types of URPL, as well as potential therapeutic options for each of those sub-types is paramount.

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The role of extravillous trophoblasts and uterine NK cells in vascular remodeling during pregnancy

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Successful embryo implantation requires both a receptive endometrium and competent blastocysts. After implantation, the maternal decidua undergoes a series of changes, including uterine spiral artery (SA) remodeling to accommodate the fetus and provide nutrients and oxygen for the fetus to survive. Uterine spiral arteries transform from small-diameter, high-resistance arteries to large-diameter and low-resistance arteries during pregnancy. This transformation includes many changes, such as increased permeability and dilation of vessels, phenotypic switching and migration of vascular smooth muscle cells (VSMCs), transient loss of endothelial cells (ECs), endovascular invasion of extravillous trophoblasts (EVTs), and presence of intramural EVT, which are regulated by uterine NK (uNK) cells and EVTs. In this review, we mainly focus on the separate and combined roles of uNK cells and EVTs in uterine SA remodeling in establishing and maintaining pregnancy. New insight into related mechanisms will help us better understand the pathogenesis of pregnancy complications such as recurrent pregnancy loss (RPL) and preeclampsia (PE).

KEYWORDS

spiral artery remodeling, extravillous trophoblast (EVT), uterine natural killer cells, recurrent pregnancy loss (RPL), preeclampsia (PE)

Introduction

Competent blastocyst implantation is a critical step in the initiation of pregnancy, in which the mother and the semi-allogenic fetus surprisingly coexist peacefully. Successful implantation involves several intricate processes, such as hormonal regulation, the invasion of fetal trophoblasts and well-coordinated maternal decidual immune cell subsets (1). After the

implantation, these factors subsequently contribute to uterine spiral artery (SA) remodeling, which includes several processes that are orchestrated by the disruption of vascular smooth muscle cells (VSMCs), the transient loss of endothelial cells (ECs), intravasation or extravasation by interstitial or endovascular trophoblast, and amorphous fibroid deposition containing intramural extravillous trophoblast cells (EVTs) (2). SA remodeling transforms uterine spiral arteries from low-flow, high-resistance vessels to high-flow, low-resistance vessels. Increased blood flow in the intervillous space promotes oxygen and waste exchange between the fetus and mother (3). uNK cells and EVTs are indispensable factors in SA remodeling and they play a vital role in the decidua- or trophoblast-associated remodeling (2). During the process of implantation, there is expansion and activation of uNK cells and EVTs at the maternal-fetal interface (4). Disruptions in either of these populations may contribute to preeclampsia (PE) and recurrent pregnancy loss (RPL) (5, 6). This review focuses on the interaction between uNK cells and EVTs, and their respective impacts on uterine SA remodeling. The pathogenesis of PE and RPL related to dysregulated function of uNK cells and EVTs will also be discussed.

EVT differentiation and invasion

The blastocyst mainly consists of two structures called the inner cell mass and the trophectoderm, which differentiate into fetus and placenta, respectively (7). During the formation of the placenta, the trophectoderm transforms into mononuclear cytotrophoblasts (CTBs), which form placental villi through branching morphogenesis. In floating villi, which is bathed in maternal blood, CTBs fuse into multinuclear syncytiotrophoblasts (STBs) and form the syncytial layer, where a vast range of functions such as production of pregnancy hormones and clearance of fetal waste products are fulfilled (8, 9). CTBs at branched anchoring villus tips have a proliferative phenotype and differentiate into EVTs, which have an invasive, cytokine-secreting phenotype, forming a stratified structure called the cell column. EVTs in the distal region of the cell column invade the decidua up to the inner third of the myometrium (10). EVTs that migrate into the maternal decidua are called interstitial EVTs and further develop into endovascular trophoblasts that migrate through the spiral arteries (11).

EVT invasion is both stimulated and inhibited by contact with a number of different maternal cell types in the decidua. Lack of decidua may lead to excessive EVT invasion in placenta accreta, while inadequate EVT invasion is associated with pregnancy complications such as preeclampsia (PE) and recurrent pregnancy loss (RPL) (12). Invasive EVT plays an essential role in the SA remodeling process. A number of factors including cytokines, chemokines, and environmental oxygen have been reported to stimulate or inhibit the differentiation/invasion of EVT. CCR1 is expressed on human trophoblasts and its ligands, CCL5 and CCL2, are expressed by decidual tissue. The chemokine-CCR1 system could promote EVT migration and induce the initiation step of trophoblastic invasion toward maternal tissue (13). Some chemokines such as CXCL14 and CXCL6 could inhibit trophoblast invasion through the downregulation of MMP-2 and MMP-9 activity (14, 15). Roser et al. developed a database to predict the interactions between receptors and their respective ligands based on single-cell sequencing of the maternal-fetal interface between 6-14 weeks (16). The researchers confirmed the presence of PDL1 (also known as CD274) in EVTs and identified new inhibitory interactions between KLRB1 (Killer cell lectin like receptor B1) and TIGIT (T cell immunoreceptor with Ig and ITIM domains) on uNK cells and CLEC2D (C-type domain family 2 member D) on EVTs, which suggested that the damaging effects of maternal uNK cells on fetal EVTs was circumvented in the microenvironment of the maternal-fetal interface (16).

Uterine NK (uNK) cells have a significant impact on placentation by regulating the invasion of trophoblasts cells into the decidua basalis and spiral artery remodeling, considering the high abundance of uNK cells in the decidua in the first trimester and their association with EVT through mechanisms including cytotoxicity, local cytokine production or induction of trophoblast apoptosis (17). uNK cells can regulate trophoblast invasion and angiogenesis by releasing IL-8 and interferon-inducible protein-10 upon contact with trophoblasts (18). Coculture of uNK cells with CTB or HTR8 cells significantly promoted trophoblast invasion *via* the secretion of IL-8 and HGF (19). Wang et al. confirmed that IL-22 produced by uNK cells and decidual stromal cells (DSCs) significantly promoted trophoblast proliferation and viability (20). The differential secretion and expression of chemokines/cytokines and their respective receptors could also induce selective leukocyte trafficking to mediate trophoblast invasiveness and placental angiogenesis (21).

Actually, not all species have typical invasive trophoblasts like humans. For example, trophoblast invasion is not extensive in mouse pregnancy, which have a haemochorial placenta with vascular remodeling restricted to the decidua. However, in humans, SA remodeling can extend into the inner myometrium. Similar to human placentation, the rat possesses hemochorial placentation with inherently invasive trophoblasts and EVT-guided transformation of SA remodeling (22). Trophoblast cell invasion is shallow in mice compared with the extensive intrauterine infiltration of rat, thus the rat is regarded as an excellent *in vivo* model for exploring the functions of uNK and EVT (23).

Phenotypes and subsets of uNK cells during pregnancy

uNK cells constitute the majority of the cell populations at the maternal-fetal interface during the first trimester of human pregnancy. uNK cells account for 70% of local leukocytes, while

peripheral blood NK (pNK) cells constitute 15% of circulating leukocytes (24, 25). Given the large number of uNK cells in the decidua during pregnancy, the important role of these cells needs to be further explored. There are also differences in the phenotypes of pNK cells and uNK cells. pNK cells are mainly $CD56^{dim}$ and express high levels of CD16, while uNK cells are mostly $CD56^{bright}$, $CD16^-$ cells (26). CD16 is the Fc receptor and can recognize antibody-coated cells and induce NK cells to release cytotoxic cytokines, which is called NK-mediated antibody-dependent cellular cytotoxicity (27). $CD56^{bright}$, $CD16^-$ NK cells secrete cytokines such as TGF-β while $CD56^{dim}$, $CD16^+$ NK cells are more cytotoxic (28). The main function of uNK cells is to release cytokines/chemokines that induce angiogenesis and placentation, while the main function of pNK cells is immune defense against infections and tumors through their cytolytic activity and production of cytokines, such as IFN-γ and TNF (29). Granule content and organization of uNK cells are different from those of pNK. Although uNK cells are poorly cytotoxic, they have large granules filled with perforin and granzymes. Meanwhile, pNK cells from healthy individuals are more cytotoxic and produce more cytokines with the increase of granule size (30). Granules in uNK cells are significantly larger in size and fewer in number compared with those in pNK cells (31, 32).

Diverse subsets of NK cells are dependent on different marker genes expressed on cells or diverse cytokine profiles in both humans and mice. A study using flow cytometry showed the production of type 1, type 2, type 3 and regulatory cytokines in uNK cells (33). uNK cells were grouped as NK1, NK2, NK3 and NKr1 according to the different cytokines they released. NK1 cells are Th1 (Type 1 T helper cells) cytokine-producing cells while NK2 cells release Th2 (Type 2 T helper cells) cytokines. TGF-β is produced by NK3 cells and IL-10 is released by NKr1 cells (34). Similar to the Th1/Th2/Th3/Tr1 (Type 1 T regulatory cells) paradigm, which plays an important role in balancing T cell-mediated immune stimulation and immune tolerance, the NK1/NK2/NK3/NKr1 paradigm is critical in the maintenance of normal pregnancy (35). Immature NK2 cells, which produce IL-13 and IL-5, eventually develop into NK1 cells that release IFN-γ. NK3 cells are the central population of uNK cells. The number of decidual NK3 cells and NKr1 cells that can produce TGF-α-related immunosuppressive factors is increased during normal pregnancy but decreased in miscarriage (33). In contrast, the number of decidual NK1 cells is increased in patients with miscarriage. The subpopulation of NK cells is changed from an NK3-predominant state to an NK1-predominant state in patients with miscarriage (33).

Yang et al. divided uNK cells into cytotoxic NK cells, tolerant NK cells, regulatory NK cells and memory NK cells (36). Cytotoxic NK cells are mainly $CD27^-CD11b^+$ NK cells, which play an essential role against infection at the maternal-fetal interface during pregnancy. Memory NK cells are termed pregnancy-trained decidual NK (PTdNK) cells and have high expression of LILRB1 and NKG2C. The incidence of placental dysfunction decreased during repeated pregnancies compared with that in first pregnancies, which may be partly attributed to the activation of PTuNK cells. VEGFα released by PTuNK cells can promote vascular remodeling (37). Regulatory NK cells can secrete cytokines and chemokines, such as $CD49a^+Eomes^+$ NK cells. Tolerant NK cells are immature cells that may eventually develop into other subsets of NK cells (36).

KIR/HLA interactions

uNK cells have been reported to be involved in the guidance of trophoblast invasion as well as uterine SA remodeling during pregnancy *via* direct interaction between ligands and receptors or cytokine production (38). KIRs are paired receptors expressed on NK cells with both activating and inhibitory functions. Most KIRs are inhibitory, so that they were initially thought as inhibitory receptors and named "Killer-cell Inhibitory Receptors". When A limited number of activating receptors within this family were found, both activating and inhibitory groups were termed "killer-cell immunoglobulin-like receptors" (KIRs) (39). The groundbreaking "missing self" hypothesis, firstly put forward by Karre and Ljunggren, stated that inhibitory KIRs could recognize self-major histocompatibility (MHC) class I surface molecules and protect the target cells against the cytotoxic activity of NK cells, which is essential in facilitating self-tolerance (40, 41). The KIR gene family is currently composed of 15 genes (KIR2DL1, KIR2DL2, KIR2DL3, KIR2DL4, KIR2DL5A, KIR2DL5B, KIR3DL1, KIR3DL2, KIR3DL3, KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS4, KIR2DS5, KIR3DS1) and 2 pseudogenes (KIR2DP1 and KIR3DP1), which are named based on the number of Ig-like domains and the length of the cytoplasmic tail (42). Generally, inhibitory receptors have a long cytoplasmic tail and are labeled as "L", while activating receptors have a short cytoplasmic tail and are labeled as "S". Only the KIR2DL4 receptor is exceptional and have both activating and inhibitory signals (43).

Human leukocyte antigen (HLA), also known as major histocompatibility complex (MHC), are the most polymorphic loci in the human genome, encoding the human MHC class Ia (HLA-A, HLA-B, and HLA-C), class Ib (HLA-E, HLA-F, HLA-G and HLA-H) and class II (HLA-DR, DQ, DM, DO and DP) molecules (44). EVT express non-classical HLA-G, HLA-E, HLA-F and polymorphic HLA-C molecules instead of the typical class I or class II molecules in normal pregnancy. Meanwhile, other trophoblast cell types have no expression of HLA genes (45, 46). HLA-C allotypes, including the C1 or the C2 epitope, act as ligands for KIRs. Inhibitory KIR2DL1 receptors and activating KIR2DS1 receptors bind to the C2 epitope, while inhibitory KIR2DL2/3 receptors bind to the C1 epitope (47, 48). The binding strength of inhibitory receptors is

higher than that of activating receptors upon binding to the same epitope. HLA-G are unusual ligands binding to KIR2DL4 (49). HLA-F can be expressed in two ways on the cell surface, including open conformers binding to KIR3DS1 and KIR3DL2 on NK cells, as well as peptide-bound HLA-F binding to the leukocyte immunoglobulin-like receptor (LIR) family, ILT2 and ILT4 (50, 51). HLA-F can also be expressed intracellularly in leukocytes (52). Disrupted interactions of KIR expressed on uNK cells with HLA expressed on EVT may disturb the balance between immune defense and immune tolerance and contribute to pregnancy complications (53, 54).

With the development of technology, Vento-Tormo et al. used nearest-neighbor cluster analysis of single-cell transcriptomics from first trimester decidua to redefine uNK cells into three major subsets including uNK1, uNK2 and uNK3, which all co-express the tissue-resident markers CD49a and CD9 (55). uNK1 is the only subset that express LILRB1, which is the receptor binding to HLA-G molecules expressed on EVT. Inhibitory receptors KIR2DL1, KIR2DL2 and KIR2DL3 and activating receptors KIR2DS1 and KIR2DS4 are KIRs that have high affinity for HLA-C and have higher expression levels in the uNK1 subset. The HLA-E receptors, including activating NKG2C and NKG2E as well as inhibitory NKG2A, are specifically expressed on uNK1 and uNK2 cells instead of the uNK3 subset. uNK1 cells secrete higher levels of CSF1, which bind to the receptor CSF1R expressed on EVT and macrophages. The above results suggest that uNK1 play a vital role in interacting with EVT and responding to EVT (55). uNK2 and uNK3 secrete more XCL1 chemokines than uNK1, which have interactive receptors both on maternal dendritic cells and fetal EVT (32). uNK3 cells express high levels of CCL5, while CCR1, the receptor for CCL5, is expressed on EVT. The expression pattern of the CCL5-CCR1 ligand-receptor complex suggests that uNK3 may also play a role in regulating EVT invasion (13).

Innate lymphoid cells (ILCs) are effectors and regulators that play a major role in innate immunity and tissue modeling and repair (56). Uterine NK cells are the best characterized member of the ILC family, which are detectable in decidua in the early phases of pregnancy (57). Huhn et al. have identified five main subsets of ILCs with different functional activities and diverse capacities to produce cytokines during first trimester using mass cytometry, including uterine NK cells (uNK)1-3, ILC3 and proliferating NK cells (32). They also demonstrated that uNK1 had lower responses in missing self assays, but higher responses to stimulation by cross-linking activating KIR2DS4, which may be associated with changes in granule content and organization followed by increased KIR expression in uNK1 (32, 55, 58). Further, Vazquez et al. found two novel decidual ILC subsets (C10 and C2) that express low T-bet and divergent Eomes levels through dimensionality reduction coupled with clustering using term human decidua samples (59). Combined with data of recent single cell sequencing of first trimester decidua (55), they proposed the continuity of decidual ILC subsets across pregnancy from early pregnancy through late gestation (59).

The role of uNK cells in uterine spiral artery remodeling

Vascular remodeling is a crucial step in the establishment and maintenance of pregnancy. Previous studies have shown that SA remodeling mainly depends on EVT (60, 61). However, some other evidence demonstrates that uNK cells dominate the initiation of the remodeling process, while EVTs play a secondary role (62, 63). Early uterine SA remodeling only includes the process of extracellular matrix degradation, transient loss of vascular ECs, and vascular smooth cell separation (2). This process is called decidua-associated remodeling, followed by trophoblast-associated remodeling (2). "Decidua-associated" or "Trophoblast-independent" remodeling is a maternal-mediated phase that precedes EVT invasion (2). Endothelial swelling and separation of medial VSMCs occurred in partially remodeled SA without intramural or endovascular EVTs, suggesting an early stage of SA remodeling prior to invasion of vessel wall by EVTs (64). Further study in pseudo-pregnant mice showed that early SA remodeling was independent of EVTs. Compared to normal pregnancy, disappearance of EVTs in pseudo-pregnant mice did not disturb early SA remodeling (65). The accumulation of uNK cells at the maternal-fetal interface during pregnancy suggests that uNK cells play an important role in the process of uterine SA remodeling. However, the exact function of uNK cells in SA remodeling is not fully understood. The possible role of uNK cells in SA remodeling may be to mediate the disorganization and clearance of VSMCs and endothelial cells (66).

The proportion of uNK cells in the first trimester is significantly higher than the proportion of uNK cells at term pregnancy (67, 68). uNK cells are an important source of growth factors and cytokines (18, 69). Therefore, uNK cells have two main functions, including promoting vascular remodeling and regulating trophoblast invasion (18, 70). Robson et al. demonstrated that uNK cells from early human pregnancy can induce morphological changes of VSMCs and breakdown of extracellular matrix component (71). The primary role of VSMCs is to control vessel contraction, thus regulating blood pressure and blood vessel tone. During the process of partially SA remodeling, VSMCs transformed from a contractile phenotype to a more synthetic or dedifferentiated phenotype. Markers of contractile phenotype are α-SMA, calponin, H-cal, MyHC and smoothelin, while the synthetic marker is osteopontin (64). VSMCs exhibit plasticity and can dedifferentiate in response to changes in local environmental cues including cell-cell and cell-matrix interactions and various inflammatory mediators during vascular development or vascular injury (72, 73).

uNK cells surround the spiral arteries and secrete many factors such as Angiopoietin (Ang) -1, Ang-2, VEGF-C, and IFN-γ, which can disrupt the integrity of VSMC and mediate extracellular matrix degradation (71). uNK cells can also secrete a variety of metalloproteinase (MMP) such as MMP2 and MMP9, which could degrade gelatin and break down collagen in the spiral artery model (74). A recent study showed that when VSMCs were cultured in uNK cell conditioned media for 24 h, the expression of VSMC markers such as calponin, smooth muscle protein 22-alpha (SM22α), smooth muscle alpha actin (αSMA), and myosin heavy chain 11 (MYH11) was significantly inhibited, suggesting that active uNK cells could affect VSMCs dedifferentiation. The dramatic differentiation of VSMCs eventually contributes to the loss of VSMCs, which is an important step in SA remodeling (75). IFN-γ derived from uNK cells could upregulate the LncRNA Maternal expressed gene 3 (MEG3) and increase the expression of MMP-2, thus promoting VSMCs apoptosis and migration (76). A subsequent study showed that miR-361-5p targeted by lncRNA MEG3 played an important role in inhibiting VSMCs proliferation and promoting VSMCs apoptosis (77).

The role of EVTs in uterine spiral artery remodeling during pregnancy

It has been proposed that the remodeling of both VSMCs and ECs was mediated by apoptosis through Fas signaling pathway or apoptotic cytokines (78–80), but Bulmer et al. demonstrated that SA remodeling involved the disorganization, rounding and migration of VSMCs in an EVT-dependent manner, instead of the apoptosis of VSMCs (81). Further, others have demonstrated that VSMCs only undergo apoptosis once they have undergone phenotypic switching and migrated away from the vessel wall, both processes require EVTs (64, 71, 82, 83). The uNK cells attract EVT toward the vessels through angiogenic growth factors and cytokines, thus attracting the VSMCs away from the vessel wall into the decidual stromal, where they undergo apoptosis and are phagocytosed by decidual macrophages (82). Upregulated MEG3 in trophoblasts could induce VSMC apoptosis and impair trophoblast migration by inactivating the PI3K/Akt pathway, suggesting that MEG3 could inhibit SA remodeling by EVT-mediated VSMCs loss and inhibiting EVT invasion (84). In addition, exosomes derived from EVTs also have many effects on SA remodeling and immune balance. Exosomes may promote the migration of VSMCs out of vessel walls and lead to uterine SA remodeling (85).

It was previously believed that EVTs completely replaced the endometrium in SA remodeling and acquired endothelial cell (EC) - like characteristics (86, 87). However, a comprehensive review by Pijnenborg et al. questioned the existing dogma of ECs mimicry by EVTs and demonstrated that EVTs and ECs coexisted in a fully remodeled SA with double labeling immunohistochemistry (2). Bulmer et al. further demonstrated that there was no evidence that EVTs replaced the endothelium of the vessel wall and ECs were never completely lost in the vessels. It was the presence or absence of an endovascular EVT plug that partly determined the extent of coverage of the SA lumen by ECs (88). In the presence of endovascular EVTs, ECs undergo morphological change and become rounded and discontinuous, which morphology is similar to EVT. This may explain that why ECs are mislabeled as EVTs. In fully remodeled SA, ECs showed a more typical morphology, which are elongated and flattened against the underlying extracellular matrix (ECM) (88).

The role of EVTs play in the completion of arterial remodeling is also highly dependent on the ability of migration/invasion into the decidual stroma. Inadequate trophoblast migration and invasion or induced trophoblast apoptosis and decreased proliferation disrupt angiogenesis and vascular remodeling. Our team has also done a series of work in terms of trophoblast invasion and migration. We firstly identified the transcription factor YY1 (Yin Yang 1), which could promote trophoblast invasion by targeting matrix metalloproteinase-2 (89). Besides we also revealed that EIF5A1 (Eukaryotic translation initiation factor) could promote trophoblast migration and invasion by binding directly to ARAF mRNA, thus activating the integrin/ERK signaling pathway (90).

uNK cells can regulate SA remodeling indirectly *via* modulating EVT invasion. Pollheimer et al. (91) revealed that the pro-invasive effect of uNK cells on EVTs was in a stage-dependent way. The conditioned media of uNK cells harvested from later gestational age within the first trimester (10-13 weeks' gestation) could promote EVT invasion, while uNK cell-derived conditioned media from an earlier developmental stage did not have an effect on EVT invasion. This can be reflected by another experiment showing that the number of circulating EVTs peaked in 12-13 gestational weeks (92). EVTs with strong invasive ability invade the uterine arteries and form plugs in the lumen of the uterine arteries, which eventually disintegrated and migrate into the maternal blood. The peaked number of EVTs in 12-13 gestational weeks may be related to the pro-invasive effect of uNK cells on EVTs in 10-13 gestational weeks.

Cell-to-cell crosstalk between uNK cells and EVTs during pregnancy

The direct or indirect interaction of uNK cells with EVT has been shown to facilitate the induction and maintenance of immune tolerance, protect the placenta against pathogen infection, and promote SA remodeling (58, 93, 94).

Immune tolerance

Fetuses carrying paternal human leukocyte antigen (HLA) are semi-allogenic to mothers. However, they coexist peacefully.

Fascinating adaptations both on maternal or fetal side rely on fine-tunings of decidual immune cells, decidual stromal cells, and trophoblasts to direct away from immune rejection towards immune tolerance (95). Maternal-fetal interface is important during the establishment of pregnancy-associated immune homeostasis. MHC molecules such as HLA-C, -G, and -E expressed by EVTs have been demonstrated to be ligands of either activating or inhibitory uNK cell receptors, providing a delicate balance between tolerance of trophoblast from maternal immune response and cytolytic activation of uNK (96). HLA-G is mainly expressed in EVT and believed to be a critical factor to prevent maternal immune attack to semi-allogenic fetus. Upon encountering fibronectin, which is a major component of uterus, EVT increased the expression of HLA-G. HLA-G^+ EVT did not elicit a profound cytokine response by uNK, thus HLA-G^+ EVT has the greater ability to induce maternal tolerance compared with HLA-G^- EVT (97). HLA-G is also expressed either on the surface or inside of the uNK. uNK containing surface HLA-G account for about 2.5% of the total number of uNK and they acquire surface HLA-G through forming synapses with HLA-G^+ EVT. When virus attacked, uNK were activated and resulted in the disappearance of internalized HLA-G as well as restoration of cytotoxicity. The interaction of EVT with uNK formed a HLA-G cycle of trogocytosis, endocytosis and degradation, which process contributed greatly to immune tolerance and antiviral immunity (98). Although HLA-G has been proved to be related to the induction of immune tolerance, polymorphic HLA-C can elicit a maternal immune response. It has been reported that engagement of activating KIR with HLA-C can reduce the risk of pregnancy complications, possibly through providing specific immunity to viral and bacterial pathogens (48). Specifically, interaction of KIR2DS1 with HLA-C2 contributes to uNK activation, while interaction of KIR2DL1 with HLA-C2 leads to inhibition of cytotoxicity of uNK cells (99). NKG2A could engage with its ligand HLA-E to regulate placental function and immune adaptation, thus inhibiting preeclampsia (PE) occurrence in human and mice (100).

The expression of HLA-C, G, E and F is distinct in three trimesters of pregnancy, suggesting that different types of EVT have distinct ability to interact with uNK cells and influence NK cell maturation and function (101–103). The expression of HLA-C is highest in the first trimester, while the expression of HLA-G is highest in the term pregnancy. HLA-E is only expressed in the first trimester (46). Only the first trimester EVT, instead of the term trimester EVT, upregulated the cell surface expression levels of HLA-C and HLA-G in response to the stimulation of the proinflammatory cytokine IFN-γ (103). Besides, different levels of HLA-E and HLA-G may regulate uNK responses by influencing the expression of HLA-E receptors (NKG2A/C) and HLA-G receptors (KIR2DL4, LILRB1 and LILRB2) (103). HLA-F is highly expressed on normal EVT of the first trimester placenta and becomes intracellular and weaker in the second and term trimester (46). This result is totally contrary to an earlier study, which showed that there was an increase in expression of HLA-F from the second trimester to term trimester and HLA-F was expressed only in the cytoplasm during the first trimester, after which HLA-F moved to the cell surface with the progression of pregnancy (104). This controversy needs to be further investigated. Zhang et al. found that there was an increased number of EVTs around uNK cells in the first trimester compared to the second trimester and EVTs could decrease the activating receptor NKG2D on uNK cells (105). In the second trimester, uNK cell function was inhibited through the loss of interactions between uNK cells and EVTs, which contributed to immune tolerance (105).

Immune defense

Placenta is not only an organ that provides nutrients and exchanges oxygen and gas between fetus and mothers, it is also a powerful physical barrier to prevent transmission of virus and microorganism by initiating innate immune response. A strong association between intrauterine pathogen infection and pregnancy complications such as preterm birth, preeclampsia and abortion has been demonstrated by several studies (106–108). Epidemiological evidence shows that pregnant women are more susceptible to viral infection possibly due to immune tolerance (109).

Upon encountering cytomegalovirus (CMV)-infected decidual stromal cells, uNK cells change phenotypes and become more cytotoxic through both the NKG2D and the CD94/NKG2C or 2E activating receptors, which increases antiviral immune responses (110). A study has also demonstrated that uNK expressing activating KIR2DS1 acquired higher cytotoxic function when exposed to human cytomegalovirus (HCMV)-infected decidual stromal cells (DSC), especially when DSCs express HLA-C2 (58). However, CMV also resides and replicates in the trophoblasts (111, 112). In contrast to infected DSC, uNK were unable to secrete cytotoxic cytokines or degranulate upon encountering with CMV-infected primary EVT (58). Interestingly, a significant loss of HCMV-infected EVT was observed upon coculture with uNK even without cytokine production or degranulation (58). This phenomenon was further explained by a recent study, which demonstrated that uNK cells killed intracellular bacteria through transferring Granulysin to EVT *via* nanotubes without killing EVT, which is independent of degranulation and cytokine secretion (94). Although uNK-secreted growth factors and cytokines are important factors to defense infection and facilitate placentation, direct transfer of cytosolic proteins and nutrients through nanotubes could also contribute to uNK regulation of placentation.

SA remodeling

SA remodeling is a crucial process during pregnancy to provide enough blood supply to meet the demands of the

growing fetus. The surrounding niche cells including uNK and EVT and uterine environment including hormones and oxygen tension play an important role in SA remodeling during pregnancy. *In vitro* experiments of VSMC cell line demonstrated the high efficiency of uNK and EVT in inducing VSMCs dedifferentiation (113).

The endocrine system and the immune system act synergistically during implantation and maintenance of pregnancy. For example, studies in mice show that uNK cells play an important role in the modification of uterine blood vessels *via* an IFN-γ pathway. The IFN-γ production of uNK cells could be inhibited *via* glucocorticoid receptor, which is cross-reacted with progesterone, further inducing immune tolerance during pregnancy (114, 115). Human decidual stromal cells will upregulate the expression of IL-15 mRNA during progesterone-induced decidualization, which may influence uNK proliferation, differentiation and production of cytokines (116). Estrogen also plays an important role in regulating the functions of human uNK cells through mediating uNK cell migration and promoting secretion of CCL2 from uNK cells, which facilitates uNK cell-mediated angiogenesis (117). The HLA-F mRNA expression level is upregulated under the stimulation of progesterone, while knockdown of the progesterone receptor downregulates HLA-F expression level, suggesting a role of progesterone in regulating HLA-F expression and EVT invasion, further impacting the process of SA remodeling (118).

In both humans and rodents, the primary role of uNK cells is in regulating early stages of spiral artery remodeling, which increases blood supply to the placenta and is essential to set the foundation for maternal nutrient and oxygen delivery to the placenta for fetal growth. Blood flow to the human intervillous space does not begin until 10 to 12 weeks of pregnancy (119). Placental PO2 values measured in the 12-13 weeks were significantly increased compared with those obtained at 8-10 weeks, which suggest that the increase of placental PO2 at the end of the first trimester is related to the establishment of continuous maternal blood flow in the intervillous space (120). The change of oxygen tension at the maternal-fetal interface is thought to have secondary influence on EVT invasion. Physiologically hypoxic conditions in the first trimester, which is 2%-3% oxygen, are believed to promote embryo implantation and trophoblast invasion compared to normoxic conditions (121). The above results show that uNK cells have the potential to indirectly regulate trophoblast invasion and SA remodeling through regulation of oxygen tension at the maternal-fetal interface.

A study suggested that placental vascularity might depend on the allogeneic interaction between maternal KIR on uNK cells and paternal HLA-C expressed by trophoblasts. The interaction induces the secretion of proangiogenic factors including Ang-1 and Ang-2 and the release of proinflammatory cytokines, such as IL-8, IL-10, interferon-γ (IFN-γ), tumor-necrosis factor-α (TNF-α) and macrophage inflammatory protein (MIP) (18). The interaction between HLA-G and ILT2 could also contribute to the release of proangiogenic factors (122). HLA-G expression could be inhibited by miR-133a, which might impair the angiogenic and invasive functions of uNK cells (123). It has also been demonstrated that uNK cells can transform into a senescent phenotype upon interacting with HLA-G from trophoblasts during pregnancy (122, 124). The NK cells of senescent phenotype would produce pro-angiogenic factors that regulate trophoblast invasion and spiral artery remodeling (125). HLA-F is highly expressed on the surface of invasive EVT both ex vivo and *in vitro*. The expression of HLA-F on the surface of invasive EVT is increased compared to that of the non-invasive proliferating EVT, suggesting a role of HLA-F in EVT invasion and SA remodeling (46, 126) (Figure 1).

Dysregulated uNK cell and EVT functions in the pathogenesis of RPL

RPL is a critical reproductive complication. An individual with this disease suffers at least two consecutive pregnancy losses. RPL has relatively high morbidity and affects approximately 1% to 2% of women (127). Common causes of pregnancy loss include anatomical anomalies and genetic, endocrine and immunological factors. However, the reasons for almost half of the cases remain unexplained (128). A lack of inhibition of uNK cells and dysregulated EVT migration and invasion may partly contribute to the occurrence of RPL (129).

Omnia et al. found an abnormal increase in both CD158b (inhibitory KIR) and CD161 (activating KIR) among all NK cell subsets in the peripheral blood and decidua of patients with recurrent pregnancy loss. The cytotoxic function of $CD16^+$ NK cells is related to the expression of CD161 (130). These findings confirmed the hypothesis of Zhu et al. that the immune system is disturbed by increased expression of inhibitory and stimulatory KIRs in patients with miscarriages (131). The expression of HLA-G is decreased in women with RPL (132). A recent finding by Zhou et al. showed that EVT-derived HLA-G interacted with ILT2 in uNK cells and promoted the expression of PBX1, which is a transcription factor with critical functions. PBX1 is mainly expressed in $CD49a^+$ tissue resident NK cells and can promote the transcription of growth-promoting factors (GPFs) that contribute to fetal development. In addition, there was a decrease in the number of NK cells in PBX1-mutant mice, suggesting that PBX1 might indirectly modulate the immune microenvironment by downregulating uNK cells. Impaired $CD49a^+PBX1^+$ uNK cells may be related to an increased risk of RPL (133). Consistently, Li et al. (134) found lower levels of $CD49a^+$ in patients with RPL than in healthy controls. However, numerous studies have found that there was an increased population of uNK cells in the luteal-phase endometrium of women with RPL (135–138). An increase in uNK cells and their production of cytokines and angiogenic factors promoted endometrial angiogenesis, which could lead to increased level of oxygen, thus increasing oxidative stress (139, 140). Consistently, Chen et al. found that the number of micro-blood

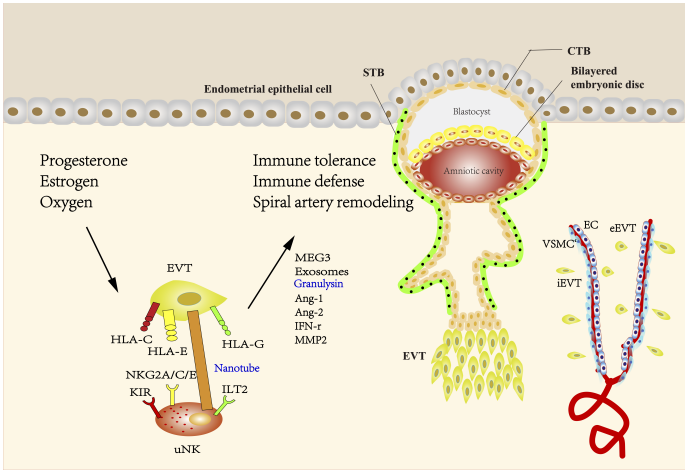

FIGURE 1
The interaction between EVT and uNK cells during pregnancy. The blastocyst mainly consists of two structures called the inner cell mass and the trophectoderm, which differentiate into fetus and placenta, respectively. During the formation of the placenta, the trophectoderm transforms into mononuclear CTBs, which have two differentiation ways. CTBs can fuse into multinuclear STBs and form the syncytial layer. Proliferative but non-invasive CTBs can switch to invasive but non-proliferative EVT, forming a stratified structure called the cell column. EVTs that migrate into the maternal decidua are called interstitial EVTs (iEVT) and further develop into endovascular trophoblasts (eEVT) that migrate through the spiral arteries. The direct or indirect interaction of uNK cells with EVT has been shown to facilitate the induction and maintenance of immune tolerance, protect the placenta against pathogen infection, and promote SA remodeling. Uterine environment including hormones and oxygen tension at the maternal-fetal interface plays an important role in regulating EVT and uNK cells during pregnancy. HLA-C, -G, and -E expressed by EVTs have been demonstrated to be ligands of either activating or inhibitory uNK cell receptors, providing a delicate balance between tolerance of trophoblast from maternal immune response and cytolytic activation of uNK. HLA-G is mainly expressed in EVT and believed to be a critical factor to prevent maternal immune attack to semi-allogenic fetus, while polymorphic HLA-C can elicit a maternal immune response. The interaction between HLA-G and ILT2 can also contribute to the release of proangiogenic factors. Engagement of activating KIR with HLA-C can reduce the risk of pregnancy complications, possibly through providing specific immunity to viral and bacterial pathogens. NKG2A/C/E could engage with its ligand HLA-E to regulate placental function and immune adaptation. uNK cells surround the spiral arteries and secrete many factors such as Ang-1, Ang-2, and IFN-γ, which can disrupt the integrity of VSMC and mediate extracellular matrix degradation. IFN-γ derived from uNK cells could upregulate the MEG3 and increase the expression of MMP-2, thus promoting VSMCs apoptosis and migration. Exosomes derived from EVTs may promote the migration of VSMCs out of vessel walls and lead to uterine SA remodeling. uNK cells can transmit granulysin into EVT *via* nanotubes and kill intracellular bacterial without damaging EVT. (uNK cells, uterine natural killer cells; EVT, extravillous trophoblast; CTBs, cytotrophoblasts; STBs, syncytiotrophoblasts; iEVT, interstitial EVTs; eEVT, endovascular trophoblasts; SA, spiral artery; HLA, human leukocyte antigen; ILT2, Ig-like transcript 2; KIR, Killer-cell immunoglobulin-like receptors; NKG2A/C/E, CD94/NK group 2 member A/C/E; Ang-1, Angiopoietin-1; IFN-γ, interferon-γ; VSMC, vascular smooth muscle cell; MEG3, maternally expressed 3; MMP2, matrix metalloproteinase-2).

vessels in the decidual tissues of patients with RPL during the peri-implantation period were increased compared with those of the normal fertility group (141). Based on the above evidences, we propose that different numbers of uNK cells in the endometrium before and after pregnancy have a distinct effect on the pathogenesis of RPL.

Although the interaction between KIR and HLA-C is critical in understanding the role of NK cells in RPL, nonspecific MHC class I inhibitory receptors such as TIM-3 (T-cell immunoglobulin and mucin domain-3) also partly explain the occurrence of RPL (142). TIM-3 is expressed on the surface of pNK cells and could induce the secretion of anti-inflammatory cytokines and promote immune tolerance at the maternal-fetal interface. Patients with RPL have a decreased percentage of TIM-3$^+$ CD56$^+$ cells in the peripheral blood (143). Besides, studies in human and mice showed that autophagy levels might affect the process of placentation through regulating trophoblast invasion and NK cell residence in the decidua, which provided new insight into the treatment of RPL (144, 145). Tan et al. revealed that suppressed trophoblast autophagy increased the secretion of IGF-2 (Insulin growth factor-2), which induced the differentiation of NK cells from a less cytolytic phenotype to cells with high killing activities. IGF-2 could also downregulate PEG10 (paternally expressed 10) levels, which inhibited trophoblast invasion. Moreover, autophagy suppression disturbs the function of memory uNK cells during the first pregnancy, which could attack newly formed trophoblasts during subsequent pregnancies and increase the incidence of RPL (146).

Dysregulated uNK cell and EVT functions in the pathogenesis of PE

PE is a common pregnancy complication due to poor placental development (147). PE clinically manifests as

proteinuria and new-onset hypertension after 20 weeks of pregnancy. There are two types of PE, including early-onset (before 34 weeks) and late-onset (after 34 weeks). PE is the main reason for the morbidity and mortality of mothers and fetuses (148). However, the pathogenesis of PE remains unclear.

PE progression is divided into two stages: placental ischemia derived from disrupted SA remodeling in the first trimester and subsequent inflammation and hypertension resulting from excessive release of antiangiogenic factors such as sENG (soluble endoglin) and sFLT1 (soluble fms-like tyrosine kinase 1) in the second and third trimesters (149, 150). It is speculated that the changes in the number and subsets of uNK cells and inadequate trophoblast invasion may damage SA remodeling and further contribute to the occurrence of PE (151).

Zhang et al. found that the number of $CD56^{+}$ $CD3^{-}$ uNK cells was increased, and activation markers on uNK cells such as IFN-γ (interferon gamma), IL-8 and CD107a were obviously dysregulated in PE (152). The expression of TGF-β in the decidua of patients with PE was negatively correlated with VEGF levels and activation markers expressed on uNK cells (152). Thus TGF-β might play a critical role in the pathogenesis of PE. Another study showed that the reduced population of uNK cells was associated with the occurrence of PE through an altered cytokine environment, which might lead to defective trophoblast invasion and SA remodeling (153). However, it was found that insufficient perfusion of uterine arteries could stimulate the activation of cytolytic NK cells, while reduced number of NK cells could rescue placental ischemia-induced damage such as hypertension and inflammation (154).

The state of SA remodeling can be reflected by uterine artery Doppler ultrasound. When SA remodeling is damaged, patients will have decreased maternal blood supply and high blood flow resistance. Thus impaired SA remodeling is represented by a high resistance index while normal SA remodeling is represented by a normal resistance index. Fraser et al. (80) found that the pro-invasive effect on EVTs by uNK cells from women with high resistance indexes was decreased compared with those from women with average resistance indexes. This is consistent with the findings of Wallace et al (5). Besides, uNK cells from women with high resistance indexes may secrete fewer pro-apoptotic factors and were less likely to induce VSMC apoptosis compared with those from women with normal resistance indexes (80).

Conclusion

The immunology of the maternal-fetal interface is extremely complex, because there are great variety of participating components and diverse interactions between fetal and maternal cells. There are still huge gaps in understanding the role of uNK cells and EVTs in SA remodeling and the interaction between uNK cells and EVTs, dysfunction of which may result in the pregnancy-related diseases. This review highlights the critical and important role that uNK cells and EVT play in spiral artery remodeling during pregnancy. Several problems remain to be resolved in the future.

1. uNK cells degranulate and secrete cytokines in response to CMV-infected decidual stromal cells, while uNK cells do not have such effect on CMV-infected EVT. Further study showed that uNK cells transmitted granulysin into EVT *via* nanotubes and kill intracellular bacterial without damaging EVT. The balance between immune tolerance and immune defense against bacterial or virus in the placenta during pregnancy needs to be further explored.

2. With the development of technology from flow cytometry to single-cell transcriptomics, we have a more profound knowledge about uNK cell subtypes. Nowadays, uNK still have not been definitively linked to pregnancy pathologies, which may be associated with the subset complexity. Different uNK subsets have diverse cytokine production and KIRs, predicting different functions. More specific definition and description of uNK subsets may provide deeper insight into the functions of precise treatment for pregnancy complications.

3. The particular KIR/HLA combinations have either a protective or detrimental role in placentation, which can predict NK cell responses and possible treatment modifications. Understanding how the interaction between KIR and HLA genes has an effect on the pathogenesis of disease is a challenging problem. The KIR expression on uNK cells is initially stable. Upon encountering allogenic fetus or invading pathogens, KIR expression will be altered to maximize the balance between self-tolerance and protection against viral infection. Besides, the expression of HLA-G, C, E and F are different during three trimesters in the pregnancy and play an important role in regulating immune response. The study of uNK KIR receptors and their interaction with trophoblast HLA-G, C, E and F help clinicians to prevent pregnancy complications such as RPL and PE.

4. The mechanism of trophoblast invasion and the migration and apoptosis of VSMCs are of great value for our comprehensive understanding of SA remodeling, facilitating the early diagnosis and treatment of pregnant complications due to dysfunctional SA remodeling such as PE and PRL. Ma et al. firstly bring up a concept of migracytosis, a cell migration-dependent process for the release of intracellular contents through migrasome, which is a newly discovered organelle (155). Researchers have demonstrated that migrasomes play a critical role in cellular communication, cell migration and early embryo development. It is very likely that migrasomes function in immune response and angiogenesis (156–158). Whether migrasomes are produced by trophoblasts and VSMCs and whether migrasomes regulate trophoblast invasion and VSMC migration at the maternal-fetal interface during pregnancy are total brand new areas remaining to be explored. Migrasomes may also play a role in cell-cell communication between uNK cells and EVT, providing a new target for regulating SA remodeling.

Recently, spatial transcriptomics have developed greatly, which can provide specific locality information combined with high-resolution transcriptome profiles to deconvolute the cellular compositions of the maternal-fetal interface microenvironment. There is no way to elucidate the cellular interactions purely based on single-cell RNA-seq data due to the absence of spatial information. Li et al. used the mouse model to identify the cellular composition and the embryonic communication with maternal decidua during early pregnancy under the guidance of spatial transcriptome technology (159). Spatial transcriptome can be further employed in the human placenta during the first, second and third trimester, which facilitates the understanding of dynamic changes of KIRs and HLA and communication between uNK and EVT. A comprehensive understanding of the interaction between decidual NK cells and EVTs can provide us with new insights into the pathogenesis of RPL and PE, which urges us to explore more effective diagnostic methods and treatments for pregnancy complications resulting from SA remodeling.

Author contributions

YL designed this study; X-WW and Y-CZ wrote the review; FW and F-JT revised the review. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Dysfunction of CCR1$^+$ decidual macrophages is a potential risk factor in the occurrence of unexplained recurrent pregnancy loss

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Recurrent pregnancy loss (RPL) puzzles 1–3% of women of childbearing age worldwide. Immunological factors account for more than 60% of cases of unexplained RPL (URPL); however, the underlying mechanism remains unclear. Here, using single-cell sequencing data and functional experiments with clinical samples, we identified a distinct population of CCR1$^+$ decidual macrophages (dMφ) that were preferentially enriched in the decidua from normal early pregnancies but were substantially decreased in patients with URPL. Specific gene signatures endowed CCR1$^+$ dMφ with immunosuppressive and migration-regulatory properties, which were attenuated in URPL. Additionally, CCR1$^+$ dMφ promoted epithelial-to-mesenchymal transition (EMT) to promote trophoblast migration and invasion by activating the ERK1/2 signaling pathway. Decidual stromal cell (DSC)-derived CCL8 was the key regulator of CCR1$^+$ dMφ as CCL8 recruited peripheral CCR1$^+$ monocytes, induced a CCR1$^+$ dMφ-like phenotype, and reinforced the CCR1$^+$ dMφ-exerted modulation of trophoblasts. In patients with URPL, CCL8 expression in DSCs was decreased and trophoblast EMT was defective. Our findings revealed that CCR1$^+$ dMφ play an important role in immune tolerance and trophoblast functions at the maternal–fetal interface. Additionally, decreased quantity and dysregulated function of CCR1$^+$ dMφ result in URPL. In conclusion, we provide insights into the crosstalk between CCR1$^+$ dMφ, trophoblasts, and DSCs at the maternal–fetal interface and macrophage-targeted interventions of URPL.

KEYWORDS

decidual macrophages, CCR1, CCL8, trophoblasts, epithelial-to-mesenchymal transition, unexplained recurrent pregnancy loss

Introduction

The development of the semi-allogeneic fetus without rejection by the maternal immune system has aroused widespread concern in reproductive immunology. Delicate maternal immunomodulation depends on the crosstalk between decidual immune cells (DICs), decidual stromal cells (DSCs), and fetal-derived trophoblast cells, which play key roles in embryo implantation, maternal–fetal immune tolerance, placenta formation, and spiral artery remodeling (1–4). Dysregulated immunology disturbs the unique maternal–fetal immune environment, causing deficient decidualization, embryo rejection, and placental malformation, which eventually result in various pregnancy complications, including unexplained recurrent pregnancy loss (URPL) (5–7). Recurrent pregnancy loss (RPL), defined as two or more pregnancy loss before 20-24 weeks of gestation, is a devastating health problem that affects 1–3% of reproductive women (8). In approximately 50% of these patients, RPL has an unknown etiology, defined as URPL (8). Immunological factors account for more than 60% of URPL cases (9). However, the underlying mechanism remains unclear.

As the second-most abundant immune cell at the maternal–fetal interface, decidual macrophages (dMφ) are multifunctional and actively participate in the establishment of a tolerogenic immune microenvironment, defense from invaders, tissue repair and remodeling, scavenging apoptotic cells, and regulation of trophoblast cell activity (10–12). Evidence from studies suggests that dMφ are enriched in the vicinity of the trophoblast invasion front and promote trophoblast migration and invasion, as well as the replacement of vascular smooth muscle cells in spiral artery remodeling (13, 14). However, dMφ from women with URPL show M1-like features with increased expression of activation molecules (such as CD80 and CD86) and inflammatory cytokines but reduced production of anti-inflammatory cytokines such as IL-10 (15). This transformation of dMφ in URPL from an immunosuppressive M2-like phenotype to a pro-inflammatory M1-like phenotype results in adverse effects on the migratory and invasive activity of trophoblast cells (16). Our knowledge of dMφ is still limited to the M1-M2 classification (17, 18), which may not be adequate for understanding their precise roles and changes in the complicated pathological context of URPL. Whether a specific dMφ subset plays a role in the occurrence of URPL? What are the regulatory factors of the subset and mechanisms by which they mediate the phenotypic and functional changes in these cells in URPL? These are the main subjects that we try to explore in this study.

CCR1, as a G protein–coupled receptor for a C-C type chemokine that is broadly expressed by various cells, including tumor cells, myeloid-derived suppressor cells, and monocytes/macrophages (19–21). CCR1, with the highest ligand promiscuity, is a crucial component in cell migration, cell differentiation, immune responses, immune regulation, and other pathophysiological processes by binding to its relative ligands, including CCL3, CCL5-9 (CCL6 and CCL9 are of mouse origin), CCL13-16 and CCL23 (22–24). Previous studies have demonstrated that CCR1 mediates the migration and recruitment of peripheral monocytes and accumulation of metastasis-associated macrophages (25, 26). Moreover, recruitment of CCR1-expressing myeloid cells promotes tumor invasion and metastasis in colorectal cancer (27). In addition, single-cell analysis revealed that CCL3–CCR1 interactions increased macrophage recruitment and anti-inflammatory patterns in tumors (20). Notably, CCR1 expression is more abundant in M2 Mφ than in M1 Mφ (28). Deletion of CCR1 results in fewer M2 Mφ during mammary gland development (29). CCL5 also directly motivates M1 polarization and inhibits M2 polarization through CCR1-mediated activation of the MAPK and NF-κB pathways in drug-induced liver injury (30). Therefore, functional regulation of CCR1 on Mφ is tissue- and ligand-specific. Few studies have reported on CCR1 in pregnancy, except for CCR1 expression in leukocytes, DSCs, glandular epithelium, and luminal epithelium by immunohistochemical staining of the decidual tissue (31). CCR1-mediated modulation on dMφ implicated in normal pregnancy and URPL remains undisclosed and is our concern.

In this study, we aimed to analyze CCR1 expression in dMφ of women with URPL and normal pregnancy and investigate the phenotype and function of $CCR1^+$ dMφ in the patient and control groups. Additionally, we assessed the regulation of $CCR1^+$ dMφ by DSC-derived CCL8. Our data demonstrate that $CCR1^+$ dMφ are preferentially enriched in the decidua from normal early pregnancies and significantly decreased in patients with URPL. More importantly, $CCR1^+$ dMφ in URPL displayed distinct transcriptional profiles and functions compared with those in normal controls. This unique dMφ subset, modulated by CCL8, contributed to the maternal-fetal immune tolerance and the normal function of trophoblast cells. In summary, we provide insights into maternal–fetal crosstalk and suggest $CCR1^+$ dMφ or CCL8/CCR1 signaling as a potential target to prevent URPL.

Materials and methods

Subjects and human sample collection

The collection and use of human tissue samples were approved by the Human Research Ethics Committee of the Obstetrics and Gynecology Hospital, Fudan University, and followed the principles of the Helsinki Declaration (0423-10-HMO). Written informed consent was obtained from all women in this study. Endometrial tissues (n=20) were collected from patients with leiomyomas during hysterectomy. Patients aged between 25 and 40 years with regular menstrual cycles who had

not received hormone therapy or took any medications were considered for this study. Normal decidua, villous tissues, and peripheral blood were obtained from women with clinically normal pregnancies (NPs; terminated for nonmedical reasons, gestational age: 6-10 weeks). All women in this study who had NPs never had spontaneous miscarriage and had at least one live birth before the current pregnancy. Decidual tissues and peripheral blood were collected from patients with URPL who received uterine curettage because of a lack of fetal heartbeat detected by ultrasound at 6-10 weeks of gestation. All URPL subjects had regular ovulatory menstrual cycles and a history of two or more consecutive miscarriages, excluding genetic, anatomic, or endocrine abnormalities; infections; immune disorders (anti-phospholipid antibody syndrome, thrombophilia, systemic lupus erythematosus, Hashimoto's thyroiditis, Graves' disease, rheumatoid arthritis); or poor health habits. Demographic details and characteristics of women with NPs (n=69) and URPL (n=25) are shown in Table 1. All samples were obtained under sterile conditions and divided into two parts: One part was immediately fixed in 4% paraformaldehyde for immunohistochemistry (IHC) studies, and the other part was immediately collected into ice-cold DMEM/F12 medium (Gibco, USA), transported to the laboratory within 30 min, and washed in 1× sterile phosphate buffered saline (PBS) for cell isolation.

Cell lines

HTR-8 cells, an immortalized first-trimester trophoblast cell line, were purchased from the Cell Bank of the Chinese Academy of Sciences, Shanghai, China, and cultured in DMEM/F12 medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA).

Isolation and culture of human endometrial stromal cells (ESCs)

Endometrium tissues were washed twice in 1× PBS and cut and digested in DMEM/F12 medium supplemented with 1.0 mg/ml type IV collagenase (Sigma-Aldrich, USA) for 30 min at 37°C with gentle agitation. The suspension was filtered through a 40-µm nylon mesh (Falcon, USA) and centrifuged at 300×g for 8 min. The collected cells were cultured in phenol red-free DMEM/F12 medium (Genom, China) containing 10% charcoal-stripped FBS (BioSun, China), 1% ITS (Oricellbio, China), and 500 ng/mL puromycin for 24 h in a 37°C humidified incubator containing 5% CO_2. Adherent cells (ESCs) were digested and resuspended in complete medium for other treatments.

TABLE 1 Demographics and clinical characteristics of the population.

	NP (n=69)	URPL (n=25)
Age (years)	29.36 ± 0.50	30.68 ± 0.71
Gestational age (weeks)	8.02 ± 0.11	8.28 ± 0.18
Gravidity	2.52 ± 0.08	3.6 ± 0.14****
Parity	1.2 ± 0.05	0.12 ± 0.07****
Number of abortions	0.32 ± 0.06	2.48 ± 0.13****
Number of live births	1.21 ± 0.05	0.12 ± 0.07****

****p < 0.0001.

Isolation and culture of human DSCs and immune cells

Decidual tissues were trimmed into 1-mm^3 segments and submerged in DMEM/F12 medium supplemented with 1.0 mg/ml type IV collagenase (Sigma-Aldrich, USA) and 150 U/ml DNase I (Sigma-Aldrich, USA). Digestion was performed for 30 min at 37°C with gentle shaking. After digestion, the cells were washed in sterile PBS and filtered through 100, 70, and 40µm sieves. The filtered suspension was centrifuged at 300×g for 8 min and the collected cells were resuspended in DMEM/F12 medium. The suspension was layered on a discontinuous Percoll density gradient (20%/40%/60%; GE Healthcare, USA) and centrifuged for 30 min at 800×g. DSCs were isolated from the 20%/40% Percoll interface, and immune cells were distributed at the 40%/60% Percoll interface. The cells were then washed in sterile PBS for twice. DSCs were cultured in DMEM/F12 medium (Genom, China) containing 10% FBS (Gibco, USA) for 24 h in a 37°C humidified incubator containing 5% CO_2. Immune cells were collected and resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% FBS for further experiments.

Isolation of $CCR1^+$ dMφ

dMφ were isolated from DICs using APC anti-human CD14 (BioLegend, USA) and Anti-APC MultiSort Kit (Miltenyi Biotec, Germany) according to the manufacturer's protocol. Next, to separate $CCR1^-$ and $CCR1^+$ dMφ, the magnetic particles were first removed from dMφ by using the MultiSort Release Reagent (Miltenyi Biotec, Germany). Then dMφ were labeled with CCR1-fluorescein isothiocyanate (FITC) antibody (BioLegend, USA) followed by subsequent incubation with anti-FITC microbeads (Miltenyi Biotec, Germany). Then, magnetic separation was applied to positively select $CCR1^+$ dMφ subset. The purity of $CCR1^+$ dMφ was greater than 90%, as measured by FCM. The cells were cultured in DMEM-F12 medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin.

Preparation of peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated from peripheral blood samples of patients with NPs and URPL using Ficoll density gradient centrifugation (Solarbio, China) at 800×g for 20 min. $CCR1^+$ peripheral monocytes (pMo) were isolated using the same method as that for $CCR1^+$ dMφ. The cells were treated with 50 ng/ml recombinant human macrophage colony-stimulating factor (M-CSF) (MedChemExpress, China).

Reagents and cell treatments

To induce *in vitro* decidualization, ESCs were plated on tissue culture plates, treated with complete medium containing 0.5 mM 8-bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP) (Sigma-Aldrich, USA) and 100 ng/ml MPA (Sigma-Aldrich, USA), and the medium was given fresh every other day and cells were treated for 4 days. The supernatant of cultured cells was collected and stored at −80°C for further analysis.

PBMCs were treated with 100 ng/ml rhCCL8 (R&D Systems, USA) with or without the administration of CCR1 antagonist (BX471) (MedChemExpress, China) at a concentration of 20 μM. A co-culture system of PBMCs and DSCs with or without the addition of 4 μg/mL CCL8 neutralizing antibody (R&D Systems, USA) was established *via* a 0.4-μm pore size Transwell co-culture system (Corning, USA). The neutralizing antibody was pretreated for 2 h. Briefly, PBMCs were placed in the lower chambers and DSCs were seeded in the upper chambers for 24 h before harvest.

A co-culture system of HTR-8 cells with $CCR1^-$ dMφ or $CCR1^+$ dMφ pretreated with control medium or recombinant human CCL8 (rhCCL8) (R&D Systems, USA) at concentrations of 10, 50, 100, and 200 ng/ml was established using a Transwell co-culture system (0.4-μm pore size, Corning, USA). Briefly, $CCR1^-$ or $CCR1^+$ dMφ were placed into the upper chambers and HTR-8 cells were seeded into the lower chambers. The ERK1/2 inhibitor PD98059 (MedChemExpress, China) was used at a concentration of 30 μM.

Flow cytometry (FCM)

Cells were washed in PBS and incubated with fluorochrome-conjugated antibodies for 30 min at 4°C for cell surface staining. The following specific anti-human monoclonal antibodies were used: PerCP-Cy5.5-conjugated anti-CD45, FITC- or APC-conjugated anti-CD14, PE-Cy7- or FITC-conjugated anti-CCR1, AF700-conjugated anti-CD206, PE-conjugated anti-CD163, anti-CD80, and BV421-conjugated anti-CD86. Cells were fixed and permeabilized with BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit (BD Biosciences, USA) according to the manufacturer's protocol. The permeabilized cells were stained for intracellular cytokines as follows: APC-conjugated anti-IL-10, anti-TGF-β, and PE-conjugated anti-IL-8. All antibodies were purchased from BioLegend. FCM was performed using CytoFLEX (Beckman Coulter, USA), and the data were analyzed using FlowJo Version 6.1 software (TreeStar, USA).

Enzyme-linked immunosorbent assay (ELISA)

The secretion of CCL8 in the cultured supernatant samples was determined using ELISA with the human CCL8 ELISA Kit (Abcam, UK) according to the manufacturer's protocol. Absorbance was measured using a spectrophotometer (Biotek, Vermont, USA) at 450 nm.

RNA isolation and quantitative real-time polymerase chain reaction

Total RNA was extracted using TRIzol reagent (Invitrogen, USA) and reverse-transcribed into first-strand cDNA (TaKaRa Biotechnology, Japan) according to the manufacturer's instructions. The synthesized cDNA was amplified using specific primers (Sagon, China) and SYBR Green (Yeasen, China) on the ABI PRISM 7900 Sequence Detection System (Applied Biosystems, USA). The reactions were run in duplicate using RNA samples. Fold change in the expression of each gene was calculated using the $2^{-\triangle\triangle CT}$ method, with actin as an internal control. According to the existing gene sequences in GenBank, primers were designed using computer assistance, and the primer sequences are shown in Table 2.

IHC

IHC was performed on specimens of the human endometrium and decidual tissues from patients with NPs and URPL and villus tissues from patients with NPs. Paraffin-embedded sections were cut to a thickness of 3 μm. Tissue slides were incubated at 60°C for 2 h, and sections were deparaffinized and rehydrated in the order of using xylene and graded ethanol (100%, 95%, 85%, 75%, and 50%). They were soaked in 0.01 M citric acid (pH 6.0) for 20 min at 95°C for antigen retrieval. The slides were then incubated in 3% H_2O_2 for 10 min, blocked with 5% BSA for 20 min at room temperature, and incubated with rabbit anti-CCL8 antibody (Abcam, UK) at 4°C overnight in a humidity chamber. The samples were stained with an anti-rabbit IgG secondary antibody for 30 min and incubated in a DAB substrate solution until the desired staining

TABLE 2 Primer sequences.

Gene	Forward sequence	Reverse sequence
CCL8	CAGTTTCCATTCCAATCACCTG	TTGGTGATTCTTGTGTAGCTCT
ACTIN	CATGTACGTTGCTATCCAGGC	CTCCTTAATGTCACGCACGAT

intensity was reached at room temperature. The sections were then counterstained with hematoxylin, washed in running tap water for 30 min, dehydrated, and cleared through a graded series of ethanol (50%,75%, 85%, 95%, and 100%) and xylene. Finally, the sections were sealed with neutral resin and analyzed using an optical microscope (Olympus BX53, Japan).

Western blot (WB)

Cell lysates were prepared in radioimmunoprecipitation assay lysis buffer (Beyotime, China) containing 1% proteinase inhibitor solution (Beyotime, China) and 1% phosphatase inhibitors (NCM Biotech, China). The protein yield was quantified using a bicinchoninic acid protein assay (Beyotime, China), and the protein was boiled with a 5× loading buffer (NCM Biotech, China). Total protein samples (20 μg) were separated using SDS–PAGE (Beyotime, China) and transferred to polyvinylidene difluoride membranes (Millipore, USA) for 1 h. Nonspecific binding sites were blocked by incubating the membranes with the QuickBlock™ Blocking Buffer (Beyotime, China) for WB for 10 min, followed by overnight incubation with primary antibodies against E-cadherin (1:5000; ProteinTech, USA), N-cadherin (1:2000; ProteinTech, USA), vimentin (1:1,000; PTM bio, China), ERK1/2 (1:1000, Proteintech, USA), p-ERK1/2 (1:1000, ProteinTech, USA), and tubulin (1:1000; Beyotime, China) diluted in blocking buffer (Beyotime, China) overnight at 4°C with gentle shaking. Primary antibodies were removed by washing the membranes three times in TBS-T, and the membranes were incubated for 1 h with secondary antibody (1:5000, arigo Biolaboratories Corp, China) at room temperature. After washing three times with TBS-T, immuno-positive bands on the blots were visualized using an enhanced chemiluminescence detection system (NCM Biotech, China).

Scratch wound healing assay

Scratch wound healing assay was performed to assess cell motility. HTR-8 cells were seeded in six-well plates. $CCR1^-$ and $CCR1^+$ dMφ were pretreated with or without 100 ng/ml rhCCL8 for 24 h. When the HTR-8 cell density reached 80%, scratches were made with 1-ml pipette tips, and wounded monolayers were washed three times with PBS, followed by co-culture with pretreated $CCR1^-$ and $CCR1^+$ dMφ in a 0.4-μm pore size Transwell co-culture system with the addition of serum-free medium. Cells were incubated with 5% CO_2 at 37°C for 24 h, and the wound healing rates were determined and photographed. The images were analyzed and calculated using ImageJ software (NIH, USA).

Cell migration assays

An 8-μm pore size filter (Corning, USA) was used to perform Transwell migration. $CCR1^+$ pMo and dMφ were seeded into the upper chamber without serum and complete medium with 10% serum with or without 100 ng/ml rhCCL8 was added to the lower chamber. Cells migrated to the lower chamber after 48 h. Cells were gently washed with PBS and fixed with 4% paraformaldehyde and then stained with crystal violet. Images were taken using a microscope and counted with Image J software.

Matrigel invasion assay

A Transwell system with 8-μm chambers (Corning, USA) in a 24-well plate was precoated with Matrigel matrix (Corning, USA) to evaluate the invasive ability of HTR-8 cells. $CCR1^-$ and $CCR1^+$ dMφ were pretreated with or without 100 ng/ml rhCCL8. Then, HTR-8 cells resuspended in serum-free medium were placed into the upper chamber and the lower chamber was filled with complete medium, $CCR1^-$ dMφ, or $CCR1^+$ dMφ. The plates were incubated at 37°C for 48 h. The inserts were washed with PBS, and non-invading cells were removed from the upper chambers. The inserts were then fixed in 4% paraformaldehyde and stained with crystal violet. The invaded cells were imaged by microscopy and quantified by counting cells in five random fields using ImageJ software.

Single-cell transcriptomics analysis

Single-cell sequencing data were downloaded from the Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/), Genome Sequence Archive (GSA) database, and ArrayExpress from EMBL-EBI. The original source of single-cell raw data is CRA002181 (32), which comes from the decidua of 15 healthy controls and 9 patients with URPL in the GSA database. The expression profile of the blood and normal decidua of early

pregnancy was derived from E-MTAB-6678 (33) in the ArrayExpress database, and endometrial samples were derived from GSE111976 (34) in the GEO database.

Data processing, high-dimensional reduction, and clustering

Fastq files of raw data were downloaded from the GSA database, and Cellranger (10X genomics) was used to process, align, and generate the feature-barcode unique molecular identifier matrices with default parameters. The gene expression matrix was analyzed using the R package Seurat (CreateSeuratObject) (35). To filter out low-quality cells, we first removed cells with detected gene numbers (<500 or >3000) and high mitochondrial content (≥10%). The expression matrix was then normalized (NormalizeData), and highly variable genes were identified by fitting the mean–variance relationship (FindVariableGenes). Next, we performed principal component analysis using highly variable genes and integrated samples using the R package Harmony (RunHarmony). The same principal components were used to embed cells in the K-nearest neighbor graph (FindNeighbors) and cluster them using the Louvain algorithm at a resolution of 0.5 (FindClusters). To label the cell clusters, we used a set of classic marker genes to annotate each cell type.

Functional enrichment and signal pathway analyses

To investigate the function of macrophage subsets, we calculated the fold change of all detected genes (FindMarkers) and performed gene set enrichment analysis (GSEA) to determine the function of macrophage subsets in the decidua and peripheral blood using the R package fgsea.

Cell–cell communication analysis

To infer cell–cell communication between DIC and other cell types, we used CellChat to calculate and visualize cell–cell communication.

Statistical analysis

Statistical analysis was performed using GraphPad Prism Version 7 (GraphPad, USA). All data were first tested for normality before statistical analysis. For parametric data, Student's t-test for two-group comparisons or one-way ANOVA for multiple group comparisons were used. For non-parametric data, Mann-Whitney test were used to compare the difference between two groups. Data are presented as mean ± SEM. The criterion for statistical significance was set at $p< 0.05$.

Results

Distinct CCR1$^+$ dMφ in patients with URPL.

To characterize dMφ during early pregnancy, two published single-cell databases were integrated and analyzed (33, 34). The results showed that dMφ from the first trimester of pregnancy had a substantially higher expression of *CCR1* than those in the proliferative and secretory endometrium (Figure 1A). Based on *CCR1* expression, we divided macrophages into CCR1$^+$ and CCR1$^-$ subsets. A comparable percentage of CCR1$^+$ Mφ was found in the proliferative and secretory endometrium; however, the infiltration of CCR1$^+$ Mφ within the first trimester decidua considerably increased approximately 2–3 folds (Figure 1B). The decidua-specific expression of *CCR1* in macrophages suggests the influence of a unique maternal–fetal microenvironment rather than reproductive hormones. We then conducted a gene ontology (GO) analysis to decipher the functional characteristics of CCR1$^+$ dMφ. Compared with CCR1$^+$ peripheral monocytes (pMo) (Figure 1C) and CCR1$^-$ dMφ (Figure 1D), CCR1$^+$ dMφ were strongly enriched in cell migration, tissue remodeling, anti-inflammatory responses, and vascularization functions. Thus, CCR1$^+$ dMφ predominantly accumulate in the uterus during early pregnancy and are characterized by an immunosuppressive propensity.

The distinct CCR1$^+$ dMφ population was clearly decreased in patients with URPL (Figure 1E). Moreover, in CCR1$^+$ dMφ from patients with URPL, genes associated with anti-inflammatory responses (e.g., *IL10*, *CD163*, *MRC1*, *TGFB1*, and *IL4*) and extracellular matrix degradation (e.g., *MMP9*, *MMP10*, *MMP14*, *MMP19*, and *ICAM1*) were less enriched, whereas those responsible for activation and pro-inflammatory responses (e.g., *CD86*, *IL1B*, *IL2*, and *TNF*) were significantly enriched (Figure 1F). These sequencing results suggest a distinct CCR1$^+$ dMφ population with a changed proportion and functional status in patients with URPL.

To validate the results from the single-cell profiling analysis, we performed FCM and found that dMφ had a significantly higher expression of CCR1 (59.98 ± 3.13) than did maternal pMo (28.04 ± 0.81) and nonpregnant endometrial Mφ (eMφ; 2.87 ± 0.79; Figure 1G). Moreover, CCR1$^+$ dMφ represented a more immunosuppressive phenotype with a higher expression of CD163, CD206, IL-10, IL-8, and TGF-β but lower expression of CD80 and CD86 than CCR1$^+$ pMo (Figure 1H). In patients with URPL, remarkably decreased CCR1 expression was detected in both dMφ and pMo (Figures 1I, J). More importantly, the immunosuppressive phenotype of CCR1$^+$ dMφ was remarkably attenuated in patients with URPL. In those with

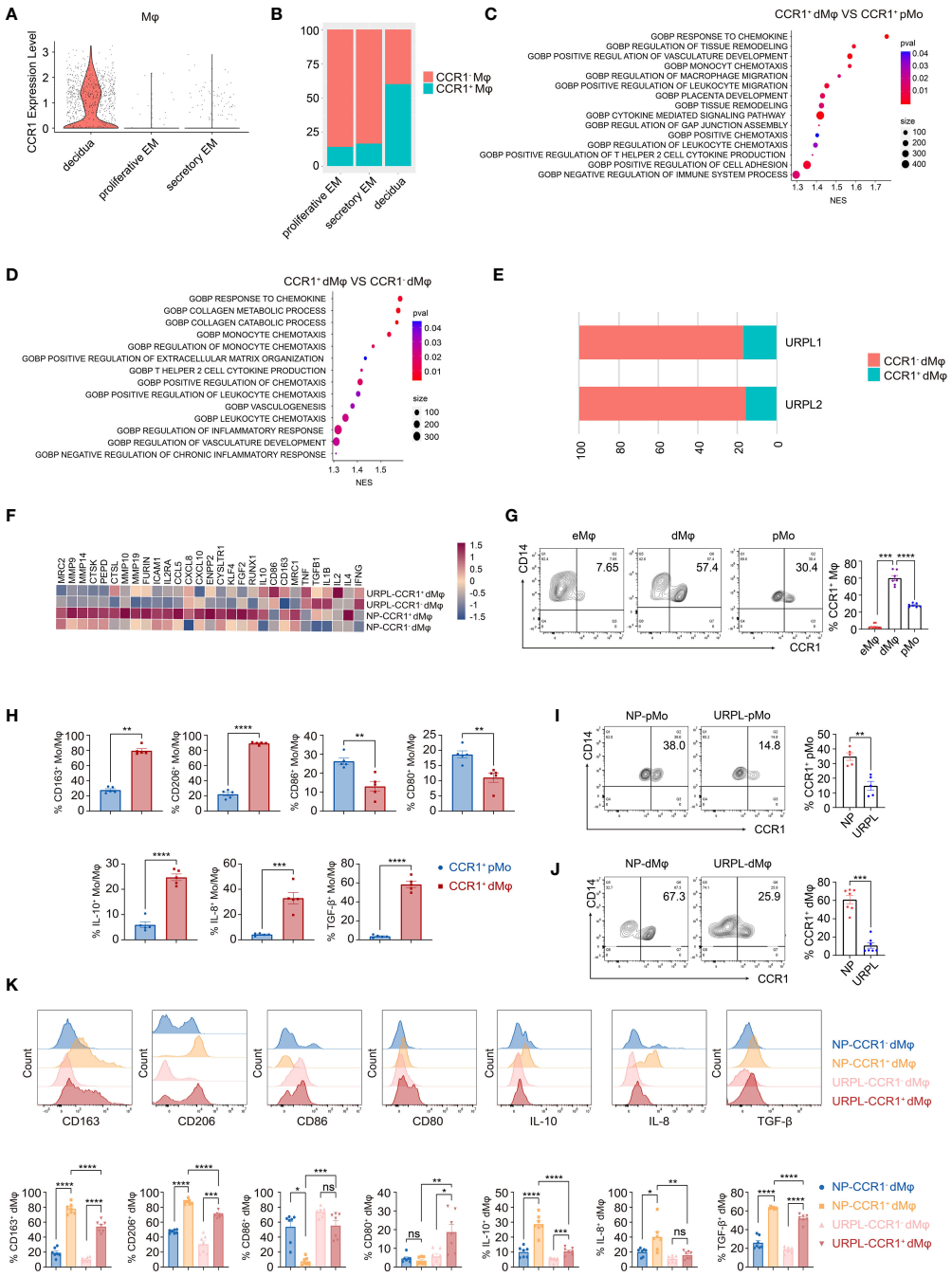

FIGURE 1
CCR1$^+$ dMφ possess a distinct phenotype in URPL patients. **(A)** The violin plot representing CCR1 expression of macrophages in the endometrium and decidua. **(B)** Histogram indicating the proportion of CCR1$^-$ and CCR1$^+$ macrophages in the endometrium and decidua. **(C, D)** Gene Ontology (GO) analysis was conducted to identify specific pathways between CCR1$^+$ peripheral monocytes (pMo) and decidual macrophages (dMφ) **(C)** and between CCR1$^-$ and CCR1$^+$ dMφ **(D)**. **(E)** Histogram illustrating the percentage of CCR1$^-$ and CCR1$^+$ dMφ in patients with unexplained recurrent pregnancy loss (URPL). **(F)** Heatmap depicting the expression of selected genes in CCR1$^-$ and CCR1$^+$ dMφ from normal pregnancies (NPs) and URPL. **(G)** Representative plots and quantification of CCR1$^+$ eMφ (n=10), dMφ (n=7), and pMo (n=7). **(H)** FCM of the expression of indicated membrane molecules and cytokines in CCR1$^+$ pMo and CCR1$^+$ dMφ. 5 samples from two independent experiments. **(I, J)** Representative plots and quantification of CCR1$^+$ pMo (n=5; 5) **(I)** and CCR1$^+$ dMφ (n=7; 7) **(J)** between NPs and URPL. **(K)** Quantification of membrane molecules and intracellular cytokines of CCR1$^-$ and CCR1$^+$ dMφ from women with NPs (n=5) and URPL (n=5). Data are presented as mean ± SEM. ns, not significant; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

NP, CCR1$^+$ dMφ exhibited a higher expression of CD163 and CD206 but lower expression of CD86 than CCR1$^-$ dMφ. In patients with URPL, CCR1$^+$ dMφ decreased the expression of CD163 and CD206 but increased the expression of CD80 and CD86, compared with CCR1$^+$ dMφ from control donors with NPs (Figure 1K). The production of anti-inflammatory cytokines IL-10 and TGF-β and a proangiogenic factor, IL-8, was substantially upregulated in CCR1$^+$ dMφ than in CCR1$^-$ dMφ of normal controls but markedly reduced in CCR1$^+$ dMφ of patients with URPL (Figure 1K). Collectively, these data demonstrate that CCR1$^+$ dMφ exhibit an immunosuppressive and anti-inflammatory phenotype that is conducive to an immune-tolerant microenvironment, whereas decreased numbers of CCR1$^+$ dMφ with dysfunction may be involved in the occurrence of URPL.

CCR1$^+$ dMφ promote epithelial-to-mesenchymal transition (EMT) to facilitate trophoblast migration and invasion

Besides the enhanced activated and inflammatory phenotype of CCR1$^+$ dMφ in patients with URPL, CCR1$^+$ dMφ in these patients have a decreased function in tissue remodeling and negative regulation on trophoblast cell migration, as revealed by single-cell sequencing analysis (Figure 2A). Next, we probed the effects of CCR1$^+$ dMφ on the biological behavior of trophoblasts. CCR1$^-$ dMφ and CCR1$^+$ dMφ were isolated and co-cultured with HTR-8 cells (a human extravillous trophoblast cell line) respectively in a non-contact Transwell system. Compared to co-culture with CCR1$^-$ dMφ, the migration and invasion of HTR-8 cells significantly increased when co-cultured with CCR1$^+$ dMφ (Figures 2B, C). A recent study demonstrated that EMT plays an important role in the regulation of trophoblast migration and invasion, and this process is promoted by M2 macrophages but inhibited by M1 macrophages (7). WB was performed to analyze EMT markers and signaling pathways involved in HTR-8 after co-culture with CCR1$^+$ dMφ or CCR1$^-$ dMφ. In the CCR1$^+$ dMφ co-culture group, the expression of the epithelial marker E-cadherin was markedly decreased, whereas that of the mesenchymal markers N-cadherin and vimentin was dramatically increased in HTR-8 cells (Figure 2D). The expression levels of E-cadherin, N-cadherin and vimentin were comparable between the HTR-8 cells cultured alone and those co-cultured with CCR1$^-$ dMφ (Figure 2D). Moreover, CCR1$^+$ dMφ co-culture increased the phosphorylation of ERK1/2 in HTR-8 cells in a time-dependent manner (Figure 2E). A specific EKR1/2 inhibitor, PD98059, was applied to the CCR1$^+$ dMφ-HTR-8 co-culture system and effectively abrogated the CCR1$^+$ dMφ-induced EMT of trophoblasts by reducing the expression of N-cadherin and vimentin but increasing the expression of E-cadherin (Figure 2F). Furthermore, the migratory and invasive activities of HTR-8 cells were determined following treatment with the EKR1/2 inhibitor. As shown in Figures 2G, H, ERK1/2 inhibition attenuated the migration and invasion of CCR1$^+$ dMφ cells. These data suggest that CCR1$^+$ dMφ induce EMT to promote trophoblast migration and invasion by activating the ERK1/2 pathway. Consistent with the decreased and dysfunctional CCR1$^+$ dMφ in patients with URPL, deficient EMT of trophoblasts was observed in patients with URPL, which was manifested as upregulated expression of E-cadherin and downregulated expression of N-cadherin in placental villous tissues, mainly in cytotrophoblasts (Figure 2I). Together, our findings suggest that dysregulated CCR1$^+$ dMφ affect EMT, leading to inadequate trophoblast migration and invasion, which is involved in the occurrence of URPL.

Upregulated CCL8 in DSCs is a candidate regulator of CCR1$^+$ dMφ

Next, we examined the possible regulator(s) of CCR1$^+$ dMφ and underlying mechanisms in early pregnancy. We investigated the interaction between dMφ and other decidual cells using single-cell sequencing analysis (33, 34). CellChat analysis of single-cell sequencing in the endometrium and decidua revealed a potentially increased interaction between the stromal cells and macrophages in early pregnancy compared with that in the endometrium (Figures 3A, B). We also compared the overall communication probabilities of the two databases (33, 34). Intriguingly, 43 out of 66 pathways were highly active, albeit at different levels, in the decidual tissues (Figure 3C). The CCL signaling pathway was activated in the decidua (Figure 3C). As depicted in Figure 3D, the CCL pathway exhibited abundant signaling interactions between DSCs and dMφ. We then analyzed changes in the expression of CCL molecules and found that the expression levels of *CCL13*, *CCL23*, *CCL28*, *CCL3L3*, *CCL4*, *CCL5*, and *CCL8* were markedly higher in the decidua than in the proliferative and secretory endometrium (Figure 3E). Specifically, only two CCL pathways, CCL8-CCR1 and CCL8-CCR2, were significantly enriched in DSCs and dMφ (Figure 3F). In addition, single-cell sequencing suggested that CCL8, a ligand of CCR1, was highly expressed in DSCs compared with that in nonpregnant ESCs (Figures 3E, G). CCL8 can bind to several receptors, including CCR1, CCR2, CCR3, CCR5, and CCR8 (24). However, except for CCR1, other receptors were scarcely expressed on macrophages from either the nonpregnant endometrium or pregnant decidua (Figure 3H). Thus, decidua-derived CCL8 is a possible ligand that interacts with CCR1$^+$ dMφ.

We then measured CCL8 expression at the maternal–fetal interface. As shown in Figure 3I, compared with the endometrium and villous, higher CCL8 expression was detected in the decidua from normal early pregnancy.

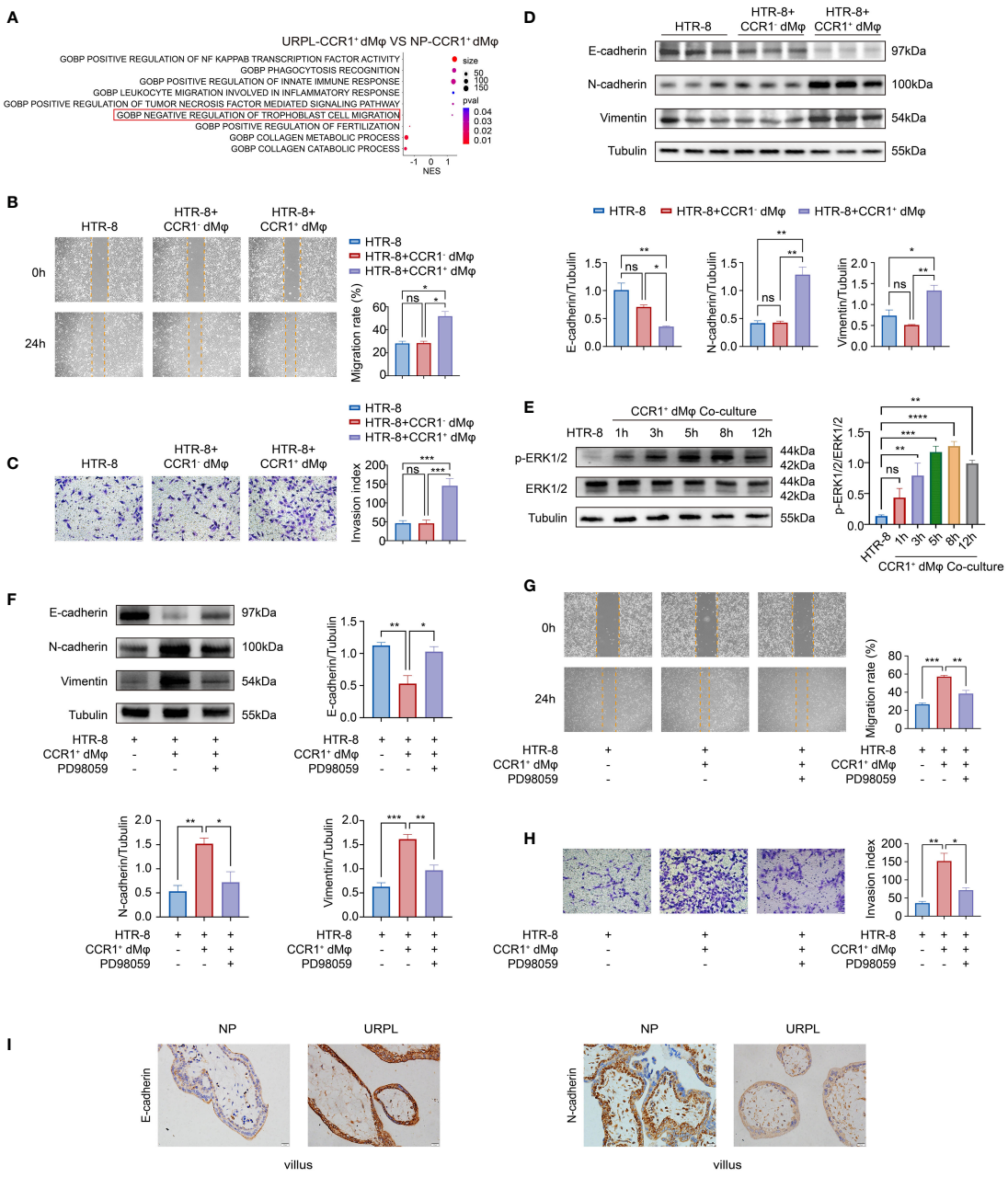

FIGURE 2
CCR1$^+$ dMφ promote the migration, invasion, and EMT process of trophoblast cells. **(A)** Selected GO pathways of the DEGs of CCR1$^+$ dMφ in NPs and URPL. **(B–D)** HTR-8 cells were co-cultured with CCR1$^-$ dMφ or CCR1$^+$ dMφ. **(B)** Scratch wound healing assay and quantitation were established to determine the migratory properties of trophoblast cells. 5 samples from two independent experiments. **(C)** Matrigel invasion assay and quantitation were performed to analyze the invasive capacity of trophoblast cells. 5 samples from two independent experiments. **(D)** Whole lysates of trophoblast cells were detected for the expression of EMT markers (n=3/group, 2 repeated experiments). **(E)** WB analysis showing the alternation of ERK1/2 pathways in HTR-8 cells co-cultured with CCR1$^+$ dMφ. Images are representative of 3 samples from 3 independent experiments. **(F–H)** HTR-8 cells were pretreated with an ERK1/2 inhibitor (PD98059) before co-culture with CCR1$^+$ dMφ. **(F)** Immunoblots showing the expression of E-cadherin, N-cadherin, and vimentin in HTR-8 cells treated as indicated. Images are representative of 3 samples from 3 independent experiments. **(G)** Scratch wound healing assay and **(H)** invasion assay were performed in HTR-8 cells treated as indicated. 5 samples from two independent experiments. **(I)** Representative immunohistochemical staining images showing the expression of EMT markers in human villous tissues from women with NPs (n=3) and URPL (n=3). The results were representative of three separate experiments. Data are presented as mean ± SEM. ns, not significant; *p < 0.05, **p < 0.01, ***p < 0.001.

Furthermore, both mRNA and protein expression levels of CCL8 were dramatically increased during *in vitro* decidualization (Figures 3J, K). However, immunochemistry staining showed that the decidua from patients with URPL exhibited markedly downregulated CCL8 expression compared with that from NPs (Figure 3L). We also identified significantly lower levels of CCL8 in DSCs from patients with URPL (Figures 3M, N). These findings indicate that elevated CCL8 during stromal cell decidualization is a possible regulator of CCR1$^+$ dMφ, and abnormal CCL8-CCR1$^+$ dMφ communication may be implicated in URPL.

CCL8 recruits and educates CCR1$^+$ pMφ into CCR1$^+$ dMφ-like immunosuppressive subsets

Next, we investigated the regulatory effects of DSC-derived CCL8 on CCR1$^+$ dMφ. We first performed a chemotaxis assay and found that CCL8, as a chemokine, potently promoted the migration of both CCR1$^+$ pMo and CCR1$^+$ dMφ (Figures 4A, B), suggesting that CCL8 contributes to the recruitment and residence of CCR1$^+$ dMφ in early pregnancy.

CCR1$^+$ dMφ was characterized by an anti-inflammatory phenotype in women with NP, which changed to an activated and less immunosuppressive status in patients with URPL. Whether CCL8 influences the unique phenotype of CCR1$^+$ dMφ during early pregnancy remains unknown. Accordingly, pMφ were treated with recombinant human CCL8 (rhCCL8), and phenotypic changes were analyzed using FCM. Higher expression of CD163 and CD206 and lower expression of CD86 were detected in CCR1$^+$ pMφ treated with rhCCL8, whereas the expression of CD80 remained unchanged. In addition, CCL8-treated CCR1$^+$ pMφ produced more anti-inflammatory cytokines, including IL-10 and TGF-β, and the proangiogenic factor IL-8 (Figure 4C). Significantly, a specific CCR1 inhibitor, BX471, was applied during the *in vitro* stimulation of pMφ with CCL8, and all CCL8-mediated effects on pMφ, including increased expression of immunosuppressive markers and anti-inflammatory cytokines, could be abrogated by BX471. These results suggest that the CCL8–CCR1 interaction is conducive to the inactive and anti-inflammatory phenotype of macrophages at the maternal–fetal interface.

Similar results were obtained in the indirect contact co-culture system of pMφ and DSCs using Transwell chambers. DSCs effectively shifted pMφ into an anti-inflammatory phenotype with a higher expression of CD163, CD206, IL-10, TGF-β, and IL-8 but lower expression of CD80 and CD86 (Figure 4D). This effect was partially blocked by a neutralizing antibody against CCL8 (Figure 4D). Collectively, DSC-derived CCL8 can recruit CCR1$^+$ pMo from the maternal periphery and induce an immunosuppressive phenotype, contributing to maternal–fetal immune tolerance.

CCL8 enhances the function of CCR1$^+$ dMφ in promoting trophoblast migration and invasion

We have demonstrated that CCR1$^+$ dMφ could promote the migration, invasion, and EMT of trophoblast cells. Whether CCL8 has an impact on this function of CCR1$^+$ dMφ is unknown. Therefore, HTR-8 cells were co-cultured with rhCCL8 preteated-CCR1$^+$ dMφ or CCR1$^-$ dMφ, followed by migration and Transwell assays. Consistent with the above results, CCR1$^+$ dMφ significantly facilitated the functions of HTR-8 cells, while CCR1$^-$ dMφ, whether treated with rhCCL8 or not, had less effects on migration, invasion or EMT process of HTR-8 cells (Figures 5A–C). Compared with CCR1$^+$ dMφ, CCL8-pretreated CCR1$^+$ dMφ showed a significantly enhanced capacity to promote trophoblast migration and invasion (Figures 5A, B). Moreover, CCL8-pretreated CCR1$^+$ dMφ co-culture further increased the expression of N-cadherin and vimentin and decreased the expression of E-cadherin in trophoblast cells (Figure 5C), indicating a more activated EMT process than that induced by CCR1$^+$ dMφ. In addition, CCR1$^+$ dMφ pretreated with different concentrations of CCL8 promoted the activation of the ERK1/2 pathway in trophoblast cells in a concentration-dependent manner (Figure 5D). These data suggest that CCL8 has a positive effect on the function of CCR1$^+$ dMφ in promoting EMT-mediated trophoblast migration and invasion.

Discussion

This study is the first one to report dMφ-specific expression of CCR1 in early pregnancy. Based on CCR1 expression, dMφ can be divided into two distinct subsets. Among them, CCR1$^+$ dMφ possessed an anti-inflammatory signature and was able to promote the migration, invasion, and EMT process of trophoblast cells through the ERK1/2 signaling pathway. Moreover, DSC-derived CCL8 was identified as a regulator of CCR1$^+$ dMφ. Through its interaction with CCR1, CCL8 recruited CCR1$^+$ monocytes from the maternal periphery and further instructed these cells into a CCR1$^+$ dMφ-like phenotype. The CCR1$^+$ dMφ-mediated function of trophoblasts was also strongly reinforced by CCL8. More importantly, the crosstalk among DSCs, CCR1$^+$ dMφ, and trophoblasts was weakened in patients with URPL, which manifested as decreased CCL8 expression in DSCs and abolished proportion of CCR1$^+$ dMφ, accompanied by a pro-inflammatory phenotypic transformation and deficient EMT of trophoblasts. Our results indicate that CCR1$^+$ dMφ play an important role in immune tolerance and trophoblast functions at the maternal–fetal interface. Dysfunctional CCR1$^+$ dMφ are closely associated with the defective crosstalk between DSCs and trophoblast cells, which is implicated in the pathogenesis of URPL (Figure 6).

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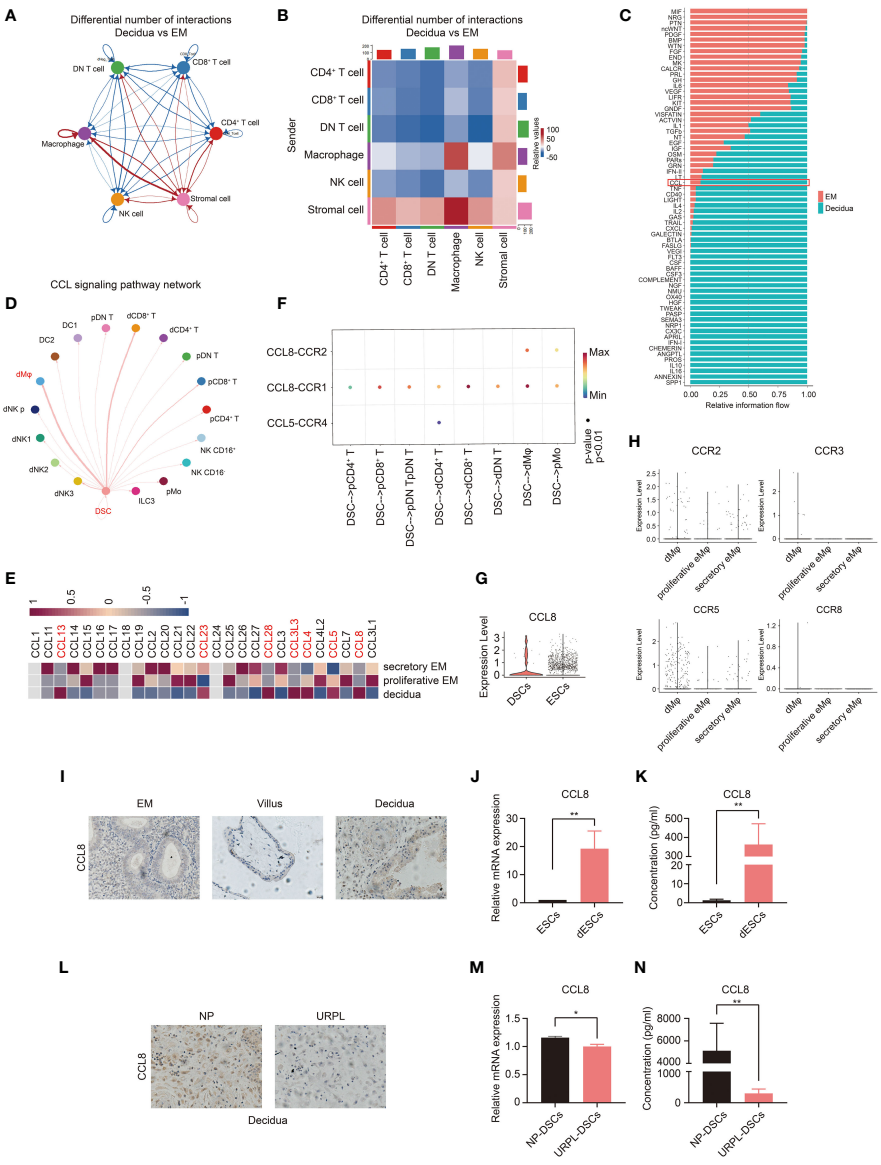

FIGURE 3
Single-cell analysis predicts the CCL8–CCR1 interaction between DSCs and macrophages during early pregnancy. **(A, B)** Number of ligand–receptor pairs between any pair of two cell populations. The edge width and shade are proportional to the indicated number of ligand–receptor pairs. **(C)** Signaling pathways were ranked based on their differences of overall information flow within the inferred networks between the endometrium and decidua. **(D)** Inferred CCL signaling networks in the decidua (circle plot). **(E)** Heatmap showing the expression of selected CCL genes in the proliferative and secretory endometrium and decidua. **(F)** The significant ligand–receptor pairs of the CCL pathway that contributed to the signaling sending from decidual stromal cells (DSCs) to immune cells. The dot color and size illustrate the calculated communication probability and p-values. **(G)** The violin plot referring to log-normalized expression values of CCL8 in DSCs and endometrial stromal cells (ESCs). **(H)** The violin plot representing log-normalized expression values of CCL8 receptors in the endometrium and decidua. **(I)** Paraffin sections of the endometrium (n=3), villous tissue (n=3), and decidua (n=3) were tested for CCL8 expression using IHC. **(J, K)** The mRNA level (n=6) and CCL8 concentration (n=10) before and after in vitro decidualization of ESCs. **(L)** Representative IHC images of CCL8 in human decidua tissues from women with NPs (n=3) and URPL (n=3). **(M, N)** The mRNA level and CCL8 concentration between DSCs from women with NPs (n=6) and URPL (n=6). Data are presented as mean ± SEM. and are representative of at least two separate experiments. *p < 0.05, **p < 0.01.

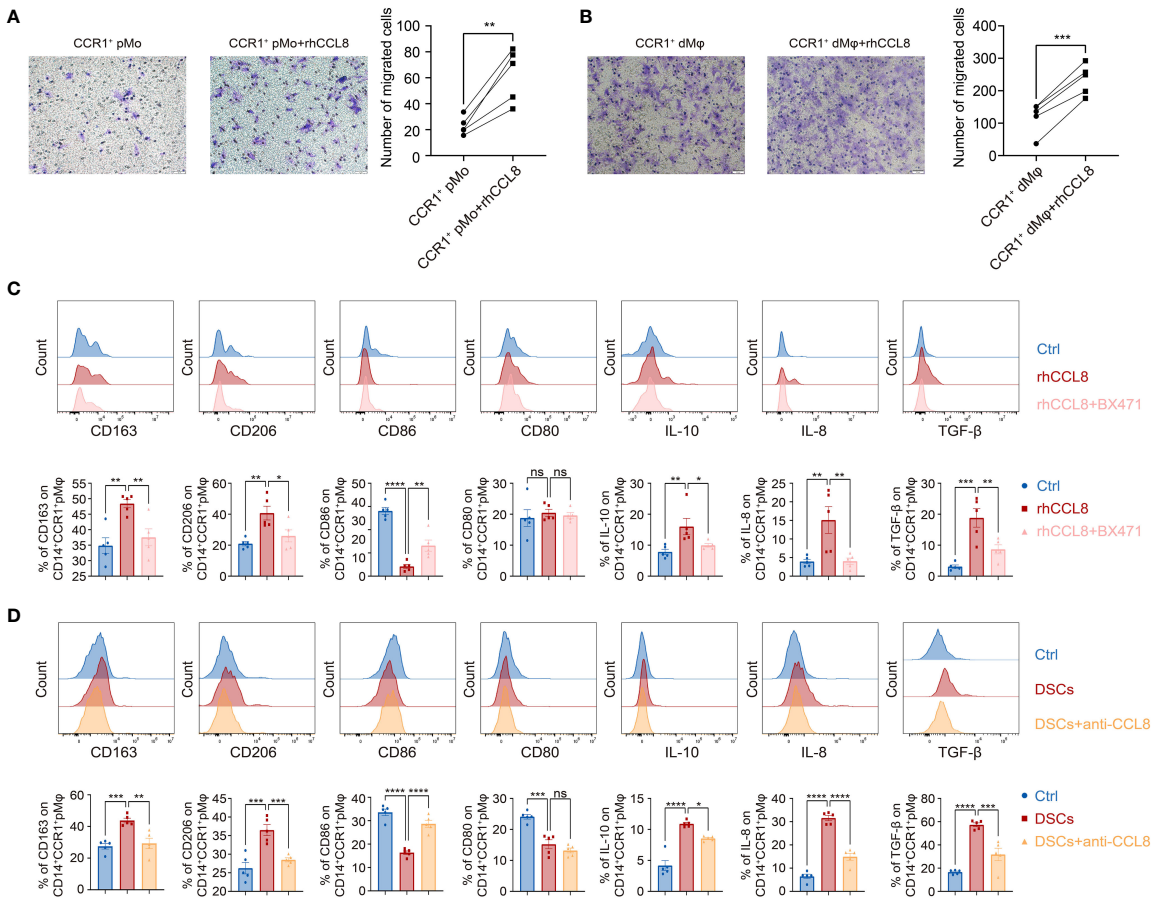

FIGURE 4
DSC-derived CCL8 recruits and educates CCR1$^+$ pMφ towards CCR1$^+$ dMφ phenotype. **(A, B)** Representative images and quantification of migration of the CCR1$^+$ pMo (n=5 per group) **(A)** and dMφ (n=5 per group) **(B)** stimulated by rhCCL8. **(C)** Representative plots and quantification of the membrane molecules and cytokines in CCR1$^+$ pMφ treated with rhCCL8 in the presence or absence of the CCR1 inhibitor, BX471 (n=5 per group). **(D)** Flow cytometric analysis and quantification of the indicated membrane molecules and cytokines in CCR1$^+$ pMφ co-cultured with DSCs in the presence or absence of the CCL8 neutralizing antibody (n=5 per group). Data are presented as mean ± SEM. and are representative of at least two separate experiments. ns, not significant; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Abnormal numbers and proportions of macrophages have been observed in early pregnancy loss, although the current results are controversial and lack a specific mechanism. Yang et al. found fewer dMφ in patients with spontaneous abortion and aborted mice compared with that in women with NPs and normal pregnant mice. One study applied IHC and reported no significant differences in the ratio of dMφ to DICs in patients with URPL (36). Recently, with the rapid development of the single-cell sequencing technology, the composition and phenotype of immune cells at the maternal–fetal interface have been further understood. Chen et al. found that dMφ showed a slightly increased ratio in patients with URPL compared with that in normal tissues (37). However, validated by both single-cell sequencing analysis and FCM, women with URPL showed a decreased dMφ population (32). In our study, we found significant enrichment of CCR1$^+$ Mφ (around 60%) in the decidua during early pregnancy and a small proportion (<10%) of this subset in the nonpregnant endometrium. However, in patients with URPL, the percentage of CCR1$^+$ dMφ was substantially reduced, suggesting that decreased total dMφ in URPL was mainly due to the decrease in the CCR1$^+$ dMφ subset. The origin of dMφ includes embryonic origin and recruitment of peripheral monocytes (38). dMφ are recruited and enriched by VEGF, CSF-1, and RANTES (39, 40) and can adhere and reside in the decidua under the action of CD74, RANKL, and lysophosphatidic acid (4, 41, 42). Here, we identified that CCL8, which was upregulated in DSCs during the first trimester, displayed an efficient chemotactic effect on both CCR1$^+$ pMo and CCR1$^+$ dMφ. Interestingly, the expression of DSC-derived CCL8 and proportion of CCR1$^+$ pMo were remarkably downregulated in patients with URPL. Thus, attenuated CCL8-mediated recruitment and residence of

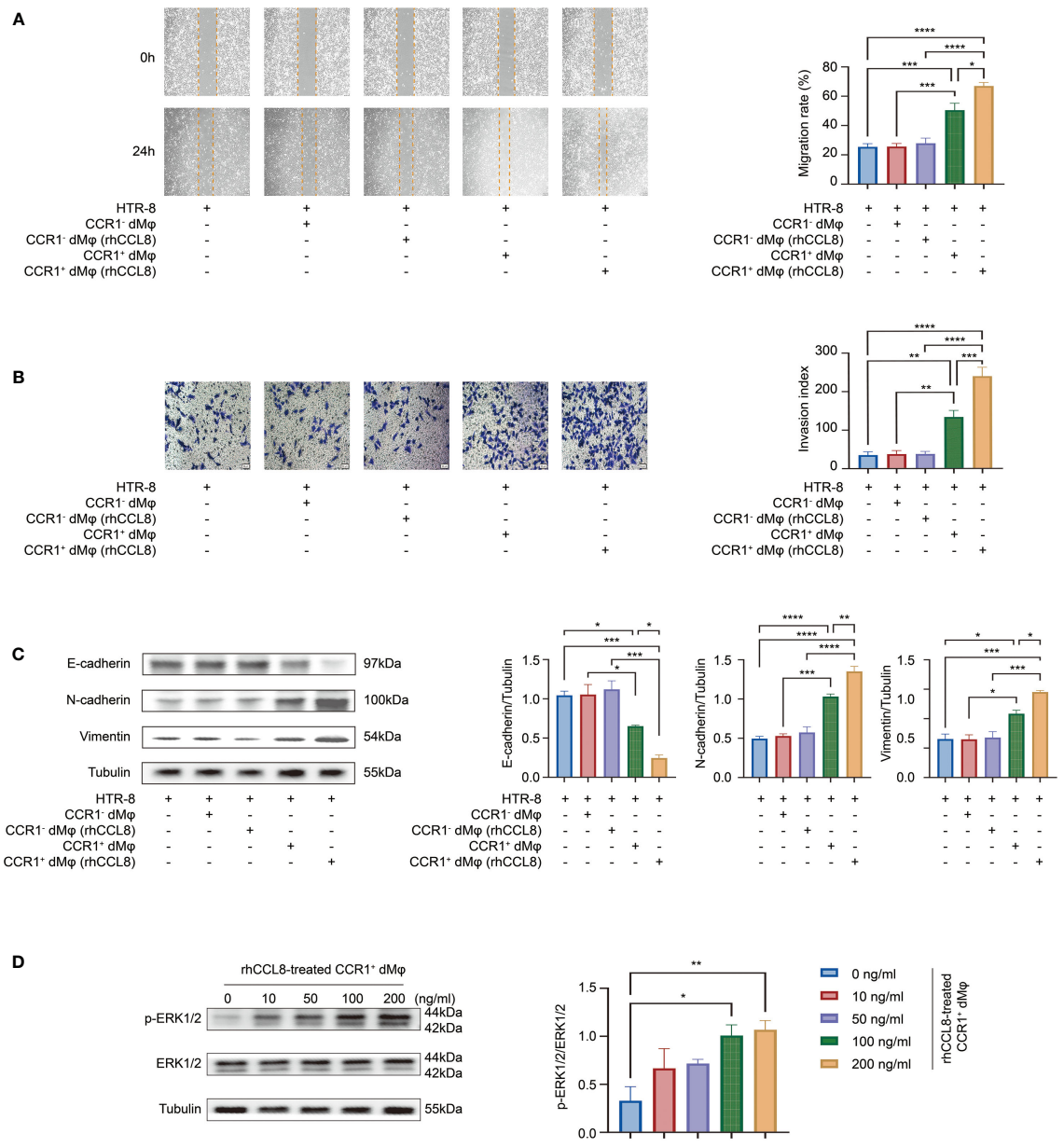

FIGURE 5
CCL8 enhances the effect of CCR1$^+$ dMφ on trophoblast functions. **(A–C)** HTR-8 cells were co-cultured with CCR1$^-$ dMφ or CCR1$^+$ dMφ pretreated with or without rhCCL8. **(A)** Scratch wound healing assay and quantitation were used to represent trophoblast migration (n=5 per group, 2 independent experiments). **(B)** Invasion assay and quantitation were applied to show the invasive activity of trophoblast cells (n=5 per group, 2 independent experiments). **(C)** WB was performed to examine the expression of E-cadherin, N-cadherin, and vimentin in HTR-8 cells treated as indicated. Images are representative of 3 samples from 3 independent experiments. **(D)** Effects of CCR1$^+$ dMφ pretreated with different dosages of rhCCL8 on the activation of the ERK1/2 pathway in HTR-8 cells. Images are representative of 3 samples from 3 independent experiments. Data are presented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

CCR1$^+$ dMφ may contribute to the decreased total dMφ in patients with URPL.

Over the years, macrophages have been categorized under the conventional M1/M2 classification, and dMφ are no exception. dMφ possess a variety of M2-type macrophage surface molecules, such as CD206 and CD163, and highly express anti-inflammatory factors; thus, dMφ are widely accepted as M2 in NP (11). Activation of dMφ toward the M1 phenotype and increased number of M1 dMφ have been correlated with the pathology of URPL (43, 44). However, with deep single-cell analysis, multiple studies have found that the expression profiles of macrophages in different tissues

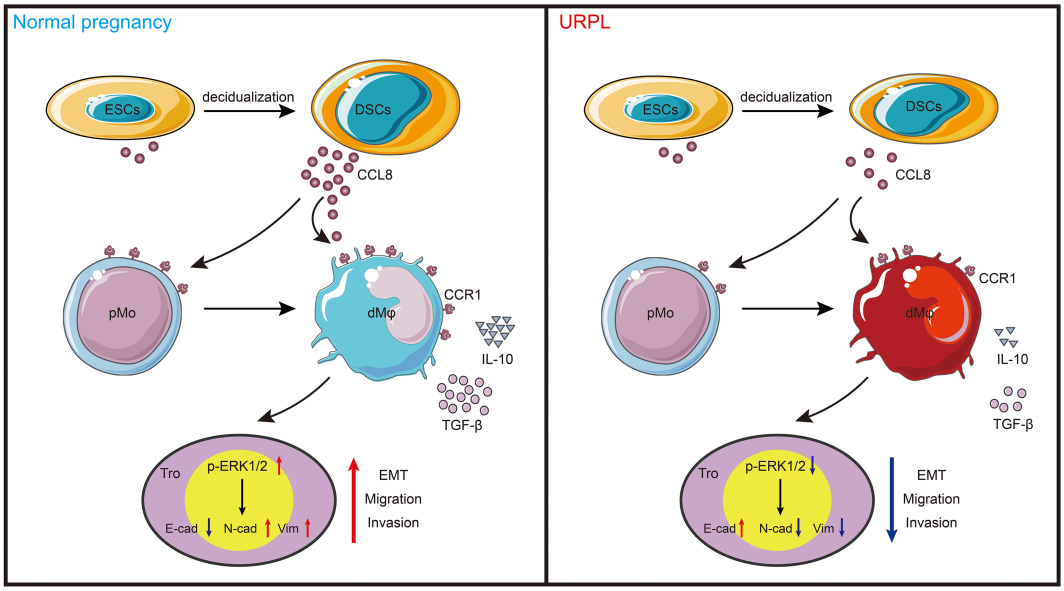

FIGURE 6
Schema of how CCR1$^+$ dMφ function at the maternal–fetal interface. CCL8 expression is increased substantially during endometrial decidualization. Through interaction with CCR1, CCL8 attractes CCR1$^+$ pMo from peripheral blood and skews them into a CCR1$^+$ dMφ-like phenotype, which displays immunosuppressive phenotype and produces more anti-inflammatory cytokines. This crosstalk further supports the migratory and invasive capacities of trophoblasts during EMT by activating the ERK1/2 pathway. Conversely, poor decidualization, characterized by reduced CCL8 secretion, results in decreased number of and dysfunctional CCR1$^+$ dMφ, which impair the normal functions of trophoblast cells in women with URPL.

display significant diversity (45–47). Here, we identified CCR1$^+$ and CCR1$^-$ dMφ in the decidua from early pregnancy. Previous reports have shown that CCR1 expression was the lowest in M1 macrophages but the highest in tumor-educated macrophages (48). Similarly, compared with CCR1$^-$ dMφ, CCR1$^+$ dMφ in early pregnancy exhibited a significant anti-inflammatory phenotype, characterized by a high expression of CD163 and CD206 and increased production of IL-10 and TGF-β. These results indicated an M2-like phenotype in CCR1$^+$ dMφ and an M1-like phenotype in CCR1$^-$ dMφ; thus, dMφ as a whole maintain immunosuppressive properties as well as defense capability. However, in women with URPL, the frequency of CCR1$^+$ dMφ was sharply decreased and the immunosuppressive status of these cells was changed to hyperactivated and inflammatory, leading to imbalanced maternal–fetal immune responses unbeneficial to pregnancy maintenance.

The function of CCR1$^+$ dMφ was also investigated in this study, which demonstrated that CCR1$^+$ dMφ promote trophoblast migration and invasion by inducing the EMT process *via* the activation of the ERK1/2 signaling pathway. This function of CCR1$^+$ dMφ was enhanced by CCL8. However, we detected deficient EMT in trophoblasts, reduced CCL8 expression in DSCs, and decreased number and dysfunction of CCR1$^+$ dMφ in URPL. Emerging evidence has shown that proper and sufficient trophoblast migration and invasion are essential for embryo implantation, placental formation, and spiral artery remodeling (49, 50). The EMT process refers to a cellular change in which the polarity and cell adhesion properties decrease and cell migration and invasion increase, which is accompanied by trophoblast invasion (51, 52). Inadequate and defective EMT process in trophoblast cells is an etiological factor associated with pregnancy complications, including URPL, preeclampsia, and intrauterine growth retardation (53, 54). The initiation and maintenance of trophoblast EMT are greatly influenced by the maternal–fetal microenvironment, including local immune cells. Through the production of IL-8 and IP-10, decidual natural killer cells can stimulate the recruitment and migration of trophoblast cells (55). Studies have found that activated M1 macrophages induced by lipopolysaccharide dampen trophoblast migration and invasion by producing high levels of TNF-α through the regulation of the E-cadherin/β-catenin pathway (16, 56). IL-10 derived from M2 macrophages can reverse the effect of TNF-α-induced poor invasive properties of trophoblast cells. Additionally, M2 macrophages can promote EMT, invasion, and migration of trophoblasts by secreting G-CSF *via* the

activation of the PI3K/AKT/ERK1/2 pathway (57). Therefore, our findings suggest that dysfunction of $CCR1^+$ dMφ may account for the downregulated EMT process in trophoblasts, which leads to limited trophoblast migration and invasion in patients with URPL.

Macrophages are highly plastic to the disturbance of homeostasis and are conditioned by the local tissue environment (58). CCR1 was initially identified as a receptor of CCL3 and CCL5. With the discovery of additional chemokines in CC categories, studies have revealed that CCR1 binds and functions in response to a range of chemokines, such as CCL7, CCL8, CCL14, and CCL15 (24, 59, 60). In our study, CCL8 potently modulated the origin, phenotype, and function of $CCR1^+$ dMφ in early pregnancy, as demonstrated by single-cell analysis and functional experiments. CCL8 is a crucial regulator of the homing of TH2 cells and drives chronic allergic inflammation by interacting with CCR8 (61, 62). Furthermore, mainly produced by airway macrophages, CCL8 elicits the movement and activation of type 2 innate lymphoid cells (ILC2) during inflammation (63). Studies investigating the roles and mechanisms of action of CCL8 in macrophages are limited. The ablation of CCL8 in mice implanted with breast cancers reversed the chemoattractant effect of M2 macrophages in a CCR2-dependent manner (64, 65). Zhang et al. detected an increase in CCL8 expression, resulting in the enrichment of myeloid cells, restoration of immune suppression, and acceleration of carcinogenesis, which were blocked by the CCL8 receptor CCR1 (48). Our results revealed that either exogenous CCL8 or DSC-derived CCL8 could promote the transformation of $CCR1^+$ pMφ into a more anti-inflammatory phenotype, which could be abrogated by the CCL8 neutralizing antibody. CCL8 improved the function of $CCR1^+$ dMφ in promoting EMT, migration, and invasion of trophoblast cells. Therefore, as a potent regulator of $CCR1^+$ macrophages, CCL8 is of great significance for establishing an immune-tolerant microenvironment and adequate trophoblast functions.

Collectively, our study identified a distinct dMφ subpopulation, $CCR1^+$ dMφ, that exhibit immunosuppressive activity during early pregnancy. The decreased frequency of CCR1 in dMφ was accompanied by downregulated inhibitory membrane molecule expression and dysfunctional anti-inflammatory cytokine production in women with URPL. We also determined that $CCR1^+$ dMφ could promote migratory and invasive traits and EMT in trophoblast cells by activating the ERK1/2 pathway. Moreover, the recruitment of $CCR1^+$ dMφ, as well as their immunotolerant phenotype and regulatory functions in trophoblasts, all depend on the interaction with DSC-derived CCL8. Our findings illustrate a distinct dialogue among $CCR1^+$ dMφ, trophoblast cells, and DSCs during early pregnancy and underscore the critical role of $CCR1^+$ dMφ subsets in URPL. These data provide insight into the immune mechanisms of URPL and potential targets for intervention.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving human participants were reviewed and approved by Obstetrics and Gynecology Hospital, Fudan University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

YS performed the experiments, analyzed data, and drafted the first version of manuscript. YL designed experiments, researched literatures and edited the manuscript. LX helped to carry out the experiments. JC assisted with data analysis. DL and MD conceived the project, supported the research and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Decidual macrophages in recurrent spontaneous abortion

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Recurrent spontaneous abortion (RSA) is defined as two or more pregnancy loss, affecting the happiness index of fertility couples. The mechanisms involved in the occurrence of RSA are not clear to date. The primary problem for the maternal immune system is how to establish and maintain the immune tolerance to the semi-allogeneic fetuses. During the pregnancy, decidual macrophages mainly play an important role in the immunologic dialogue. The purpose of this study is to explore decidual macrophages, and to understand whether there is a connection between these cells and RSA by analyzing their phenotypes and functions. Pubmed, Web of Science and Embase were searched. The eligibility criterion for this review was evaluating the literature about the pregnancy and macrophages. Any disagreement between the authors was resolved upon discussion and if required by the judgment of the corresponding author. We summarized the latest views on the phenotype, function and dysfunction of decidual macrophages to illuminate its relationship with RSA.

KEYWORDS

decidual macrophages, recurrent spontaneous abortion (RSA), M1/M2 balance, CD11clow/CD11chigh, the maternal-fetal interface

Introduction

Recurrent spontaneous abortion (RSA) is an early pregnancy complication, which is defined as two or more spontaneous pregnancy loss with the same couple (1). The European Society of Human Reproduction and Embryology (ESHRE) considers it often occur continuously prior to 24 gestational weeks (2). In recent years, RSA becomes one of the formidable challenges for the doctors and infertile patients, as it may govern the fate of the whole family. However, miscarriage is a relatively common problem, occurring in 12 to 15 percent of clinically recognized pregnancies. Its risk increases with maternal age (3) and each previous prenancy losses stepwise. Patients suffered from recurrent abortion account for 1-3% (4). Large amount of data shows that RSA patients are a high-risk population for obstetrical and perinatal complications (5, 6). As the number of miscarriages has increased, the more damage to the maternal endometrium and the

emergence of pelvic inflammatory disease, lead to secondary infertility. Equally, the risk of fetal growth restriction, placental abruption, premature delivery and stillbirth in future pregnancies are also raised. What can not be ignored are the following issues, such as venous thromboembolism, mental health and economic costs. Thus, closer surveillance of the RSA patients in late pregnancy must be introduced in clinical practice.

The causes of RSA are connected with anatomic defects, chromosomal abnormalities, immune dysregulation, thrombophilia, endocrine disease, infection, environmental and psychological factors (7, 8). Until now, approximately 50% of RSA cases remain elusive, leaving us away from an accurate examination and treatment. Actually, the follow-up studies of the exact etiology and pathogenesis are frequently difficult. Because of practical feasibility and ethical limitations, mouse models with higher conception rate and shorter gestation are always used in the studies of RSA (9). However, the relationship between unexplained RSA and immune system has increasingly drawn more attention in clinical practice. The latest research about a single-cell RNA sequencing showed macrophages have been observed in human yolk sac both morphologically and transcriptionally, which is essential for fetal development in early pregnancy (10). And numerous immunomodulatory therapies for RSA have been suggested.

Given the relationship with RSA, researches mostly support that the immunological factor is a prerequisite for a successful pregnancy (11). Compared to the more explicit role of NK cells in pregnancy (12), the roles of decidual macrophages in pregnancy have not been fully investigated. Macrophages are the second largest group of immune cells and account for 20 percent of the total leukocytes at the maternal-fetal interface (13). They participate in all physiological events in the female reproductive system, such as menstruation, implantation and deliver (14). On account of the polarization and plasticity of macrophages, they differentiate into specific phenotypes as a response to the microenvironmental stimuli (15, 16). The number and function of macrophages in the non-pregnant uterus are regulated by the estrogen and progesterone during the menstrual cycle (17). When the endometrium falls off at menstruation, macrophages with numbers peaking promote "wound healing" through phagocytosis and tissue remodel (18). Before implanting, macrophages are recruited to exhibit M1 phenotype to reply the inflammatory response resulting from seminal fluid. As extravillous trophoblasts (EVTs) begin to invade the decidua, decidual macrophages convert to a mixed profile of M1/M2 macrophages (19). Then, for the establishment of fetal immune tolerance, macrophages transform into an overwhelming M2 phenotype (20). By releasing proangiogenic growth factors such as interleukin 8 (IL-8), vascular endothelial growth factor (VEGF)-A and VEGF-C, M2 macrophages act as 'bridge cells'. They jointly facilitate unique vascularization and immunosuppression in the placental microenvironment (21, 22). In the process of tissue remodeling, decidual macrophages protect embryos from phagocytosis and infection (23, 24). Therefore, decidual macrophages are indispensable in pregnancy and its dysfunction will lead to pregnancy loss.

Phenotypes of decidual macrophages

Plasticity and polarization are landmarks of macrophages (16, 25). At the maternal-fetal interface, notable changes have occurred in the decidual macrophages. Next, we will brief the classification and possible mechanisms of macrophage in terms of its phenotype.

M1/M2

Based on their cytokines secretion, chemokines expression and functional characteristics, decidual macrophages can be classified into two subsets: classically activated macrophages (M1 macrophages), and alternatively activated macrophages (M2 macrophages) (26–28).

Bacterial lipopolysaccharide (LPS) recognition or induction of Th1 cytokines, such as tumor necrosis factor α (TNF-α), interferon-γ (INF-γ), can drive M1 polarization of macrophages. These macrophages secrete pro-inflammatory cytokines and chemokines IL-1α, IL-1β, IL-6, IL-12, TNF-α, CXCL9, CXCL10 and express surface markers CD80, CD86, TLR-2, TLR-4, and major histocompatibility complex (MHC) class II. With the capacity of presenting antigen, M1 macrophages produce T helper type 1 (Th1) responses. It is characterized by maximizing the ability of immune cells to make cytotoxic or inflammatory reaction to viral infections, tumors or grafts. In early pregnancy, M1 macrophages promote embryo implantation and protect the fetus from infection (29–33).

Moreover, IL-4 and IL-13 directly induce M2 macrophage activation, IL-10 and transforming growth factor-β (TGF-β) make macrophages polarized toward the M2 phenotype. As the part of a polarized Th2 response, M2 macrophages are involved in apoptotic cells clearance and tissue remodeling. The release of a distinct set of chemokines, such as CCL17, CCL22 and CCL24 and their corresponding chemokine receptors CCR4 and CCR3, can also cause the recruitment of Th2 cells and amplification of polarized Th2 responses. They have immunosuppressive properties with higher levels of CD206, CD209 and CD163 expression. M2 macrophages provide an immune-tolerant environment for the fetus throughout pregnancy (29, 34–36).

Macrophages are typical plastic cells which can switch phenotypes and be subject to environmental disturbances (37, 38). Therefore, it is necessary to ensure the balance of M1 and M2 macrophages, so that the embryo can implant and develop smoothly at the maternal-fetal interface (39–42).

$CD11c^{high}/CD11c^{low}$

Distinct from the traditional M1 and M2 macrophages, Houser classified decidual macrophages into CD11c high and CD11c low subpopulations on the basis of CD11c expression (43, 44). Moreover, Jiang et al. subdivided macrophages into three decidual subsets, $CCR2^{-}CD11c^{LO}$, $CCR2^{-}CD11c^{HI}$, and $CCR2^{+}CD11c^{HI}$ by flow cytometry analysis (45, 46). CD11c low decidual macrophages and $CCR2^{-}CD11c^{LO}$ subset expressed highly phagocytic receptors, such as CD209 and CD206 (43, 47). $CCR2^{-}CD11c^{LO}$ macrophages also specifically exhibits heme oxygenase-1 (HMOX1) (48, 49), which may be in favor of protecting the fetus from being affected by possible infections during the early pregnancy (42). While $CCR2^{+}CD11c^{HI}$ and $CCR2^{-}CD11c^{HI}$ subsets posses pro-inflammatory and anti-inflammatory characteristics respectively (46, 50). They maintain an immnue balance to facilitate the clearance of pathogen infection and keep the homeostasis of the maternal-fetal interface (Table 1).

Functions of decidual macrophages in normal pregnancy

Decidual macrophages have drawn remarkable attention for their functional characteristics of plasticity and polarization (16). Throughout the maternal adaptations to pregnancy, decidual macrophages also play critical roles (56). Decidual macrophages coordinate tissue remodeling and angiogenesis, induce apoptosis of damaged cells, facilitate trophoblasts invasion and suppress inflammation (57–62). Consequently, they are indispensable in contributing to the maternal-fetal immune tolerance.

As we all know, endometrial macrophages act as determinants of uterine receptivity. Owing to the characteristics of immune tolerance, they became the research focus in the medical community (63). Gorczynski pointed out that CD200 and MD-1 have immune regulatory activity toward macrophages, which is beneficial to successful pregnancy (64). If the expression of CD200R in macrophages increases, it can stimulate the activity of indoleamine2,3-dioxygenase (IDO) (65). Thus, establishing an immunosuppressive environment is necessary for successful implantation (66). Furthermore, there are many kinds of Toll receptors on decidual macrophages, such as TLR2, TLR3 and TLR4. In response to TLRs activation, decidual macrophages facilitated the secretion of pro-inflammatory cytokines IL-1β, TNF-α, IL-6, IL-8 and the production of the anti-inflammatory cytokines IL-1RA and IL-10. Among these cytokines, IL-10 was the most easily induced. Along with the higher secretion of IL-10 increased by TLRs activation, it might help sustain immune tolerance by curbing the action of pro-inflammatory cytokines (67). In addition, the inhibitory receptors expressed on invading extravillous trophoblasts, such as immunoglobulin-like transcript 2 (ILT2) and ILT4 for human leukocyte antigen (HLA)-G (68, 69), can be combined with the decidual macrophages. As a negative signal that be delivered to the decidual macrophages, they are in favor of the production of anti-inflammatory cytokines and tolerance to the trophoblast (70).

TABLE 1 Different phenotypes of macrophages during pregnancy.

Macrophage type	Characteristics	Function	References
M1 macrophages	CD80,CD86,TNF-α,INF-γ, IL-1α,IL-1β,IL-6,IL-12,NO, CXCL9,CXCL10,CCR7	Pro-inflammatory; Th1 responses; Present antigen; Promote embryo implantation; Protect the fetus from infection	(20, 29–33, 39, 40, 51, 52)
M2 macrophages	CD206,CD209,CD163,IL-10 TGF-β,VEGF,CCL17, CCL22,CCL24,CCR3,CCR4	Anti-inflammatory;Th2 responses; Immunosuppressive properties; Tissue remodeling	(20, 29, 34–36, 39, 51–54)
$CD11c^{low}$(67%)	$CD206^{hi}$,$CD209^{hi}$, expressed genes involved in regulating growth and development, as well as Extracellular matrix formation	Homeostatic function during placental growth	(43, 44)
$CD11c^{high}$(33%)	$CD206^{low}$,$CD209^{low}$, Upregulates CD1a,CD1c,CD1d; Expressed genes associated with lipid metabolism and inflammation	Antigen processing and presentation	(43, 44)
$CCR2^{-}CD11c^{LO}$ ($CD11c^{low}$, ~80%)	Exhibit the fewest inflammatory properties; highly express CD209 and MHCII.	Antigen presentation	(45, 46, 55)
$CCR2^{+}CD11c^{HI}$ ($CD11c^{high}$, 10-15%)	Facilitate the clearance of pathogen infection; maintain the homeostasis of the maternal-fatal interface.	Pro-inflammatory	(45, 46, 55)
$CCR2^{-}CD11c^{HI}$ ($CD11c^{high}$, ~5%)		Anti- inflammatory	(45, 46, 55)

Human decidual MMP-9$^+$ macrophages can degrade the extracellular matrix (ECM) and promote endovascular trophoblast invasion, and they are enriched in the vicinity of the trophoblast invasion during early pregnancy (71). IL-33, as a cytokine of the IL-1 family, is found to be associated with Th2 and M2 polarization (72, 73). It can accelerate the development of primary trophoblasts, villous cytotrophoblast (74). Granulocyte macrophage colony-stimulating factor (GM-CSF) and macrophages colony-stimulating factor (M-CSF) are secreted from first trimester decidual cells. These activated cells can promote macrophages activation, and induce extravillous trophoblasts (EVTs) apoptosis through the caspase 3/7 dependent pathway (75). Conversely, trophoblasts-derived IL-6 (76), CXCL16 (77), and hyaluronan (HA) (78) could induce M2 macrophages polarization. In addition, macrophages could also secrete exosomes or extracellular vesicles and deliver miRNAs to affect the invasion and migration capabilities of trophoblasts, thereby participating in the occurrence of RSA (51, 79).

Placental macrophages are a special M2-like polarized phenotype, which don't possess all properties of M2 cells. However, the expression intensity of CD163, CD80, CD11c, CCR5, CXCR4 on M2-like macrophages are lower than M2 macrophages. They were shown to regulate gap junction communication and promote decidualization (80).

Decidual macrophages may be the major APCs in the decidua (81). It is thought to be the sentinels of the immune system that initiate and regulate the immune response (82). M2 macrophages can wipe out infection by switching gene expression toward anti-inflammatory cytokines including IL-10, TGF-β and IL-1Ra (83). It also express high levels of scavenger receptors CD163, Stabilin-1 and c-type lectins receptors CD206 and CD209 (84). Phagocytosis of damaged and apoptotic cells are fundamental M2 macrophage functions, which also apply to decidual macrophages at the maternal-fetal interface.

The relationship between decidual macrophages and RSA

It has been fully elaborated that RSA have an immune background. What's the relationship between decidual macrophages and the aetiology of RSA? How do dysfunction of decidual macrophages lead to RSA (Figure 1)?

M1/M2 macrophages balance

As it was mentioned above, the disturbed M1/M2 macrophages balance came to light at the maternal-fetal interface of RSA (40). It seems that the M1 subtype predominates over the M2 subtype in those cases, accompanied by pregnancy complications (85, 86). M1 macrophages can suppress epithelial-mesenchymal transition (EMT), migration, and invasion of trophoblasts by transporting miR-146b-5p to directly inhibit TRAF6 expression, thereby participating in the pathogenesis of RSA (87). ChunYan Wei have tested that JAK2 inhibitor adjusted the proportion of M1/M2 macrophages, further affecting the pregnancy outcome through the CCL2/CCR2/JAK2 pathway (88). Decreased programmed death-1 (PD-1) protein expression on decidual macrophages, accompanied with reduced programmed cell death ligand-1 (PD-L1) expression on placental villi, was observed in RSA. Meanwhile, the disturbed PD-1/PD-L1 axis induced M1 differentiation (89). Knockdown of CYP26A1 in mice uterine can decrease the number of embryo implantation. It can be also discovered that the protein levels of M1 markers TNF-α, IL-6 and CD86 were significantly decreased, thus leading to the insufficient M1 polarization (90). Additionally, in the immune atlas of RSA without chromosomal aberrations, pro-inflammatory subsets of CD11c^{high} macrophages increased remarkably (91). The present research illustrated that the abnormally increased MNSFβ expression can promote the secretion of TNF-α, inducing the polarization of decidual macrophages toward a pro-inflammatory phenotype (92). More intuitive studies on mouse experiments confirmed that Cathepsin E-deficient mice displayed compromised immune reactions with higher susceptibility to bacterial infection (93).

Cytokines

Several studies have demonstrated that the expression of CD80, CD86 and HLA-DR, but not CD163 on decidual macrophages were higher in RSA patients compared to normal pregnancies, accompanied with higher production of TNF-α and lower secretion of IL-10 and IL-33 (40, 73, 94). In LPS-induced mice abortion model, the expression IL-1, IL-6, TNF-α, IFN-γ, IL-17a was significantly raised (95). Macrophage depletion was also proved to prevent CpG-induced embryonic resorption in an abortion mice model and in IL-10$^{-/-}$ mice (96, 97). The experiment as early as the 18th century has confirmed that the depletion of macrophags results in the loss of pregnancy and recurrent abortion (57). CSF-1-deficient mice displayed few decidual macrophages, with lower implantation at day 7 and 8, and always had aberrant fertility with smaller size (98). On day 0.5 or day 3.5 post-coitum, injection of diphtheria toxin (DT) to Cd11b-Dtr mouse model caused implantation failure and infertility. But implantation failure can be alleviated by administration of bone marrow-derived CD11b$^+$F4/80$^+$ monocytes/macrophages (99). And injection on day 14 and 16

led to fetal mortality without cervix ripening (100). However, it could be alleviated by administration of $RANK^+$ macrophages (101). RANKL derived from trophoblasts could make macrophage polarization to M2 by activating AKT/STAT6-Jmjd3/IRF4 signaling pathway. The knockout model of $RANK^{-}/^{-}$ mice can lead to the decreased expression of TGF-β and the increased pregnancy loss (102). Mice with uterine deficiency of high-mobility group box-1 (HMGB1) protein, showed impaired implantation and severe subfertility (103). But highly expressed HMGB1 was actively secreted by macrophages and then activated pyroptosis, leading to the occurrence and development of RSA (104).Therefore, restricting macrophages accumulation is also needed.

T-cell immunoglobulin and mucin domain containing protein 3 (Tim-3) blockade down-regulated the phagocytosis of decidual macrophages, leading to accumulation of inflammatory granulocytes and macrophages at the maternal-fetal interface (105). Therefore, high level of pro-inflammatory cytokines establishes a pro-inflammatory microenvironment and impairs normal pregnancy. It also has been indicated that dysregulation of decidual macrophages activation by regulatory T cells (Treg cells) may lead to RSA. When Treg cells regulate aberrant cell-cell contact, there will be a problem with decidual macrophages. The abnormity of decidual macrophages was indicated to be regulated by Treg cells through aberrant cell-cell contact and TGF-β secretion (106). Moreover, Jiayu Wang's studies showed that abnormally expressed USP2a may be found in the placental villous samples of RSA patients. Further studies have confirmed that TGF-β could collaborate with USP2a to promote trophoblasts migration and invasion *via* its interaction with TGFBR1 (107). Thrombospondin1 (TSP1) needs to interact with CD36, CD47 and heparin sulphate proteoglycanto enhance the ability of macrophages (108). They are engaged in regulating IL-10 secretion and boost the tolerance of the immune system at the maternal-fetal interface (109). Thus, low expression of TSP1 along with decreased IL-10 could appear in RSA.

Therapies

Owing to the uncertainty of the pathogenesis, the likelihood of recurrence, recent studies suggest that various treatment of RSA may work (110, 111). Clinicians often use progesterone to support or supplement the pregnancy, by oral, vaginal, intramuscular, or other ways. It is considered to be essential for successful embryo implantation. But now, it is increasingly becoming the psychological support to patients (112). Besides, all treatments for RSA are almost based on immunomodulation for their effects (2, 113). A meta-analysis of the treament of APS-related RSA showed that aspirin plus low-molecular-weight heparin (LMWH) can significantly reduce the rate of repeated pregnancy loss (114). Another study proved the combination of anticoagulant and anti-inflammatory could contribute to a better pregnancy outcome (115). Prednisone, hydroxychloroquine (116) and cyclosporine A (117) are also part of the clinical therapy regimen of RSA.

When a semi-allogeneic fetus appears at maternal-fetal interface, maternal tolerance is required to avoid the miscarriage. Patients with RSA may lack this capacity. Therefore, alloimmunization was born in response to the condition. It has been suggested that the effect of lymphocyte immunotherapy (LIT) was probably positive. And a higher success rate was likely observed in those immunized with paternal lymphocytes (118). Some experts recommend immunotherapy before and during pregnancy with low dose of lymphocytes. It can break the balance between Th1 and Th2 cytokines, reducing the level of Th1 cytokines (IL-2, INF-γ, TNF-α, and IL-6), while increasing the level of Th2 cytokines (IL-4, IL-10) (119). As opposed to "active immunization" with allogenic lymphocytes which was introduced previously, intravenous immunoglobulin (IVIg) was termed as "passive immunization". The effect of IVIg on Treg/Th17 cells ratio enhances Treg cells function, and thereby improve the live birth rate in pregnancy to some extent (120). The RSA mice models with intraperitoneally administration of G-CSF certified that the absence of G-CSF weakened the inhibitory effects on macrophages, leading to more M1-type differentiation and overexpressing NLRP3 inflammasomes at the maternal-fetal interface. It implies that G-CSF may improve pregnancy success rate by modulating the inflammatory state (121).

It is worth noting that immunotherapy is not a panacea for treating all patients with RSA. The choice of therapeutic plans should have certain indications (122). For unexplained RSA, we should make efforts to seek the pathogenesis. Testing for inherited thrombophilia and hyperhomocysteinemia should be performed. If necessary, screening for immunological factors such as Human Leukocyte Antigen (HLA), cytokines, antinuclear antibodies (ANA), Natural Killer (NK) cells, anti-HLA antibodies and antisperm antibodies, Lupus anticoagulant (LAC), Anticardiolipin antibodies (ACL) and anti-β2 glycoprotein-I antibodies (β2-GPI). So, further research in personalised treatment options is warranted.

Conclusion

Take together, polarized macrophages can influence the reception of maternal to embryo through the secretion of various cytokines and chemokines. The specific etiology and pathogenesis among them is very complicated, which is an emerging field that needs to be explored urgently. How to

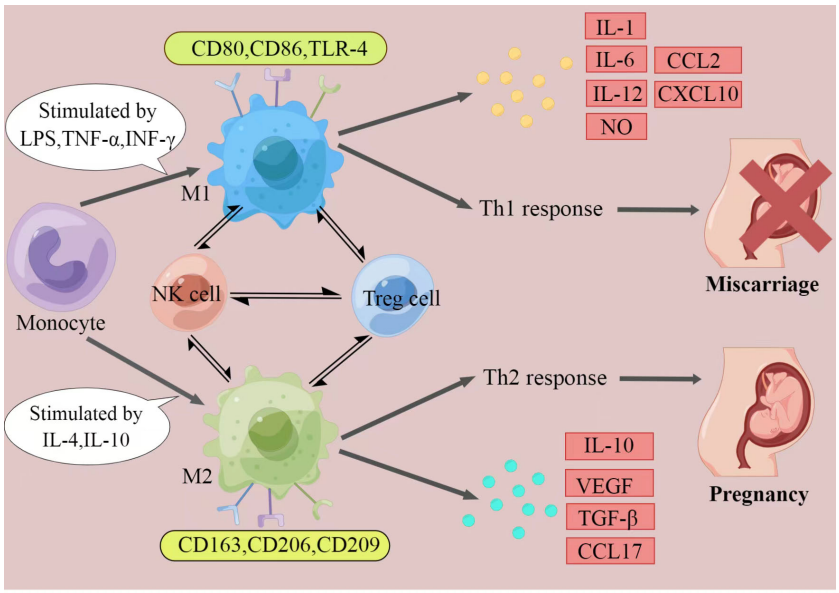

FIGURE 1
The polarization of macrophages and their characteristics. The figure displays a general principle of polarized M1 and M2 macrophage. M1 and M2 phenotypes represent two extremes of macrophage polarization and display distinct functions, thereby result in different pregnancy outcomes. In response to different stimuli, decidual macrophages undergo M1-like, or M2-like activation. M1 macrophages are stimulated by LPS, TGF-α, or IFN-γ. They express CD80, CD86, and TLR-4, secrete IL-1, IL-6, IL-12, NO, CCL2 and CXCL10, and produce Th1 responses, exert pro-inflammatory effects. In contrast, M2 macrophages are activated by IL-4 or IL-10. They express CD163, CD206, and CD209, secrete IL-10, VEGF, TGF-β and CCL17, and promote Th2 responses, provide an immune-tolerant environment for the fetus. Thus, if M2 macrophages play the major role at the maternal-fetal interface, pregnancy would continue. When M1 macrophages are absolutely dominant, it will ultimately lead to miscarriage. (Created by Figdraw).

maintain M1/M2 macrophages balance? Even we know the correlation of decidual macrophages with RSA, what can we do to help them? Collectively, there are a large number of challenges to be overcome, and further efforts are needed.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

Author contributions

Q-YZ and Q-HL wrote and discussed the manuscript. Y-YF discussed the manuscript. Q-YZ and A-FJ designed and created tables. C-ER and Y-HM reviewed the manuscript. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Insights into the immunomodulatory regulation of matrix metalloproteinase at the maternal-fetal interface during early pregnancy and pregnancy-related diseases

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Trophoblast immune cell interactions are central events in the immune microenvironment at the maternal-fetal interface. Their abnormalities are potential causes of various pregnancy complications, including pre-eclampsia and recurrent spontaneous abortion. Matrix metalloproteinase (MMP) is highly homologous, zinc(II)-containing metalloproteinase involved in altered uterine hemodynamics, closely associated with uterine vascular remodeling. However, the interactions between MMP and the immune microenvironment remain unclear. Here we discuss the key roles and potential interplay of MMP with the immune microenvironment in the embryo implantation process and pregnancy-related diseases, which may contribute to understanding the establishment and maintenance of normal pregnancy and providing new therapeutic strategies. Recent studies have shown that several tissue inhibitors of metalloproteinases (TIMPs) effectively prevent invasive vascular disease by modulating the activity of MMP. We summarize the main findings of these studies and suggest the possibility of TIMPs as emerging biomarkers and potential therapeutic targets for a range of complications induced by abnormalities in the immune microenvironment at the maternal-fetal interface. MMP and TIMPs are promising targets for developing new immunotherapies to treat pregnancy-related diseases caused by immune imbalance.

KEYWORDS

matrix metalloproteinase, maternal-fetal interface immune microenvironment, pregnancy-related diseases, matrix metalloproteinase inhibitor, immunotherapy

1 Introduction

Matrix metalloproteinase (MMP) belongs to a family of zinc (II)-dependent endopeptidases, which cleave extracellular matrix (ECM) proteins and participate in processes such as tissue remodeling and angiogenesis. From the immunological point of view, a successful pregnancy is one in which the maternal immune system can accept an embryo containing paternal antigens. Alterations in the immune environment at the maternal-fetal interface can induce immune imbalance, which can be accompanied by varying degrees of inflammatory responses, thus triggering pathological pregnancy outcomes. Tissue inhibitor of matrix metalloproteinase (TIMP), the predominant endogenous inhibitor of MMP, acts by binding MMP in a 1:1 ratio. In this review, we will provide a comprehensive overview of the immune role of MMP in the embryo implantation process and pregnancy-related diseases using data reported in PubMed and other scientific databases. Additionally, we present the possibility and potential benefits of MMP and its inhibitor as a biomarker and potential therapeutic target for pregnancy-related diseases.

2 Structure, functions, and regulations of MMP

2.1 Structure of MMP

MMP is a family of zinc(II)-dependent protein hydrolases with a common core structure that degrade the ECM, which is essential for vascular remodeling (1). Notably, zinc(II) of MMP-3 maintains its protease activity even when replaced by other ions (2). Twenty-eight types of MMP have been identified in vertebrates, and twenty-three of them are expressed in human tissues (3). MMP could be secreted by connective tissue, pro-inflammatory cells, and uterine placental cells, including fibroblasts, vascular smooth muscle (VSM), leukocytes, trophoblasts, etc.; A typical MMP consists of a pre-peptide sequence (80 amino acids), a catalytic metalloproteinase structural domain (170 amino acids), a variable length linker peptide or a hinge region, and a heme-binding protein structural domain (except for MMP-7, MMP-23, and MMP-26) (4, 5). *In vivo*, MMP generally exists as the inactive form of proMMP precursor, which is cleaved by various protein hydrolases (e.g., serine proteases, fibrinolytic enzymes, etc.), eventually producing active MMP to perform its functions (6).

2.2 Functions of MMP

Based on substrate specificity, MMP can be classified into gelatinase, collagenase, matrilysin, stromelysin, membrane-type MMP(MT-MMP), and others (4, 5). Because of the basic function of degrading ECM proteins, MMP is involved in a wide range of physiological and pathological processes in the human body. Physiologically, MMP plays a critical role in cell proliferation, migration and differentiation, tissue repair and remodulation, embryogenesis, and wound healing (7). Pathologically, MMP disorders are connected with tumor invasion and metastasis because MMP can degrade almost all protein components in ECM (8).

The degradation of ECM by MMP (mainly collagen and elastin) is the basis for the involvement of MMP in tissue damage repair. The degradation process of MMP requires the coordinated action of a Zn^{2+} active center and a water molecule (including three histidines and one glutamate), with methionine as a hydrophobic base to play a supporting role (5). During the MMP-substrate interaction, the Zn^{2+}-bound water molecule launches a nucleophilic attack on the substrate, which eventually leads to its decomposition and release of water (9).

The process of tissue injury is inevitably accompanied by the development of inflammation, during which multiple inflammatory cells and mediators are included in the alteration. Recent research suggests that the ability to regenerate tissue may be independent of the inflammatory response, and thus the correlation between immunity and tissue regenerative capacity is gradually gaining widespread attention (10). In general, there may also be an underlying immune inflammatory response during tissue remodeling in MMP. Inflammatory cytokines (Interleukin(IL)1-α, IL1-β, IL-2, IL-17, C-reactive protein, Tumor necrosis factor-α (TNF-α), etc.) can be found in healing phases of chronic venous ulcers, which are believed to stimulate the production of neutrophil gelatinase-associated lipoprotein (NGAL),thus activating MMP-9 and form MMP-9/NGAL complexes to help to heal (11, 12). The endometrial remodeling process is precisely regulated in which MMP is essential (13). In all types of the endometrium, MMP-26 was found to have cyclical changes in its expression that may be associated with the endometrial tissue remodeling process (14, 15).

During the tumor growth and invasion process, MMP may be involved in key processes that disrupt the balance of growth and anti-growth factor signaling in the tumor microenvironment and tumor neovascularization (16). Non-catalytic functions targeting MMP are now an emerging researchhotspot. For example, the cytoplasmic tail of MT1-MMP canbind to Factor inhibiting hypoxia-inducible factor-1 (FIH-1) and promoting stable FIH-1-Munc18-1-interacting protein three interaction, which enhances hypoxia-inducible factor (HIF) target gene expression, thereby promoting Warburg effect and angiogenesis in a non-proteolytic manner (17).

This review focuses on the role of MMP in early pregnancy. The primary reproductive events include endometrial decidualization, uterine spiral artery remodeling, trophoblast

cell invasion and differentiation, and placenta formation. MMP may be involved in uterine placental and vascular remodeling during normal pregnancy, as MMP is significant in tissue regulation remodeling (18).

2.3 MMP and ovarian sex hormone regulation

Apart from being expressed by cells, the expression of MMP can also be induced by various exogenous signals, such as cytokines, growth factors, hormones, and changes in cell-matrix and cell-cell interplay (7). Noticeably, during the implantation window period in early pregnancy, a large number of factors such as cytokines, adhesion molecules, and proteolytic enzymes are secreted by the endometrium under the mediation of estrogen and progesterone, which play critical roles in the identification and adhesion of the embryo and endometrium, and further regulate the process of embryo implantation. The interactions between steroid hormones and MMP need to be fully understood.

Affected by steroid hormones secreted by the ovary, the endometrium undergoes continuous cyclic exfoliation and remodeling throughout the female reproductive phase: estradiol stimulates endometrial proliferation during the proliferative phase of the menstrual cycle, while progesterone further acts on the estrogen-affected endometrium to induce its glandular secretion and stromal cell differentiation into metaphase cells (19). Endometrial MMP and TIMP expression regulations are essential for endometrial growth, rupture of circulating tissue, and pregnancy establishment, yet the mechanisms regulating the expression patterns of MMP and TIMP during the menstrual cycle have not been fully elucidated (20). Ovarian sex hormones (e.g., estrogen and progesterone) affect the expression of MMP, which in turn can coordinate with ovarian sex hormones to co-involve in the endometrial tissue remodeling and shedding process (21). Steroid hormone regulation of the MMP system includes direct or indirect regulation of gene transcription, specific changes in the expression, and action of local cytokines (20).

The relationship between estrogen and the specific expression of MMP family members remains unclear. Activator protein-1 (AP-1) transcription consists of c-Jun and c-Fosproteins, proven to be a significant regulator of multiple MMP transcription under multiple conditions (22). The promoters of most MMP genes contain AP-1 elements, and upon increased estrogen exposure, the ligand-bound estrogen receptor complex can increase the expression of the AP-1-bound transcription factors Fos and Jun (23).

Vitro research has shown that the addition of progesterone to endometrial explants or isolated stromal cells downregulates MMP expressions (24). Specifically,$10^{-8}m^{-1}$ estrogen and $10^{-7}m^{-1}$ medroxyprogesterone acetate inhibited the pro-MMP-1 secreted by cultured human endometrial stromal cells (25). MMP-3 mRNA was remarkably curbed by estrogen and progestin medroxyprogesterone acetate (10^{-8}mol/L-10^{-6}mol/L) (26). An animal experiment using an estrogen-progestin subcutaneous implantation device to mimic the proliferative and secretory phases of the menstrual cycle in de-ovulatory rhesus monkeys found that all the MMP expressions were upregulated after progesterone withdrawal and spontaneously downregulated after menstruation in the absence of progesterone effects (27). The pattern of regulations of MMP by progesterone differs from that of estrogen. First, progesterone receptors can induce AP-1 activation in the absence of ligands, and the effect of progesterone receptors on AP-1 activity was shown to be cell type-specific (28). Second, progesterone can reverse AP-1 activation, and animal experiments suggest that progesterone can inhibit estrogen-induced c-fos mRNA expression (29). Finally, progesterone also indirectly affects the expressions of MMP by regulating cytokines (19), and IL-1α released from epithelial cells induces its expression in surrounding stromal cells through paracrine and autocrine amplification loops, thereby increasing the total amount of endometrial IL-1α and triggering MMP-1 expression (30). In contrast, progesterone at the stromal cells at the mRNA level inhibits IL-1α (19).

Human chorionic gonadotropin (HCG) has both local and systemic functions in early pregnancy (31). HCG can affect embryo attachment, embryo formation, trophoblast infiltration, and other pregnancy-related processes by up-regulating leukemia inhibitory factor (LIF), vascular endothelial growth factor (VEGF), MMP-9 and other factors, and it was reported that 500 IU/ml of HCG administration inhibited intrauterine insulin-like growth factor-binding protein-1 and macrophage-stimulating factor while elevating the level of LIF, VEGF and MMP-9 (32).The rapid increase of serum progesterone due to the rise of systemic HCG level in early pregnancy also has effects on MMP; as mentioned above, the inhibitory effect on MMP of progesterone may help limit trophoblast invasion in the proper range.

3 MMP and maternal-fetal interface events

3.1 Trophoblast invasion

Trophoblast invasion is a critical process in human placental formation, involving the regulation of cell adhesion and the degradation process of the ECM, which underlies the conversion of the uterine spiral arteries (33). To dilate the vasculature and provide adequate nutrition for embryonic growth, the extravillous trophoblast invades the maternal endometrium and remodels the spiral arteries, which allows sufficient

uteroplacental perfusion (34, 35). Increased expression levels/activity of MMP-2 and MMP-9 can be found in the aorta of normal pregnant rats (36). It is suggested that these altered MMP-2 and MMP-9 may be associated with a series of cellular events at the maternal-fetal interface. EGF-mediated trophoblast invasion may be related to altered MMP-2 expression/activity (37). High levels of MMP-9 facilitate the degradation of the endometrial ECM and loosen intercellular junctions, thus favoring the invasion of extravillous trophoblast cells.With the highest levels of mRNA and protein during pregnancy at week 6-7, MMP-26 decreases gradually before reaching a minimum level at mid-gestation, and this is inconsistent with the spatiotemporal regulation of trophoblast invasive capacity (38, 39). Mishra (40) suggested that the extracellular matrix metalloproteinase inducer (EMMPRIN) may also affect embryonic adhesion and fusion with the tubular epithelium by influencing the expression of MMP-2 and MMP-14. EMMPRIN, serving as an MMP inducer, expresses membrane protein in the immunoglobulin superfamily with two heavily glycosylated extracellular structural domains (41, 42).

Notably, Nissi (43) et al. monitored serum concentrations of MMP-9, TIMP-1, TIMP-2, and MMP-2/TIMP-2 complexes in normal pregnant women at different gestational weeks, finding no statistically significant changes in levels during normal pregnancy. Few studies have been conducted on maternal serum MMP and TIMP during normal pregnancy, and further experimental studies with expanded sample sizes are needed in the future.

3.2 Angiogenesis and remodeling

Angiogenesis and remodeling are complex series of processes including recruitment, migration, proliferation, and apoptosis of vascular cells consisting of stem/progenitor cells, endothelial cells (ECs), vascular smooth muscle cells (VSMC), etc., while the ECM plays its essential role in vascular development and morphogenesis by providing matrix scaffolds, interacting with matrix receptors or providing growth factors (44). MMP regulates VSMC growth, proliferation, and migration processes critical in vascular remodeling. MMP-2 secretion is closely associated with the migration of VSMC from rat thoracic aorta cultured *in vitro*, involving the breakdown of the basement membrane and pericellular ECM, while in bovine studies, it was found that MMP-2 induces the migration of VSMC by triggering oxidized low-density lipoprotein (OxLDL)-induced activation of the sphingomyelin/ceramide pathway, which ultimately leads to smooth muscle cell (SMC) proliferation and migration, and that this MMP-2 activation process is mediated by MT1-MMP (45, 46). Additionally, animal studies demonstrate that MMP-2 mRNA is increased in the uterine artery at day 7 and day 21 of gestation in rats, which suggests its pregnancy-associated vascular remodeling role (47).

4 MMP and immune microenvironment at the maternal-fetal interface

The maternal-fetal interface is a critical site for the establishment and maintenance of normal pregnancy, where immune cell populations such as macrophages, T cells, natural killer cells, and Dendritic Cells (DCs) accumulate (48).

4.1 Macrophage

Macrophages are primary monocyte-derived intrinsic immune cells with remarkable heterogeneity and plasticity that are essential for homeostasis and host defense (49, 50). As the second most abundant population of leukocytes in the decidual cells, macrophages are actively involved in coordinating the apoptotic process during tissue remodeling, thereby preventing the release of potentially pro-inflammatory and pro-immunogenic cellular contents during secondary necrosis, current studies suggest that CD14 or CD68 can be used as immune markers to identify decidual macrophages (51, 52). The percentage of macrophages in leukocytes showed no significant change in early and mid-pregnancy, while the percentage of $CD14^+$ macrophages tended to decrease significantly by late pregnancy (53).

It is currently believed that the phenotypic characteristics of macrophages reflect the local microenvironment response, including various cytokines and other mediators secreted by adventitial cells, and that during the embryonic implantation window, macrophages establish a pro-inflammatory microenvironment for embryonic implantation (54). *In vitro*, macrophages are usually classified into M1 and M2 phenotypes: pro-inflammatory M1 macrophages based on classical activation (LPS+ Interferon-γ (IFN-γ)), NFκB pathway, JAK/STAT signaling or IFN regulatory factor (IRF) induction and IL-4 or IL-4/IL-3-induced anti-inflammatory M2-type macrophages (55–57). Induced by IL-4, IL-10 and IL-13, M2-type macrophages have the function of anti-inflammatory by producing a large amount of IL-10 and TGF-β; Besides, its immune regulatory function is critical for early pregnancy maintenance (58).

It has been proven that decidual macrophages involved in ECM degrading in vascular remodeling are mediated by MMP-3 (59). Macrophages are an important source of MMP, and inflammatory factors can regulate the expression of macrophage proteases, including MMP (16, 60). For example, the expression of MMP-9 can be induced by TNF-α at the

transcriptional level (61). Also, the MMP-9 promoter is subject to IL-18-mediated AP-1 and NF-kappaß-dependent activation (62). It was shown that MT1-MMP can control macrophage invasion by ECM components and cell surface molecular signaling (63, 64). Recent studies revealed that the transcriptional target phosphoinositide 3-kinase δ (PI3Kδ)-expressing MT1-MMP can dependently trigger Akt/GSK3 cascade signaling and ultimately restrict the expression of macrophage-derived pro-inflammatory mediators (44). The potential link between MMP and macrophages and their expression products before may be one of the directions for further work in the future.

4.2 Dendritic cell

Maternal immune cells and fetus-derived trophoblasts have bidirectional regulation interaction in early pregnancy, and polarization toward Th2 in the immune response is considered to be the key to successful pregnancy (65). Generally, DCs play a pivotal role in this process. Even though only approximately 1% of early pregnancy DCs are present in the decidual cells, they have a dual critical role as potent antigen-presenting cells mediating immune activation and immune tolerance, which is particularly important during pregnancy (48, 66–68). These paradoxical dual functions of DCs seem to depend on their different stages of differentiation: immature dendritic cells (iDC), characterized by DC-SIGN, promote T-cell tolerance, whereas $CD83^+$ mature DCs function as an inducer of T-cell immunity (69–72). It should be noted that there also existed $DEC205^+$ DCs in the metaphase stromal cells that were activated but still showed an immature state (73).

DCs are critical effectors of TGF-β activity, promoting both Peripheral T-regulatory cell production during TGF-β activation and inducing differentiation of pathogenic Th17 cells for T-cell tolerance (74). MMP may provide an alternative pathway for the proteolytic activation of potential TGF-β *in vivo*. An *in vitro* experiment demonstrates that MMP-9 depends on CD44 for cell surface localization in TA3 mouse breast cancer cells, activating TGF-β2 and TGF-β3, which promote cell invasion and angiogenesis (75).

4.3 Natural killer cell

Natural killer cells (NK cells), which account for more than 70% of metaphase leukocytes in early pregnancy, are represented by the $CD56^+CD16^-$ phenotype (48). According to the type of cytokines secreted, NK cells can be divided into NK1 (mainly secreting IFN-γ, TNF-α) and NK2 (mainly secreting IL-4, IL-5, IL-10, IL-13). However, the number of uterine NK (uNK) cells in the placenta decreases in late pregnancy (76). The complete spectrum of MMP expressed by NK cells and macrophages has not been determined, while *in vitro*, experimental studies have identified NK cells and macrophages expressing MMP-2, MMP-7, MMP-9, MMP-11, MMP-16, MMP-19, and TIMP (77). Current clinical studies suggest that protein array studies of $CD56^+$uNK cells collected at 8-10 weeks of gestation indicate that uNK cells are the primary producers of angiogenic growth factors, but uNK cells collected at 12-14 weeks of pregnancy are the primary producers of cytokines (78).

With strong secretion of pro-inflammatory factors, angiogenic factors, etc., previous studies have reported a regulatory interaction of decidual NK cells (dNK cells) in trophoblast invasiveness through the secretion of IL-8 and interferon-inducible protein-10 (IP-10), which further stimulate CXCR1 and CXCR3-mediated pathways and thus exert regulatory effects (79). It has been suggested that IL-6 and IL-8 secreted by $CD56^+$uNK cells and $CD14^+$ macrophages are involved in uterine spiral artery remodeling either by secreting IFN-γ (80, 81).

Exploration of the functions of immune cells at the maternal-fetal interface throughout pregnancy is almost impossible due to the limited access to specimens, and the speculated role of immune cells at the maternal-fetal interface in human early pregnancy is revealed in Figure 1. Existing studies have adequately demonstrated their essential part during pregnancy, and an in-depth understanding of their interactions can assist in identifying potential immune risks in pathological pregnancies and thus responding to them.

5 MMP and pregnancy-related diseases

5.1 Preeclampsia

Insufficient remodeling of the uterine spiral arteries is one of the necessary pathological changes in Preeclampsia (PE). The association with an acute atherosclerotic response may be one of the interactions by which inadequate spiral arterial remodeling induces placental malperfusion, in which arteries undergo an atherosclerotic process, form intimal plaques, and enter a vicious cycle of ischemia and reperfusion with an extensive intravascular inflammatory response (82, 83). Dysregulated endogenous immune responses may lead to excessive inflammatory responses, such as a significant increase in pro-inflammatory cytokines in patients with PE;At the same time, a significantly increased risk of PE in pregnant women who are in a state of inflammatory overreaction early in pregnancy (84). Abnormal immune factors inducing trophoblast under invasion and endothelial cell dysfunction are considered significant causes of PE, including innate and adaptive immune factors (85, 86). Studies have shown that pregnant women with human immunodeficiency virus infection have a lower incidence

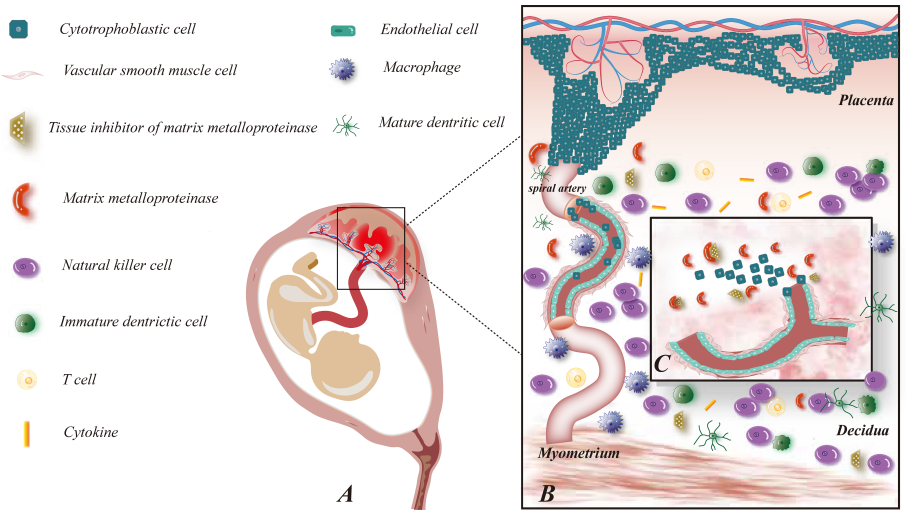

FIGURE 1
Maternal-fetal interface immune microenvironment on early pregnancy and role of MMP on embryo implantation. **(A)** This figure demonstrates the relative location of the fetus and its appendages on the maternal uterus; **(B)** This figure shows the composition of the maternal-fetal interface immune microenvironment, including fetal-originated cytotrophoblast cell, immune cell subsets: NK cells, macrophages, T cells, DC cells, cytokines, etc., maternal uterine spiral artery: vascular smooth muscle cells and vascular endothelial cells, MMP and TIMP families; **(C)** This figure illustrates the enlarged view of the functions of MMP on early pregnancy. MMP and TIMP assist the process of trophoblast invasion and uterine spiral artery remodeling by degrading ECM.

ofPEor hypertensive pregnancy (HTN-Preg), providing evidence for a strong association between organismal immunity and PE (87).

The level of MMP-2 expression in patients with PE remains controversial: some studies have found high levels of MMP-2 in the plasma or amniotic fluid of patients with PE or subsequent development of PE, while others have suggested that there is no statistical difference in the level of MMP-2 expression (88–91). However, the results of most experimental studies showed low expression or low activity of MMP-9 in serum and placental tissues of patients with PE (92). Moreover, the decrease in MMP-9 is found to be more pronounced in patients with early-onset PE (93). These findings suggest that abnormal expression of MMP-2 and MMP-9 may be involved in the pathogenesis of PE. Since MMP has a significant proteolytic effect on ECM, downregulation of MMP expression in patients with PE may lead to impaired growth, proliferation, and migration of SMC, thus interfering with the process of uterine spiral artery remodeling, which is consistent with the findings of Li (94) et al. on increased collagen content in the uterus, placenta, and aorta in mice with a model of PE. Another study showed a decrease in MMP-2 expression levels in the placenta of patients with severe PE under hypoxic conditions, mediated by the Nodal/ALK7 signaling pathway, acting in coordination with taurine upregulated protein 1 (TUG1) to achieve an invasive impaired trophoblast outcome (91).

There is extensive evidence that placental ischemia and hypoxia promote the release of a variety of active growth factors, including pro-angiogenic factors such as VEGF and placental growth factor (PlGF), as well as anti-angiogenic factors such as soluble fms-like tyrosine kinase-1 (sFlt-1), anti-inflammatory cytokines TNF-α as reactive oxygen radicals (ROS), IL-6, and HIF (18, 95). VEGF is a supergene family derived from a platelet growth factor, while sFlt-1 is a soluble antagonist of VEGF, both of which function importantly in the regulation of angiogenic homeostasis (96). An elevated sFlt-1/PIGF ratio is observed in patients with late-onset PE, and several authors have proposed sFlt-1/PIGF as an early predictor of PE (97–100). Studies support sFlt-1 as a potential upstream mechanism linking placental ischemia and reduced MMP-2 and MMP-9 content in HTN-Preg, of which VEGF can reverse this reduction in MMP content induced by sFlt-1 (94). Removal of circulating sFlt-1 in patients with early PE by a plasma-specific dextran sulfate column may reduce urinary protein and prolong pregnancy with no significant adverse fetal effects (101). sFlt-1/PIGF offers new ideas for optimizing the management of PE.

In addition to the abnormal remodeling process of spiral arteries, PE is also closely associated with inflammatory immune hyperactivation. Previous studies have found an excess of macrophages in placental biopsy specimens from patients with PE and that these excess macrophages tend to be located in and around the spiral arteries, separating them from trophoblast cells rather than in the stroma surrounding the spiral arteries and extravillous trophoblast (52). Apoptosis of extravillous trophoblast cells induced by macrophages through inflammatory mediators such as TNF-α is associated with

damaged intravascular trophoblast invasion in PE (102). Altered MMP-9 expression in the serum of patients with PE is associated with type I TNFR, suggesting an underlying inflammatory process, especially in early PE (103). The immune mechanisms underlying the association of MMP with the development of PE are not yet clear. Here we propose the following hypothesis based on the available evidence: differential expression levels or activity of MMP (especially MMP-9) are induced by mutual promotion with various inflammatory factors (e.g., TNF-α, IL-6), causing damage to vascular ECs, and the release of ROS in hypoxic conditions to stimulate oxidative stress-inducing vascular endothelialand smooth muscle cell dysfunction (Figure 2).

It is also worth mentioning that MMP is involved in atherogenesis by taking an essential role in the immune response and vascular inflammation (5). The balance between synthesis and degradation of ECM components is crucial for plaque stability, and MMP, in addition to its role in degrading the ECM of patients with atherosclerosis, reflects a systemic inflammatory response whose imbalance with TIMP may be the result of changes in the environment of pro- and anti-inflammatory factors in advanced clinical stages of coronary artery disease (104). However, most of these studies were oriented towards the assessment of the role of carotid or coronary arteries, and whether MMP exerts the same effect during atherosclerosis of the uterine spiral arteries at the maternal-fetal interface in patients with PE is not yet supported by precise experimental results, which may be one of the directions worthy of future research.

5.2 Recurrent spontaneous abortion

Recurrent spontaneous abortion (RSA) is a severe reproductive disorder of pregnancy that remains an incomplete problem in obstetrics and gynecology (105). In

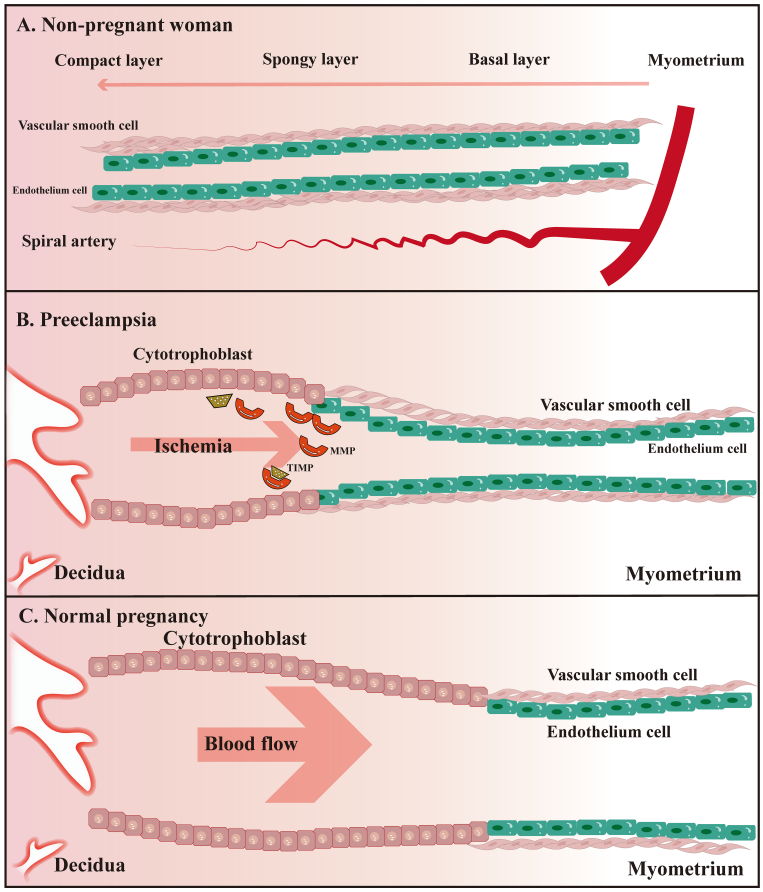

FIGURE 2
Schematic diagram of uterine spiral artery remodeling in non-pregnant women, PE, and normal pregnant women. **(A)** The uterine spiral arteries extend into the functional layer of the endometrium, of which the lumen diameter changes periodically under the influence of ovarian hormone levels; **(B)** Remodeling of the uterine spiral arteries is insufficient compared to normal pregnant women; **(C)** Physiological remodeling of the uterine spiral arteries is witnessed with the thickened spiral arteriole, enlarged lumen, and increased blood flow speed.

addition to the removal of the N-terminal structural domain by hydrolases and regulation by endogenous inhibitors, the regulation of MMP at the gene transcription level is influenced by various cytokines and growth factors, which may closely related to abortion.

VEGF plays a vital role in embryo implantation by participating in placental development and improving endometrial tolerance. It acts mainly through binding to tyrosine kinase receptors, including Flt-1, kinase insert domain receptor (KDR, also termed VEFGR-2), Flt-4 (also termed VEGFR-3), with Flt-1 also being expressed in macrophage cell lineage cells (106–108). Lash (109) explored changes in the expression of VEGF and its receptors in the endometrium of women possessing a history of recurrent miscarriage and proposed that the expression level of VEGF-A was decreased. In another study,He and Chen (110) detected the amount of VEGF protein in the early chorionic villous tissue of patients with recurrent miscarriage by western blot and found that the amount of VEGF protein was downregulated in the early chorionic villous tissue of patients with recurrent miscarriage (0.79 ± 0.40) compared to the control group (1.01 ± 0.37). The association of the ERK-VEGF/MMP-9 signaling pathway with the epithelial-mesenchymal transitionprocess can be observed in primary hepatocellular carcinoma cells (111, 112). Indeed, the underlying cause of VEGF dysregulation in recurrent miscarriage remains unknown, and genetic variation may be one of the potential causes (113). Yan, Fang (33) showed that the MMP2-735T allele and the MMP9-1562T allele might be associated with RSA risk. Among them, the MMP9-1562T allele was also associated with preterm birth (114). Due to the critical role of VEGF and MMP in the placental implantation process, in an analysis of blood and follicular fluid from women with multiple implantation failures,Benkhalifa (115) suggested that circulating MMP-7 and VEGF could serve as potential predictive biomarkers for recurrent implantation failure.

IFN-γ is a soluble dimeric cytokine,which are higher in the peripheral blood of non-pregnant women with recurrent miscarriage than in the healthy population, suggesting IFN-γ as a potential risk factor for patients with RSA (116, 117). This is consistent with the finding that IFN-γ reduces MMP-2 secretion and trophoblast invasiveness (118). Recognizing the relationship between maternal Th1/Th2 cytokines and unexplained recurrent spontaneous abortion (URSA) helps in the early diagnosis of URSA as well as treatment monitoring, and IFN-γ/IL-4 in the early diagnosis of URSA reduces the rate of missed diagnoses (119, 120). In addition to IL-4, IL-6, IL-1, and IL-12 can be found at altered levels in patients with RSA (121–123) (Table 1).

5.3 Trophoblastic disease of pregnancy

Gestational trophoblastic disease (GTD) is caused by allogeneic embryo transfer and includes a series of interrelated disorders, staphyloma, invasive staphyloma, choriocarcinoma, placental site trophoblastic tumors, and epithelioid trophoblastic tumors (136). Gestational trophoblastic diseases are characterized by vascular abnormalities in the trophectoderm with an imbalance in the expression of MMP and its inhibitors (137).

Several clinical studies have shown that the imbalance between activation and inhibition of MMP-2 plays an important role in the pathogenesis, progression, and metastasis of GTD and that MMP-2 is predominantly expressed in the syncytial trophoblast of gravida (138), as well as a higher positive rate for MMP-2 and TIMP-2 in gestational trophoblastic tumors compared to normal villi (139). In addition, those with malignant potential had higher MMP-9/TIMP-1 ratios than those without malignant transformation and normal villous tissues, illustrating its potential as a predictor (140). In an *in vitro* study using 0,5,10,25,50,100,200 μg/L IL-12 to treat human choriocarcinoma cell line JEG-3, it was observed that the overall expression level of MMP-9 was reduced by IL-12 treatment compared to the control, but increased with increasing IL-12 concentration, while application of 5 μg/L IL-12 observed that MMP-9 expression was downregulated with time (0,24,36,48,72 hours) (141). IL-12 coordinates the involvement of MMP-9 in cell invasion in a dose- and time-dependent manner and is one of the possible mechanisms of choriocarcinoma development.

MMP may be involved in the invasive and metastatic potential of choriocarcinoma, which has high expression of MMPand low expression of its inhibitors. Compared to choriocarcinoma, placental site trophoblastic tumor has low expression of MMP and increased expression of inhibitors of MMP, which explains the lower invasiveness of placental site trophoblastic tumor compared to choriocarcinoma (142).

6 Therapeutic potential of MMP and inhibitor in pregnancy-related diseases

The role of MMP in the maternal-fetal interface makes it a promising target for immunotherapy. Overall, MMP is inhibited by both endogenous and exogenous inhibitors, with TIMP acting as the predominant endogenous inhibitor of MMP (143).

TIMP N-terminus folds as a single unit with TIMP attached to the active sites of MMP to inhibit its functions, and TIMP-1, TIMP-2, TIMP-3, and TIMP-4 homologous TIMP have been identified, of which TIMP-1 and TIMP-3 are glycoproteins (144). Intriguingly, in general, a single TIMP can inhibit multiple MMP with different effects, e.g., TIMP-1 can act simultaneously on MT1-MMP, MT3-MMP, MT5-MMP, and MMP-19 (145). Furthermore, TIMP-3 can even inhibit metalloproteinases other than MMP (146). The inability to specifically target specific MMP may be one of the reasons for

TABLE 1 Interleukin levels in normal human pregnancy and recurrent spontaneous abortion.

Interleukin(IL)	Specimen	Number(n)	Normal Pregnancy (pg/ml)	RSA (pg/ml)	Trend	Method	Reference
IL-1β	Placenta	15:15	1.00*	53.58*	↑	qPCR	(123)
IL-2	Peripheral blood mononuclear cell	32:21	378.6 ± 64.9	1829.4 ± 514	↑	ELISA	(124)
IL-4	Serum	135:135	22.72 ± 15.34	8.76 ± 2.60	↓	ELISA	(125)
IL-6	Decidua	40:35	–	–	↑	RT-PCR	(126)
IL-6	Serum	40:60	0.6 ± 0.2	6.7 ± 0.9	↑	ELISA	(127, 128)
IL-10	Serum	25:24	307.7 ± 188.6	144.0 ± 106.5	↓	ELISA	(117, 129)
IL-12	Serum	18:29	8.00	12.40	↑	ELISA	(121)
IL-15	Placenta	15:12	1.00*	31.70*	↑	qRT-PCR	(130)
IL-17	Serum	40:60	1.5 ± 0.1	62.7 ± 7.8	↑	ELISA	(127)
IL-18	Placenta	15:15	1.00*	4.90*	↑	qPCR	(131)
IL-22	Decidua	11:9	5.1(3.1-5.8)**	2.9(2.0-4.4)**	↓	qRT-PCR	(132)
IL-25	Trophoblast cell	20:11	3.85(3.6-4.51)#	5.18(4.46-5.76)#	↓	qPCR	(133)
IL-27	Decidua	18:16	–	–	↓	qRT-PCR	(134)
IL-33	Decidua	6:6	–	–	↓	qPCR	(135)
IL-35	Serum	40:60	89.36 ± 33.5	54.48 ± 3.1	↓	ELISA	(127)

Values represent means ± standard deviation. Numbers shows n(normal pregnancy):n(recurrent spontaneous abortion).
* means the concentration multiplier of RSA based on normal pregnancy, which is considered to be base 1.
** means the results are calculated in ΔΔCt, and # in ΔCt. The range of quartiles is shown in parentheses.

the multiple side effects seen in clinical trial studies related to TIMP. This problem seems to be solved by monoclonal antibodies with high specificity and affinity for specific MMP, such as monoclonal antibodies REGA-3G12 and REGA-2D9 that react specifically with MMP-9 without cross-reacting with MMP-2 (5).

Currently, there are extensive mechanistic studies and preliminary clinical attempts regarding MMP and its inhibitor in intestinal inflammatory diseases, vascular diseases, fibrotic lesions, and tumor-related diseases. Serum MMP-3 and MMP-9 levels have been considered good markers of ulcerative colitis (UC) and inflammatory bowel disease (IBD) associated with some clinical stages (147–149). The selective MMP inhibitor ND-322 in a melanoma orthotopic mouse model can inhibit tumor growth and metastatic processes by targeting MMP-2 and MT1-MMP, providing a new avenue for adjuvant treatment options for aggressive melanoma (150). In addition, EMMPRIN is an attractive target in the treatment of oncological diseases due to its pro-angiogenic and pro-metastatic properties. Walter, Simanovich (42) designed a novel epitope-specific antibody against EMMPRIN that inhibits the secretion of MMP-9 and VEGF, shifting the tumor microenvironment of macrophages from an anti-inflammatory microenvironment dominated by TGF-β to one that is less immunosuppressive, thus allowing stimulated macrophages to perform antibody-dependent cytotoxic effects (ADCC) and kill tumor cells. There are clinical applications for EMMPRIN antibodies, such as Licartin, which has been approved by the Chinese Food and Drug Administration as a therapeutic anti-hepatocellular carcinoma radioimmune agent that is effective in reducing recurrence of hepatocellular carcinoma and prolonging survival in patients with advanced hepatocellular carcinoma after *in situ* liver transplantation (OLT) (151). Application of 50 μM concentration of docosahexaenoic acid (DHA) in human breast cancer cell line MDA-MB-231 resulted in 80% cell growth inhibition observed, while DHA inhibited breast cancer proliferation *in vitro* mainly by blocking the Cox-2-PGE2-NF-κB cascade to achieve inhibition of MMP-2 and MMP-9 transcription (152). DHA, a typical ω3-polyunsaturated fatty acid (ω3-PUFA), is one of the important unsaturated fatty acids in the body, generally from fat-rich fish, and is now widely recommended in clinical applications for nutrient

supplementation and preventing preterm birth in pregnant women (153, 154), for which the preventive function is supported by *in vitro* experiment, animal experiment and clinical study (155–157). These findings provide crucial preclinical evidence for using DHA in chemoprevention to overcome potential therapeutic options for the corresponding cancers.

In the field of female pregnancy, MMP and its inhibitors have also shown surprising promise for application. Currently, the most effective treatment for PE remains the termination of pregnancy, while the incidence of preterm births will inevitably increase. Measuring the expression levels of MMP-2 or MMP-9 in serum or amniotic fluid during pregnancy may serve as a new biomarker for predicting or monitoring PE while quantifying changes in the activity of MMP by, for example, measuring protein hydrolysis products in serum may also help in the diagnosis, condition monitoring and treatment evaluation of patients with PE. Immunotherapeutic approaches play an active role in RSA (158). Since the underlying cause of RSA is still unknown, several new therapeutic approaches have been proposed to treat RSA, including low-molecular heparin, corticosteroids, intravenous immunoglobulin, or leukapheresis, but none of them have proven their effectiveness with large-scale data to date (116). Immunotherapy regimens based on MMP may be able to give a new direction to RSA. Interestingly, patients with RSA may be associated with metabolic dysregulation, such as hyperglycemia, which can affect MMP/TIMP regulation, which may provide new evidence to support clinical glycemic regulation in RSA patients (159, 160). Exploring the expression studies of MMP and TIMP members in different gestational trophoblastic diseases can help screen potential molecular biological markers for GTD diagnosis, determine the degree of disease malignancy and prognosis, and also provide possible therapeutic targets. Selective inhibitory antibodies related to MMP could be used for future treatment of gestational trophoblastic diseases. Particular inhibitory antibodies to MMP-9 and MMP-14 have been developed and shown to be effective in inhibiting tumor growth and metastasis (161), but their clinical efficacy is currently uncertain.

Despite the significant progress now acquired in MMP inhibitor research, doxycycline is the only MMP inhibitor approved by the FDA (44). Currently, MMP inhibitor therapies have not been applied in clinical practice in obstetrics and gynecology. Until we fully understand their potential mechanisms and corresponding pharmacokinetic profiles in embryo implantation and pregnancy-related diseases, the related clinical applications of MMP inhibitor immunotherapy should be cautious.

7 Conclusions

MMP can be secreted by various cells and is involved in processes such as tissue remodeling and angiogenesis. This paper covers the immunomodulatory mechanisms of MMP and its inhibitors at the maternal-fetal interface. However, due to the difficulty of obtaining specimens at all stages of gestational age, most existing studies have focused on maternal-fetal interface studies at early gestation or delivery, and how MMP plays a role in mid-and late pregnancy has not been elucidated, which may be one of the future research directions. This paper offers the possibility of using MMP and TIMP as targets or clinical protocols for immunotherapy in pregnancy-related diseases. However, future challenges, such as preparation of specific targeting agents and clinical side effects beyond expectations, still need to be addressed.

Author contributions

MJ conducted the literature search, drew the images, and completed the first draft manuscript in collaboration with XC. HQ, WH, YZ, and DL helped prepare the manuscript. DW, YJ, and AL revised and edited the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Uterine NK cells underexpress KIR2DL1/S1 and LILRB1 in reproductive failure

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A significant proportion of recurrent miscarriage, recurrent implantation failure and infertility are unexplained, and these conditions have been proposed to have an etiology of immunological dysfunction at the maternal-fetal interface. Uterine Natural Killer cells (uNK) comprise three subsets and are the most numerous immune cells found in the uterine mucosa at the time of implantation. They are thought to play an important role in successful pregnancy by regulation of extravillous trophoblast (EVT) invasion and spiral artery remodelling. Here, we examine the frequency, phenotype and function of uNK1-3 from the uterine mucosa of 16 women with unexplained reproductive failure compared to 11 controls with no reproductive problems, during the window of implantation. We report that KIR2DL1/S1 and LILRB1 expression is lower in the reproductive failure group for both uNK (total uNK, uNK 2 and 3) and pNK. We also show that degranulation activity is significantly reduced in total uNK, and that TNF-α production is lower in all uNK subsets in the reproductive failure group. Taken together, our findings suggest that reproductive failure is associated with global reduction in expression of uNK receptors important for interaction with HLA-C and HLA-G on EVT during early pregnancy, leading to reduced uNK activation. This is the first study to examine uNK subsets during the window of implantation in women with reproductive failure and will serve as a platform to focus on particular aspects of phenotype and function of uNK subsets in future studies. Further understanding of uNK dysregulation is important to establish potential diagnostic and therapeutic targets in the population of women with unexplained reproductive failure.

KEYWORDS

NK cells, endometrium, pregnancy, ILC, recurrent miscarriage, infertility, recurrent implantation failure, reproductive failure

Introduction

Subfertility affects up to 1 in 7 couples and is commonly defined as failure to conceive after regular unprotected sexual intercourse for 12 months (1, 2). Recurrent miscarriage (RM) can be defined as loss of two (3, 4), three (5) or more clinical pregnancies and affects 1-2% of couples trying to conceive. Recurrent implantation failure (RIF) is a term used to describe failure to conceive after several attempts of *in vitro* fertilization (IVF). Similar to recurrent miscarriage, it has variable definitions which involve failure to achieve pregnancy after two (6) or three (7–9) fresh or frozen cycles with transfer of good quality embryos in women under the age of 40. Although these three conditions represent different clinical entities, a significant proportion of the cases in each group remain unexplained. Indeed, 1 in every 4 cases of subfertility do not have a cause identified after routine investigations (unexplained infertility: UI) (2, 10) and up to 50% cases of recurrent miscarriage (11).

It has been suggested that these conditions may have an immune etiology, resulting from dysfunctional molecular mechanisms involving the immune system at the maternal fetal interface. Indeed, immune dysregulation has been proposed to cause defective implantation and deep placentation resulting in a spectrum of pregnancy disorders such as recurrent miscarriage, pre-eclampsia, intra-uterine growth restriction and preterm labour, collectively termed "Great Obstetrical Syndromes" (12, 13). In line with this, the term "reproductive failure" was coined based on the theory that RM and RIF have a common pathogenic pathway involving a complex interplay of hormonal and immunological factors to ensure optimum endometrial receptivity (14). Here, we use the term "reproductive failure" to refer to women with UI, RM and RIF.

Uterine natural killer (uNK) cells are the most abundant immune cells found in placental bed of first trimester pregnancy, accounting for up to 70% of immune cells (15, 16). It is widely recognized that uNK are important for the development of the placenta by facilitating trophoblast invasion and vascular remodelling (17, 18). However, there is still uncertainty about how NK cells are associated with reproductive failure. Initially, it was thought that uNK, similar to peripheral blood NK cells (pNK), exert cytotoxicity and have the potential to kill invading trophoblasts (19, 20). However, this was quickly shown to be unlikely because uNK are less able to kill classical NK cell targets than pNK and have almost no ability to kill trophoblasts (21).

Immunogenetic studies have revealed interesting discoveries about interactions between uNK receptors KIR, LILRB1 and CD94/NKG2 heterodimer with their ligands HLA-C, HLA-G and HLA-E, respectively, on extravillous trophoblast cells (EVT). The most compelling evidence comes from studies on KIR/HLA-C interaction. KIR expressed by uNK can confer either an activating or inhibitory signal, which in turn is dependent on the genes inherited by each individual. The genetic polymorphism of KIR can be simplified by considering two main KIR haplotypes, KIR A and KIR B. The genetic polymorphism of KIR can be simplified by considering two main KIR haplotypes, KIR A and KIR B. KIR A is comprised of mostly inhibitory genes such as KIR2DL1 and KIR2DL3. KIR B contains the inhibitory genes KIR2DL1 and KIR2DL2, but in addition contains activating genes such as KIR2DS1 and KIR2DS2. The ligand for 2-domain KIR are HLA-C molecules, which are expressed by all EVT and carry either a C1 or C2 epitope. The combination of maternal KIR and paternally derived HLA-C in fetus can be widely diverse, resulting in net activation or inhibition of NK cells (22). In a study of women with RM and RIF undergoing assisted reproduction using their own or donor oocytes, women with a KIR AA genotype had lower livebirth rates compared to those with KIR AB or KIR BB genotypes, suggesting that reduced uNK activation is associated with negative reproductive outcomes (23). The livebirth rate was also decreased as fetal HLA-C2 load is increased which has implications for future clinical utility in selection of fetus with HLA-C that is "compatible" with maternal KIR. (23). However, this is still considered to be at the pre-clinical stage owing to several limitations of present evidence and practical considerations in the clinical setting (22).

On the other hand, it has been proposed that increased uNK number or cytokine production could cause excessive premature angiogenesis which in turn cause harmful oxidative stress to the feto-placental unit (24). Research on uNK cytokine production has been inconclusive, with conflicting reports on which cytokine is excessive or deficient in reproductive failure (25). Potential reasons for this are the use of different assays to measure cytokine level, addition of other cytokines (such as IL-2 or IL-15) into culture to recover immune cells which alters their cytokine production profile, and use of decidual samples collected after miscarriage potentially introducing the confounding factor of inflammatory processes that occur after a miscarriage (13).

Since the landmark discovery of three uNK subsets by single-cell RNA sequencing (scRNAseq) (26), it has become apparent that these subsets exhibit different phenotypes and function and thus are likely to have different roles in pregnancy (18). A better characterization of the three uNK subsets was identified to be essential to the field (18). We have recently characterized these uNK subsets throughout the normal human reproductive cycle (27) and have shown that all three subsets upregulate KIR and LILRB1 expression during first trimester. uNK also have the ability to increase cytokine production during the mid-secretory phase of the menstrual cycle, which aligns with period of embryo implantation. Further scRNAseq data have demonstrated that uNK1 frequency is lower and uNK3 higher in women with RM compared to pregnant controls (28, 29). However, these studies were performed in decidua collected after miscarriage which may have been affected by inflammatory processes thus not reflecting the immune environment before the miscarriage. Therefore, the question remains, how do uNK subsets behave in the endometrium of women with reproductive failure? Is one subset more affected than the others? Elucidating the answer to this may help us to focus research on a particular uNK subset to understand the pathophysiology behind reproductive failure. Therefore, we aim to evaluate differences in receptor expression, activation/cytokine production and proportion of matched pNK and uNK subsets in women with reproductive failure compared to controls and in women with ongoing compared to no ongoing pregnancy after *in vitro* fertilization (IVF).

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Materials and methods

Primary tissue

Collection of human tissue was approved by Solihull Research Ethics Committee (study number: 22/WM/0041). Inclusion criteria for patient groups were as follows: Infertility defined as women below 40 years old with failure to conceive after regular unprotected intercourse for more than one year, RM defined as loss of two or more clinical pregnancies and RIF defined as absence of pregnancy after two or more fresh or frozen transfer of good quality embryos. Exclusion criteria were any other causes identified for the reproductive failure after routine investigations including uterine, tubal, ovarian, male factor, endocrinological, parental chromosomal abnormalities and haematological (e.g. thrombophilia and antiphospholipid syndrome) factors. Inclusion criteria for the control group were women below 40 years old undergoing coil insertion for contraception with a regular menstrual cycle between 25 to 35 days and no history of reproductive problems. Exclusion criterion was usage of any coil or hormonal contraception within one month prior to sampling. The research participants' characteristics are summarised in Table 1.

Matched peripheral blood and endometrial samples were obtained for 16 reproductive failure patients (9 UI, 4 RM, 3 RIF) and 11 controls during mid-luteal phase. Endometrial biopsies were obtained by Pipelle sampler, immediately suspended in RPMI and processed within 2 hours. For the control group, endometrial samples were obtained before coil insertion for contraception between day 18-23 and mid-luteal phase was confirmed by histological dating and serum progesterone level. For the patient group, samples were obtained seven to nine days after ovulation as determined by serial urine luteinizing hormone (LH) tests.

For extraction of peripheral blood lymphocytes (PBL), whole blood was layered onto Histopaque (Sigma Aldrich), centrifuged (700 x*g* 21°C, 20 minutes) and the enriched PBL were washed twice (500 x*g*, 10 minutes, 4°C) with Dulbecco's Phosphate-Buffered Saline (Life Tech). For endometrial lymphocytes extraction, endometrial tissue was pressed through 100 µm cell strainer, pelleted (700 x*g*, 10 minutes, 4°C), resuspended in Dulbecco's Phosphate-Buffered Saline supplemented by 10% Fetal Calf Serum (Sigma Aldrich), passed through 70µm strainer, and layered on Histopaque to isolate immune cells as above. 0.2 to 1 x10^6 endometrial lymphocytes and 1 x10^6 PBL were allocated per condition of either fresh stain or culture.

TABLE 1 Research participant characteristics.

	Day of cycle	Length of cycle	Days post LH surge	Age	Gravida	Parity	Previous pregnancies	Previous IVF cycles	Pregnancy outcome	Serum pro-gesterone	Assessment
UI	22	28	9	32	0	0	0	1	Pregnant	17.9	Phenotype, function,
UI	20	28	7	39	0	0	0	0	Not pregnant x2	51.7	Phenotype, function,
UI	22	28	9	34	0	0	0	0	Pregnant	NI	Phenotype, function,
UI	22	33	7	36	0	0	0	0	Not pregnant	36.2	Phenotype, function,
UI	23	33	7	36	0	0	0	0	Not pregnant x2	NI	Phenotype, function,
UI	20	28-32	8	35	0	0	0	1	Miscarriage	NI	Phenotype, function,
UI	22	28	7	38	0	0	0	0	Pregnant	33.5	Phenotype, function,
UI	20	28	7	36	0	0	0	1	Not pregnant	35.7	Phenotype, function,
UI	21	28	7	35	0	0	0	0	Not pregnant	NI	Phenotype, function,
RM	20	28-30	7	39	3	0	x3 miscarriages	0	FAE	NI	Phenotype, function for PBL only
RM	22	28	9	34	3	0	x3 miscarriages	x2 IVF	Not pregnant	31.8	Phenotype, function
RM	20	25-32	9	32	2	0	x2 miscarriages	0	No outcome	NI	Phenotype, function

(Continued)

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TABLE 1 Continued

	Day of cycle	Length of cycle	Days post LH surge	Age	Gravida	Parity	Previous pregnancies	Previous IVF cycles	Pregnancy outcome	Serum pro-gesterone	Assessment
RM	20	28	7	36	5	2	x4 miscarriages	0	Pregnant	39.7	Phenotype, function
RIF	25	30	7	39	1	1	X1 livebirth	x3 IVF and ICSI	Pregnant	39.5	Phenotype, function
RIF+UI	17	28	7	33	0	0	0	x1IVF, x1 ICSI	Not pregnant	42.1	Phenotype, function
RIF +RM	21	30	7	35	0	0	x2 miscarriages	x3 IVF	Miscarriage	NI	Phenotype, function
Control	20	28-30	NI	27	0	0	N/A	N/A	N/A	23	Phenotype, function
Control	19	28	NI	30	0	0	N/A	N/A	N/A	51	Phenotype, function
Control	21	28	NI	40	4	3	3 livebirths, 1 miscarriage	N/A	N/A	28	Phenotype, function
Control	18	28	NI	25	0	0	N/A	N/A	N/A	NI	Phenotype, function
Control	19	28-30	NI	33	1	1	1 livebirth	N/A	N/A	22	Phenotype, function
Control	17	28-30	NI	32	0	0	N/A	N/A	N/A	13	Phenotype, function
Control	21	28	NI	39	3	2	2 livebirths	N/A	N/A	NI	Phenotype, function
Control	19	28	NI	27	0	0	N/A	N/A	N/A	24.2	Phenotype, function
Control	19	28	NI	31	0	0	N/A	N/A	N/A	NI	Phenotype and function for EL only
Control	19	30	NI	35	3	3	3 livebirths	N/A	N/A	NI	Phenotype and function for EL only
Control	24	33	NI	25	0	0	N/A	N/A	N/A	8.1	Phenotype, function

UI, unexplained infertility; RM, recurrent miscarriage; RIF, recurrent implantation failure, LH, luteinizing hormone; IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection; FAE, freeze all embryos; N/A, not applicable; NI, no information; PBL, peripheral blood lymphocytes; EL, endometrial lymphocytes.

Short-term culture for measurement of cytokine production

Both PBL and endometrial lymphocytes were suspended in RPMI supplemented with antibiotics, non-essential amino acids 10% fetal calf serum, 1 μM sodium pyruvate, 2.5 mM HEPES and 50 μM β-mercaptoethanol (all from Gibco) and divided into unstimulated and stimulated wells. Anti-CD107a BV605 (100 μl/ml; clone H4A3, Biolegend), Brefeldin (10μg/ml) and Monensin (2μM/ml) were added to all wells and Phorbol 12-myristate 13-acetate (PMA) (50 ng/ml) and ionomycin (1μg/ml) into the stimulated wells only. Cells were incubated for 4 hours at 37°C then harvested and stained.

Flow cytometry

The following anti-human antibodies were used for surface staining: Anti-CD56 Brilliant Violet (BV) 650 (clone NCAM 16.2, BD Bioscience), anti-CD39 BV421 (clone A1, Biolegend), anti-CD3 BV711 (clone SK7, Biolegend), anti-CD103 BV785 (clone Ber-ACT8, Biolegend), anti-CD16 Alexa Fluor(AF)700 (clone 3G8, Biolegend), anti-CD9 phycoerythrin(PE)/Dazzle 594 (clone HI9a, Biolegend), anti-CD49a PE/Cy7 (clone TS2/7, Biolegend), anti-CD45 allophycocyanin (APC) (clone HI30, Biolegend), anti-CD94 PE (Clone HP-3D9, BD Bioscience), anti-CD94 PECy7 (Clone DX22, Biolegend), anti-CD158a/h (KIR2DL1/DS1) VioBright 515 (clone REA1010, Miltenyi Biotec), anti-CD158a (KIR2DL1) PE

(clone HP-DM1, Biolegend) anti-CD158b (KIR2DL2/DL3) APC vio 770 (clone REA 1006, Miltenyi Biotec), CD85j (ILT2 or LILRB1) Peridinin chlorophyll protein (PerCP)-eFluor 710 (clone HP-F1, Thermo Fisher Scientific). The following anti-human antibodies were used for intracellular cytokine staining: anti-IL-8 PE (clone G265-8, BD Bioscience), anti-IFN-γ APCvio770 (clone REA600, Miltenyi Biotec), anti GM-CSF PERCP/Cyanine 5.5 (clone BVD2-21C11, Biolegend), anti-TNFα FITC (clone MAb11, Biolegend).

Cells were incubated with fixable viability dye (Live/Dead Fixable Aqua Dead Cell stain kit, LifeTech) and surface antibodies (15 minutes, 4°C). For intracellular staining, human FoxP3 buffer (BD Biosciences) was used according to manufacturer's instructions for fixation and permeabilization before staining with intracellular antibodies (30 minutes, 4°C). Excess antibodies were washed off (5 minutes, 500 *xg*, 4°C) between each incubation and twice after the final incubation with intracellular antibodies.

Statistical analysis

Data were acquired on an BD Fortessa and analysed using FlowJo (Tree Star, Ashland, OR). In order to ensure reproducibility of results, CS&T and application settings were used. Statistical analysis was performed using PRISM (GraphPad Software Inc.). Data were assessed for normality using Shapiro-Wilk tests to determine whether a parametric or a non-parametric statistical test was appropriate. The appropriate statistical test was used to compare patient and control groups as specified in figure legends. The Holm-Bonferroni correction for multiple hypothesis testing was applied to reduce family-wise error rate and both uncorrected (p) and corrected (p') values are given in figures and legends. Fisher's exact test was used to test for significant differences in proportions of patients and controls expressing *KIR2DS1*.

Results

No difference in uNK or pNK frequencies in women with reproductive failure compared to controls

In women with recurrent miscarriage, assessment by scRNAseq on decidual samples after miscarriage found that uNK1 level is lower but uNK3 level is higher compared to controls, although these findings may represent inflammatory changes subsequent to fetal demise (28, 29). Therefore, we evaluated proportion of uNK and its subsets from mid-luteal phase endometrial samples as total uNK, uNK1, 2 and 3 as proportion of CD45+ lymphocytes and the latter three as proportion of total uNK. NK cells were identified as live CD3-CD56+ lymphocytes, and CD49a was used to distinguish uterine from circulating NK cells. The uNK subsets were subsequently identified by CD103 and CD39 expression (26) (Figure 1A). pNK were identified by conventional gating strategy; CD3-CD56+ cells with gating by CD56 expression level and on CD16 to distinguish $CD56^{bright}$ and $CD56^{dim}$.

In keeping with our previous finding at this stage in the menstrual cycle (27) uNK1 frequency was lower than that of uNK 2 and 3 in the endometrium in both control and UI/RM/RIF groups (Figure 1B). However, there was no significant difference in frequency of total uNK or uNK subsets in reproductive failure compared to control group (Figure 1B). Similarly, there was no significant difference in pNK (Figure 1C). Additionally, no significant difference was detected even when each subgroup of reproductive failure was considered separately (data not shown).

In our previous meta-analysis, it was found that CD16+ cells, which are likely to represent $CD56^{dim}$ pNK, are more frequency in the endometrium of women with recurrent miscarriage compared to controls (25). Therefore, we examined the frequency of CD3-CD56+CD49a- NK cells in the endometrium, since these more accurately represent pNK. Overall, no significant difference was detected in reproductive failure compared to control group (Figure 1D).

KIR2DL1/S1 and LILRB1 expression are lower in women with reproductive failure

KIR, LILRB1 and CD94/NKG2 heterodimer are uNK receptors that interact with HLA-C, HLA-G and HLA-E on EVT respectively (30). These receptors are present in all uNK subsets throughout the menstrual cycle and in all trimesters of pregnancy, albeit at different frequencies. Previously, we have shown that KIR2DL1/S1 and LILRB1 were upregulated in all three uNK subsets during first trimester, suggesting that these receptors are important for cross-talk with EVT at the time of placentation (27).

Here, we assessed KIR2DL1/S1, KIR2DL2/S2/L3, LILRB1 and CD94 expression on total uNK, uNK1, uNK2, uNK3 from mid-luteal phase endometrial samples and $CD56^{bright}$ and $CD56^{dim}$ pNK from matched peripheral blood samples in women with reproductive failure (RM, RIF or UI) compared to controls attending the clinic for contraceptive coil fitting. Representative histograms of staining from each receptor are shown in Figure 2A.

KIR2DL1/S1 expression was significantly lower in total uNK in the reproductive failure group, with further investigation of expression in subsets revealing lower expression in all three uNK subsets, although this only reached statistical significance for uNK2 and 3 (Figure 2B) In peripheral blood, KIR2DL1/S1 was also observed to be significantly lower in the reproductive failure group among $CD56^{dim}$ but not $CD56^{bright}$ cells (Figure 2B). Immunogenetic studies have found that maternal KIR haplotypes lacking KIR2DS1 are associated with failures of early pregnancy (31, 32), so one possibility is that this finding reflects a reduction in the proportion of individuals who have the *KIR2DS1* gene in the patient group, compared to controls. To investigate this, we accessed cryopreserved PBL from the patient group to determine if the patient expresses KIR2DS1 or not (Supplementary Figure 4 and Supplementary Table 1). 56% (9/16) of the patient group expressed any KIR2DS1, similar to the 47% (33) or 44% (31) *KIR2DS1* positive previously reported in UK cohorts. Cryopreserved PBL were not available from the original control group, but in recognition of the fact that the population at our hospital in London may differ from that elsewhere in the UK, we compared the frequency of KIR2DS1 positivity in our patient cohort with a group of women with no history of reproductive

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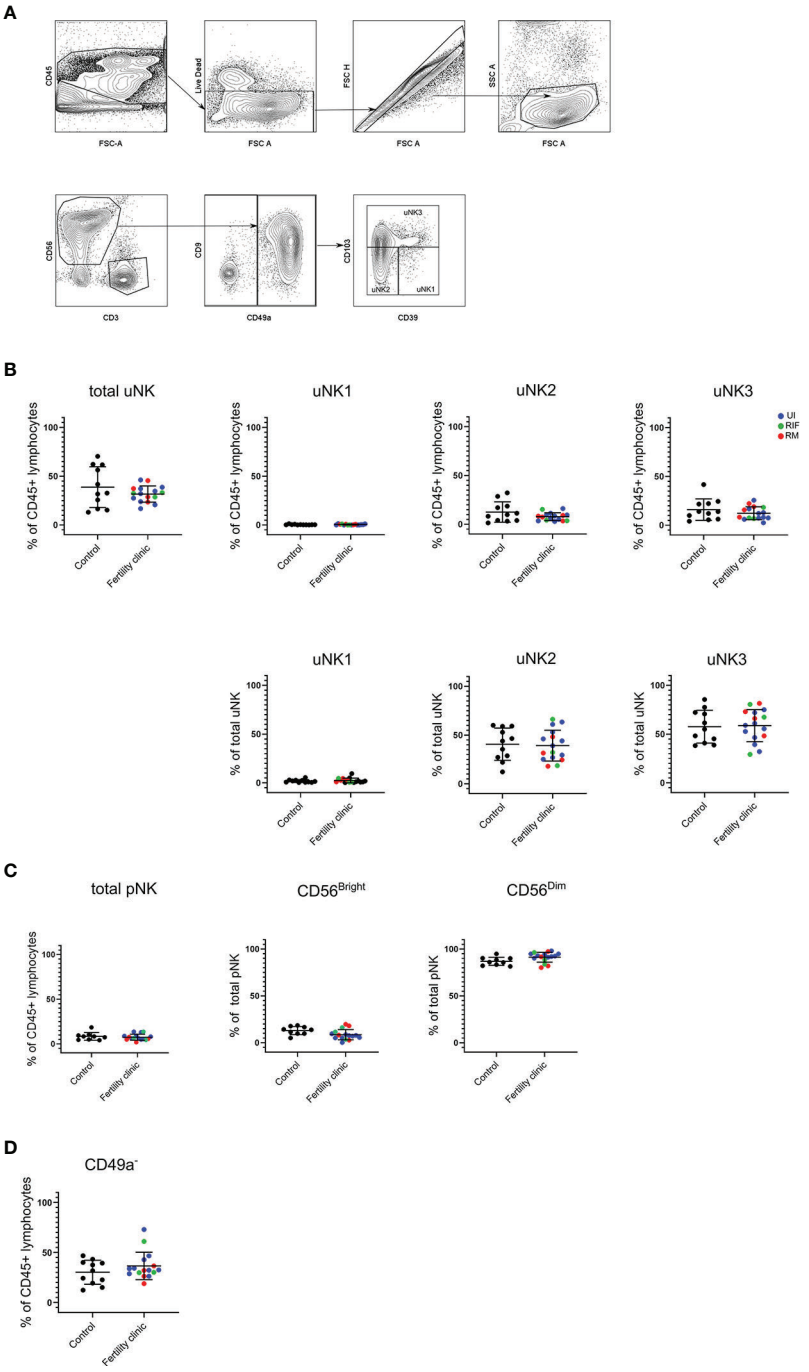

FIGURE 1
uNK and pNK proportion in unexplained subfertility, recurrent miscarriage and implantation failure compared to controls **(A)** FACs gating strategy used to identify three uNK subsets (representative example shown) **(B)** Graphs of total uNK from CD45+ lymphocytes then frequency of each uNK subset (uNK1, -2, -3) as proportion of CD45+ lymphocytes and proportion of total uNK. **(C)** Graphs of total pNK from CD45+ lymphocytes then frequency of $CD56^{Bright}$ and $CD56^{Dim}$ as proportion of total pNK. **(D)** Graph of CD3-CD56+CD49a- lymphocytes as proportion of total uNK. Means and standard deviations are shown for controls (n=11) shown in black and patients from fertility clinic including unexplained infertility (UI; n=9) showed in blue, recurrent miscarriage (RM; n=34) shown in red and recurrent implantation failure (RIF; n=3) shown in green. Statistical testing was done using unpaired t-test for normal or Mann-Whitney U test for non-normal distribution and showed no significant difference in any of the comparisons.

problems recruited from our hospital: these were 65% (13/20) KIR2DS1 positive. Therefore a lower proportion of patients was positive for KIR2DS1 than controls, although this finding was not significant (p=0.73).

LILRB1 was also significantly lower in total uNK and all three uNK subsets in the reproductive failure group, although this only reached statistical significance for uNK2 and 3 (Figure 2D). In the blood, LILRB1 expression was lower in for the reproductive failure group for both $CD56^{dim}$ and $CD56^{bright}$ pNK (Figure 2D). However, no trend was noted for KIR2DL2/S2/L3 (Figure 2C) or CD94 (Figure 2E) in either uNK or pNK.

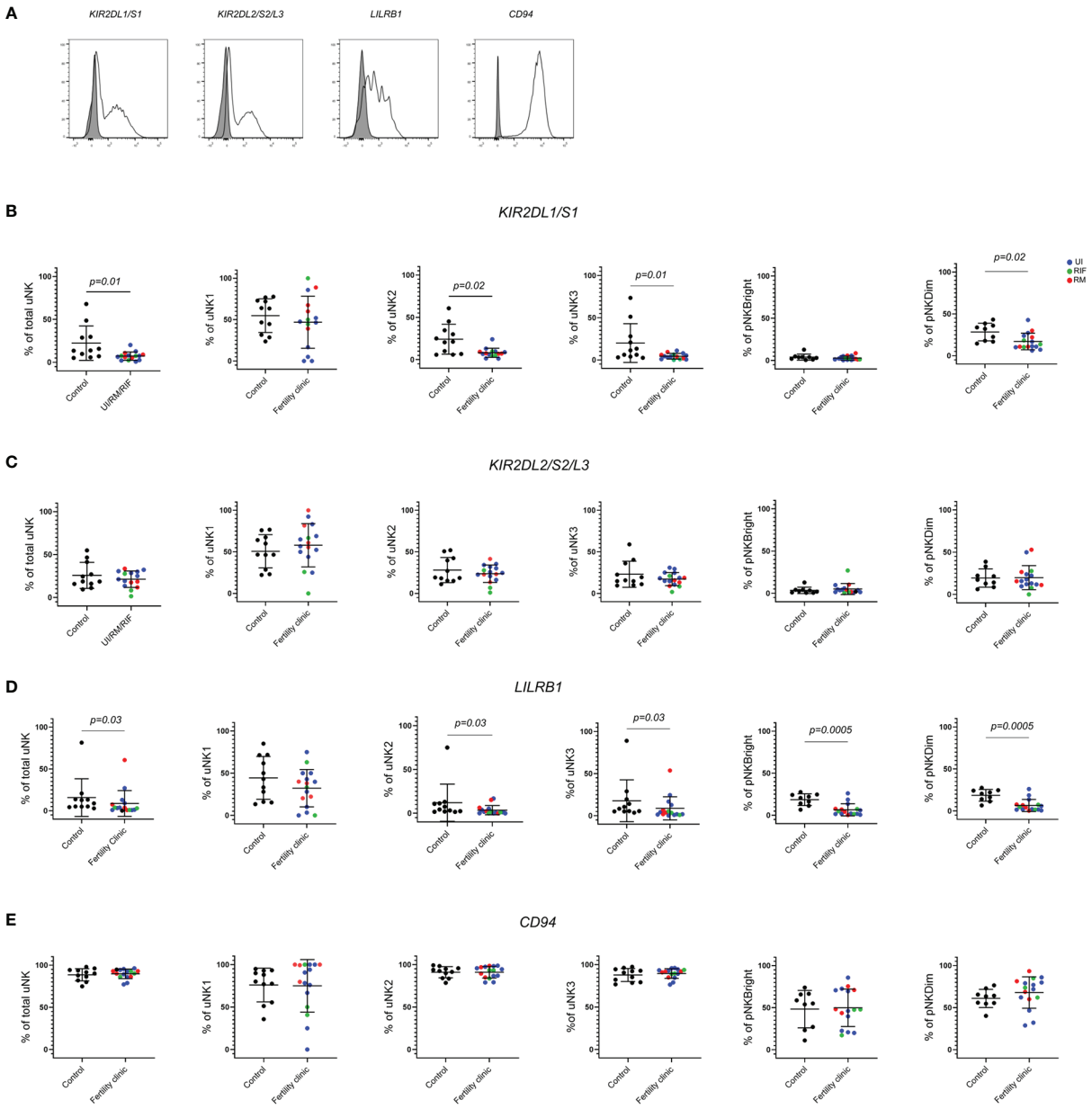

FIGURE 2
KIR2DL1/S1 and LILRB1 expression is lower in women with reproductive failure. **(A)** Uterine and peripheral NK cells taken from control and UI, RM and RIF groups were freshly stained for phenotypic markers. Representative staining of two samples from total uNK are shown in clear alongside fluorescence minus one control in grey. Graphs showing frequencies of KIR2DL1/S1 **(B)**, KIR2DL2/3 **(C)**, LILRB1 **(D)** and CD94 **(E)** on uNK and pNK. Means and standard deviations are shown for controls (n=11) shown in black and patients from fertility clinic including unexplained infertility (UI; n=9) showed in blue, recurrent miscarriage (RM; n=4) shown in red and recurrent implantation failure (RIF; n=3) shown in green. Statistical testing was done using unpaired t-test for normal or Mann-Whitney U test for non-normal distribution and p-values are shown on the graphs. Holm-Bonferroni correction for multiple hypothesis testing showed no significance except for LILRB1 on pNKBright and pNK Dim which remained significant at p'=0.003.

uNK are less activated in women with reproductive failure

To examine the ability of uNK to degranulate and produce cytokines, they were cultured for 4 hours in the presence of the protein transport inhibitors Brefeldin and Monensin. The ability of cells to respond directly *ex vivo* was determined by culturing them without stimulation, whereas their potential to respond was determined by adding PMA/I as a strong non-specific stimulus. Degranulation, as measured by the internalisation of anti-CD107a is a good measure of uNK activation (34, 35) and using this measurement we have previously found that uNK are most active at the time of implantation during secretory phase and first trimester (27). CD107a trended upwards in secretory phase and was significantly higher in first trimester compared to third trimester in uNK2 and 3 after stimulation. We also found that TNFα, IFNγ and IL-8 production across all uNK subsets is highest after stimulation with PMA and ionomycin during secretory phase (27).

Here, we replicated the assessment for functional responses with and without stimulation on uNK and pNK from women with

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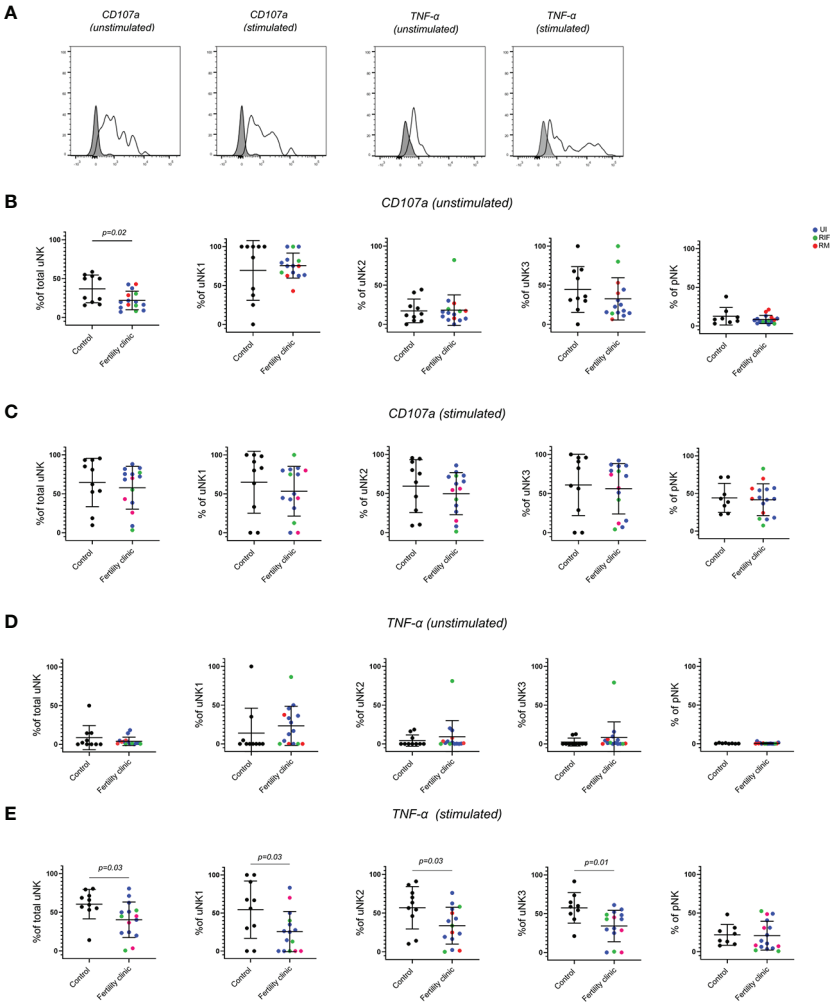

FIGURE 3
uNK are less active in women with reproductive failure. **(A)** Uterine and peripheral NK cells taken from control and UI, RM and RIF groups were cultured with or without PMA and ionomycin stimulation. Representative staining of one sample from total uNK is shown in clear alongside fluorescence minus one control in grey. Graphs showing frequencies of CD107a **(B, C)** and TNF-α **(D, E)** on unstimulated and stimulated uNK and pNK. Means and standard deviations are shown for controls (n=11) shown in black and patients from fertility clinic including unexplained infertility (UI; n=9) showed in blue, recurrent miscarriage (RM; n=3) shown in red and recurrent implantation failure (RIF; n=3) shown in green. Statistical testing was done using unpaired t-test for normal or Mann-Whitney U test for non-normal distribution and p-values are shown on the graphs. Holm-Bonferroni correction for multiple hypothesis testing showed no significance for all the comparisons.

reproductive failure compared to controls (Figure 3). Strikingly, we observed significantly lower degranulation by total uNK in women with reproductive failure, compared to controls, without stimulation (Figure 3B). When uNK subsets were considered separately, the reduction was most pronounced on uNK3 although this did not reach statistical significance. However, no discernible difference was seen for degranulation by total uNK or uNK subsets with stimulation (Figure 3C). A paired comparison of CD107a expression with and without stimulation in controls and patients revealed that CD107a was generally increased with stimulation, as expected, but it was significantly reduced in uNK1 of fertility patients only (Supplementary Figure 3).

Among all the cytokines (TNFα, IFNγ, IL-8 and GM-CSF) that were assessed, TNFα emerged as the only one that was significantly lower in all three uNK subsets in the reproductive failure group with stimulation (Figures 3D, E) Notably, none of the functional changes in uNK were mirrored by pNK. (Figure 3 and Supplementary Figure 1)

No difference in phenotype or function of uNK in women with ongoing versus no ongoing pregnancy after IVF

Next, we prospectively followed up women from the reproductive failure group to obtain data on their pregnancy outcome after IVF. We compared women who had an ongoing pregnancy (OP) with women with no ongoing pregnancy (NOP). The latter group consisted of women who did not fall pregnant as well as women who fell pregnant but miscarried. Due to the small numbers from each subgroup, they were pooled into the NOP group. The same phenotypic and functional parameters as above were assessed.

No significant difference was seen in any of the KIR (Figures 4A, B), LILRB1 (Figure 4C) or CD94 (Figure 4D) expression in either uNK or pNK. However, a trend of lower KIR expression was seen in total uNK and uNK subsets for the NOP group. On removal of the two datapoints of women who fell pregnant but miscarried from the

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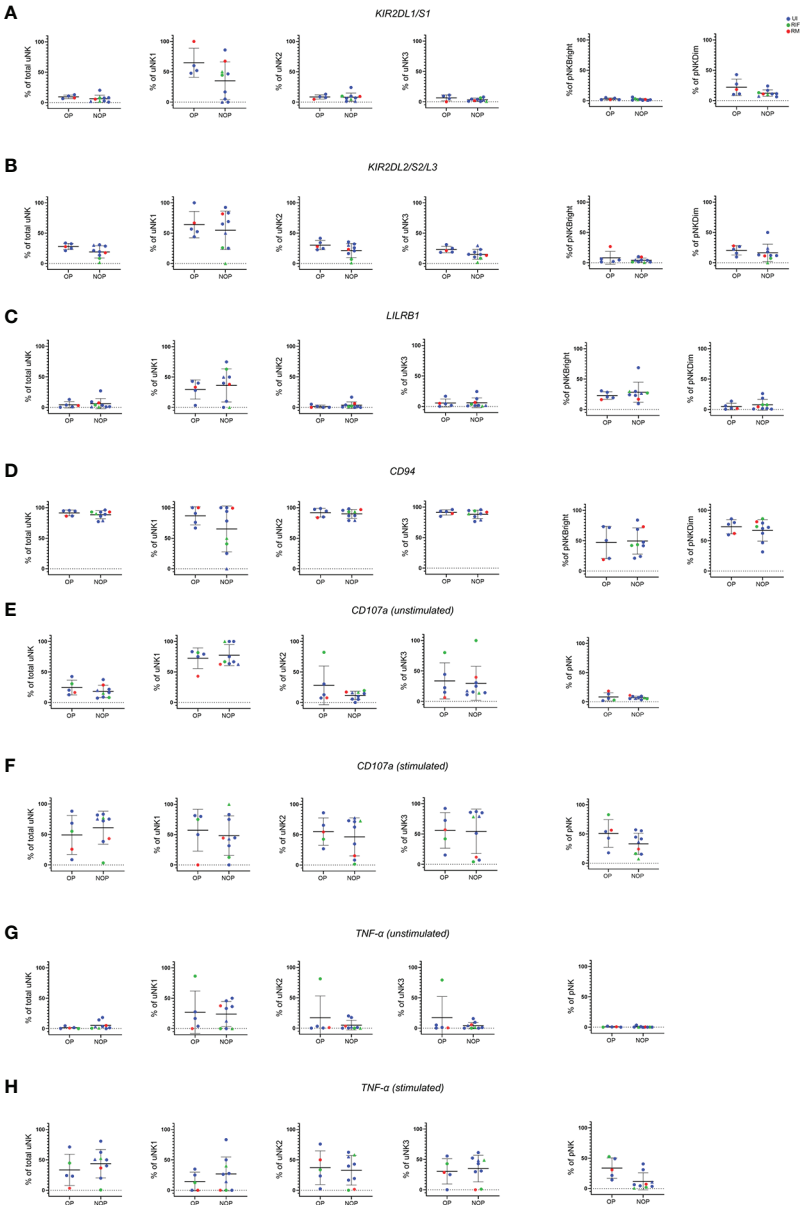

FIGURE 4
No difference in phenotype or degranulation in patients who had ongoing pregnancy compared to no ongoing pregnancy after IVF. Means and standard deviations are shown for KIR2DL1/S1 **(A)**, KIR2DL2/S2/L3 **(B)**, LILRB1 **(C)**, CD94 **(D)**, CD107a **(E, F)**, and TNF-α **(G, H)** with and without stimulation. NOP group consist of n=7 who did not become (represented as dots) and n=2 who became pregnant but miscarried (represented as triangles). Unexplained infertility (UI) is showed in blue, recurrent miscarriage (RM) is shown in red and recurrent implantation failure (RIF) is shown in green. Statistical testing was done using unpaired t-test for normal or Mann-Whitney U test for non-normal distribution and showed no significance in all of the comparisons.

NOP group, KIR2DL2/S2/L3 was significantly lower specifically in the uNK3 subgroup (data not shown). No other significant difference emerged from similar sensitivity analysis on the other receptors. In terms of functional activity, no significant difference was observed in CD107a (Figures 4E, F), TNF-α (Figures 4G, H), or any of the cytokines examined (Supplementary Figure 2).

Discussion

In this study, we have examined the frequency, phenotype and function of pNK and uNK subsets from timed endometrial samples obtained from mid-luteal phase in women with reproductive failure compared to controls. In contrast to previous studies of endometrial samples from UI, RM or RIF patients, which analyzed total CD3-CD56+ cells, here we also present data on the three uNK subsets individually.

Our previous systematic review showed significantly raised uNK in women with RM and RIF when considering total CD56+ NK cells in the endometrium during mid-luteal phase, but not in decidua collected after miscarriage (25). In women with RM, two recent studies using scRNAseq and mass cytometry demonstrated reduced uNK1 and raised uNK3 level in RM group (28, 29), albeit the studies compared termination samples to those collected after miscarriage, so

the results could be confounded by inflammatory changes occurring subsequent to fetal demise (13). In comparison, we did not observe any difference in the frequency of either CD56+ uNK or uNK subsets. One possibility could be that our cohort included patients with a mixture of types of reproductive failure, although each subgroup did not show any difference when considered separately. Another possibility is that this small study was not sufficiently powered to detect a difference, although no emerging trend was apparent from our results. A third possibility is that the comparison is not perfect because the timing of samples for control group was done by serum progesterone and histological dating instead of urine LH tracking, which increases the variance in the control group, thus reducing the power to detect differences. However, it should also not be discounted that the lack of difference is partially due to significantly lower level of uNK1 in the endometrium compared to decidua in first trimester, therefore no inherent difference in the frequency of endometrial NK cells may be present (27).

We found significantly lower expression of KIR2DL1/S1 in uNK subsets in the reproductive failure group compared to controls, and these findings were mirrored in pNK. Our finding in pNK is consistent with previous work which has reported a reduction in the expression of KIR2DL1 expression by pNK associated with reproductive failure (36–39), although we did not find a reduction in KIR2DL2 expression, which has also been reported (36). Studies on uNK KIR expression have thus far included samples retrieved after termination of pregnancy stratified to high and low risk of resistance index on uterine artery doppler (40) or post-surgical management of miscarriage (41), which reported reduction in KIR2DL1/S1 but no change in KIR2DL2/L3/S2 associated with worse pregnancy outcomes. Therefore our findings that uNK and pNK from UI, RM and RIF patients express lower levels of KIR2DL1/S1 are in line with previous work looking at other disorders of pregnancy.

Our finding that KIRD2L1/S1 expression was reduced on both uNK and pNK suggests that the deficiency in KIR expression is not tissue-specific. Early immunogenetic approaches demonstrated that women with KIR AA genotype in combination with fetus carrying HLA-C2 epitope are at higher risk of disorders of placentation associated with pre-eclampsia, and identified KIR2DS1, specifically, as protective (33, 42). Similarly, in IVF patients maternal KIR AA genotype is associated with higher miscarriage rate compared to KIR AB and BB, and this risk is augmented by presence of HLA-C2 on fetus (23, 32). Furthermore, *in vitro* studies found that stimulation of the activating KIR2DS1 and KIR2DS4 on uNK release GM-CSF that promoted trophoblast invasion (34, 35), although there is some evidence that this production of GM-CSF is not occurring in response to KIR2DS1 ligation by EVT (43).

A key question is whether the underexpression of KIRD2L1/S1 we observed is due to individuals who lack the *KIR2DS1* gene being overrepresented among patients compared to controls: our finding that the proportion of patients who do not express KIR2DS1 is similar to that in the general population suggests that this is not the case but recruitment of larger cohorts is needed to confirm this. Studies in larger cohorts should also examine the expression of KIR2DL1 and -S1 separately, to determine which of these KIRs, specifically, is reduced in reproductive failure. It is also important to consider factors other than simple presence of absence of genes which could impact KIR expression. These could include NK developmental processes that are influenced by genetic factors outside the KIR locus, such as the influence of HLA on NK cell education (44) or HCMV seropositivity, which is known to increase KIR expression on peripheral NK cells (45). HCMV seropositivity could also be relevant because uNK can respond to HCMV-infected stromal and trophoblast cells in a manner that is at least partially mediated by KIR2DS1 (46), highlighting a potential for KIR2DS1 expression to protect against HCMV-mediated early pregnancy failures. A limitation of our study was that HCMV serostatus for the donors was not available.

We also observed reduced LILRB1 expression in both uNK and pNK from reproductive failure patients. Although LILRB1 ligation inhibits pNK, recent research has found that ligation of LILRB1on uNK by HLA-G promotes growth factor secretion by uNK which in turn promotes development of the fetus through the placenta (47). A subset of uNK which express NKG2C and LILRB1 has been reported in multiparous women (48). It has been suggested that these cells could account for the greater likelihood of success in second and subsequent pregnancies (49) and if so, this would be consistent with a role for LILRB1 in optimizing placental development. For women with reproductive failure, studies on LILRB1 have mostly concentrated on pNK in RM patients (39, 50) except for one study on uNK using termination samples stratified by uterine artery resistance (51). On balance, and in line with our findings, these studies reported reduced expression of LILRB1 in women with high risk of reproductive problems. KIR2DL4 has also been proposed to recognize HLA-G although this line of evidence is controversial (13). Future studies are warranted to investigate this relationship in view of the lower expression of LILRB1 in our cohort of reproductive failure patients.

We found significantly lower degranulation by total uNK in the reproductive failure group, although no one subset accounted for this reduction, and this finding lends weight to the hypothesis that underactivation of uNK is associated with reproductive failure. Unexpectedly, degranulation was decreased by stimulation in uNK1 of fertility patients: uNK2, uNK3 and pNK in fertility patients, as well as all NK subsets in controls, increased degranulation in response to stimulation, as expected. This observation could suggest that reduced ability of uNK1 to be activated despite stimulation contributes to the pathophysiology of reproductive failure. Notably, reduced activation was specific to uNK as there was no evidence of reduced degranulation in pNK from reproductive failure patients. This is a clinically important finding and complements our previous report that pNK and uNK numbers were not correlated (27), challenging the growing tendency among IVF clinics to test pNK numbers and function.

Similarly, the only cytokine that was altered between controls and reproductive failure patients was TNFα, whose production after stimulation was reduced specifically in all uNK subsets, but not in pNK, from reproductive failure patients. This finding was in line with an earlier report that uNK from mid-luteal biopsies display reduced production of TNFα in women with RM Fukui et al. (52). On the other hand, there are reports that RM is associated with increased uNK production of TNFα and IFN-γ, although a major limitation is that all of these studies were performed on decidua collected after miscarriage hence associated with the confounding factor of inflammatory processes occurring after fetal demise (53–57). There

is some evidence that TNFα inhibits EVT invasion by mechanisms such as trophoblast apoptosis, inhibition of proliferation and reduced matrix metalloprotease production (58, 59). However, more recently it has been suggested that implantation is a pro-inflammatory process, in which case TNFα production might be expected to be beneficial (60). In support of this theory, our previous results demonstrated that TNFα and IL-8 production by uNK peak in secretory phase, potentially indicating a role for these cytokines during the window of implantation (27). Here, we have shown deficiencies of uNK activation and TNFα production during mid-luteal phase in the reproductive failure group which supports the hypothesis that pro-inflammatory cytokines are important during the implantation window.

Taken together, our findings suggest global reduction of NK cell receptors important for cross-talk with HLA molecules on EVT leads to reduced activation specifically in uNK, which in turn impairs implantation and placental development, although some other studies have suggested that RM is associated with excess of NK cell activation (61–63). However, our finding that expression of KIR and LILRB1, and uNK degranulation, is not predictive of successful pregnancy outcome sounds a note of caution about this interpretation, since it could suggest that the phenotypic and functional differences we observe are an effect, rather than a cause, of repeated failures of implantation. The insight gained from our findings will aid the direction of future research on uNK subsets in women with reproductive problems. Further larger cohorts should be recruited for studies on phenotype and function of uNK subsets, with a focus on KIR genotype and phenotype as well as uNK activation.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author. Raw data and analysis files have been deposited at the Open Science Framework, doi: 10.17605/OSF.IO/Y2PCD.

Ethics statement

The studies involving human participants were reviewed and approved by Solihull Research Ethics Committee. The patients/participants provided their written informed consent to participate in this study.

Author contributions

EW, MJ and VM designed the study. EW, DN, KM and JN-T participated in recruitment of patients and collection of clinical samples. PB performed histological dating. EW and AC carried out laboratory experiments. EW and VM analysed results and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1108163/full#supplementary-material

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A combination of the HLA-DRB1*03 phenotype and low plasma mannose-binding lectin predisposes to autoantibody formation in women with recurrent pregnancy loss

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Introduction: It is documented that a series of autoantibodies can be detected with increased frequency in women with recurrent pregnancy loss (RPL) and they may impact the pregnancy prognosis negatively. It is unknown whether the autoantibodies per se or the basic immune disturbances underlying autoantibody production, are the reason for this association. Our group has previously found that some genetically determined immunological biomarkers are associated with RPL and the same biomarkers are also in various degrees known to predispose to autoantibody production. The aim of this study was to clarify whether the RPL-associated immunogenetic biomarkers are associated with positivity for three major classes of autoantibodies associated with RPL.

Methods: In 663 patients with RPL in whom we had results for HLA-DRB1 typing and plasma mannose-binding lectin (p-MBL) measurement, it was investigated whether there is a correlation between positivity for the autoantibodies: anticardiolipin antibodies, β2 glycoprotein I antibodies, and lupus anticoagulant (jointly called antiphospholipid antibodies), thyroid-peroxidase antibodies, and antinuclear antibodies and each of the HLA-DRB1 alleles HLA-DRB1*03 or HLA-DRB1*07 either alone or in combination with low p-MBL defined as ≤500 µg/l.

Results: Although slightly higher frequencies of positivity of two or more autoantibodies were seen in patients with either p-MBL ≤500 µg/l or being positive for HLA-DRB1*03, none were significantly associated. However, in patients with the combination of low p-MBL and HLA-DRB1*03, presence of at least one autoantibody was significantly more frequent than in patients with no such combination (OR= 2.4; 95% CI 1.2-5.0, p = 0.01). In an analysis of which autoantibodies were most strongly associated with the low p-MBL/HLA-DRB1*03 combination, antinuclear antibodies were significantly more frequent in these

patients (OR 2.0; 95% CI 1.0-3.9, p=0.05) whereas the other autoantibodies were also positively but more weakly associated with this combination.

Discussion: In conclusion, to clarify the pathogenetic background, underlying immunogenetic factors should be examined in autoantibody positive RPL patients (as well as other patients with autoimmune diseases) but the genetic background may be complex.

KEYWORDS

HLA class II, mannose-binding lectin, autoantibodies, antiphospholipid antibodies, recurrent pregnancy loss, habitual abortion

1 Introduction

Autoimmune diseases are manifestations of immune system dysregulation caused by a humoral or cell-mediated immune response against self-antigens and consequently loss of self-tolerance. This heterogenous group of diseases shares some common pathophysiological mechanisms and risk factors which involve genetic, environmental, immunological, and hormonal factors that contribute to the tissue injury and clinical manifestations (1). Among the most relevant and studied genetic components in autoimmune diseases are the loci coding for human leucocyte antigens (HLA) class I and II, which are located on the short arm of chromosome no. 6. In particular, HLA class II alleles - and more specifically HLA-DRB1 alleles - are considered to be more strongly involved in the susceptibility to or protection against specific autoimmune diseases than HLA class I (2, 3). However, strong associations between HLA alleles and autoantibody positivity also exist, and antibody positivity often precedes clinical manifestation of autoimmune disease by several years or decades. Therefore, one may question whether the primary role of HLA genes is predisposition to the disease through non-humoral pathways or antibody development that secondarily leads to autoimmune diseases (4).

One example of an association between HLA alleles and autoantibody positivity, is anti-citrullinated protein antibodies in patients with rheumatoid arthritis. These antibodies are mainly found in patients with a specific 5 amino acid motif located in the β-chain of the HLA-DRB1 alleles that predisposes to rheumatoid arthritis (5). Other examples are that specific HLA-DQ alleles are associated with disease-specific transglutaminase 2 antibodies in celiac disease (6); HLA-DRB1*0401 is strongly associated with autoantibodies against insulin and islet antigen 2 proteins (7); while the HLA class II haplotype HLA-DRB1*03-DQB1*02 is associated with glutamic acid decarboxylase antibodies in type 1 diabetes mellitus (8). However, the genetic background for development of autoimmune disease and autoantibody production is complex. In addition to the HLA genes, non-HLA related genetic factors and environmental factors play a role (2).

One of the non-HLA genetically determined biomarkers of autoimmunity is mannose-binding lectin (MBL) deficiency, which is mainly caused by the interaction of several gene polymorphisms on chromosome no. 10. Plasma (p-)MBL deficiency has been associated with the development and severity of several autoimmune diseases such as lupus erythematosus and rheumatoid arthritis (9–11).

In accordance with the Recurrent Pregnancy Loss (RPL) guideline of the European Society of Hunan Reproduction and Embryology (12), RPL is in this study defined as 2 or more consecutive pregnancy losses but the definition of RPL varies between different RPL guidelines and specialist societies (12, 13). In about 50% of patients, thrombophilia or endocrine, anatomic, or genetic risk factors can be found, while in the remaining subgroup of patients an aberrant immune profile is often found (14, 15). This involves autoantibody positivity or disrupted leucocyte subset distribution or function. Anti-nuclear antibodies (ANA), anti-thyroid peroxidase (TPO) antibodies and anti-phospholipid (APL) antibodies are associated with significantly increased odds ratios (OR) for RPL (16–20). Maternal carriage of HLA-DRB1*03 (21) and HLA-DRB1*07 (22) alleles and p-MBL deficiency (23, 24) have also been reported to be associated with RPL.

Due to the well-known association between HLA class II alleles and autoantibody production, as well as the association between p-MBL deficiency and some autoimmune diseases, we found it important to investigate whether autoantibody formation is an intermediate variable between the two RPL-associated genetic factors HLA-DRB1 and low p-MBL and RPL.

2 Materials and method

2.1 Population

Patients with RPL consecutively referred to the Center for Recurrent Pregnancy Losses in Western Denmark (in the following called the RPL Center) from January 2016 to August 2022 were included. RPL was defined as two or more consecutive pregnancy losses. Patients with known chromosomal abnormalities (N=11),

Abbreviations: β2GPI, β2-glycoprotein-I; aCL, anti-cardiolipin antibody; ANA, Anti-nuclear antibodies; APL, anti-phospholipid; ART, assisted reproductive technology; CI, confidence interval; HLA, human leucocyte antigens; ICSI, intra-cytoplasmatic sperm injection; IVF, *in-vitro* fertilization; LA, lupus anticoagulant; OR, odds ratio; p-MBL, plasma mannose-binding lectin; PCR, polymerase chain reaction; RPL, recurrent pregnancy loss; TPO, thyroid peroxidase.

uterine malformations (N=12), or missing analysis for autoantibody or HLA-DRB1 (N=22) were excluded. All patients were diagnosed at the RPL Center and all participants had given an oral and written consent to the investigators for storing their data in an RPL database. During the diagnostic work-up, information on the general health, gynaecologic and obstetric history, and lifestyle was collected as well as a routine blood sample analyzed for several factors including p-MBL level, HLA-DRB1 genotype, and presence of APL antibodies (lupus anticoagulant (LA), anti-cardiolipin antibody (aCL), β2-glycoprotein-I (β2GPI) antibody), ANA, and anti-TPO. Patients were diagnosed as APL syndrome positive if they were positive for LA or had aCL or β2GPI antibody (levels ≥35 kU/I) detected twice three to four weeks apart. The patients' ethnic origin was not noted in the database but the prevalence of non-Caucasians was estimated by the clinicians to be <2%. As patients referred to the RPL clinic are recommended not to get pregnant when waiting for their diagnostic work-up, only 10.2% of patients were pregnant in the first trimester at the time the blood sample was collected.

2.2 Ethical issue

Data was extracted from the RPL database (North Region Approval Number: 2018-5). Since only data on routine investigations and interventions in the RPL Center were analysed and reported, no permission from the ethics committee was required.

2.3 Blood sample analysis

The p-MBL level was determined with the enzyme-linked immunosorbent assay (ELISA) method as described by Nørgaard-Pedersen et al. (23). A low p-MBL level was defined as ≤500 µg/l, which is the cut-off value used routinely in Danish laboratories.

HLA-DRB1 typing was performed by the FluoGene system combining polymerase chain reaction (PCR) using sequence-specific primer (PCR-SSP) with the speed of endpoint fluorescence detection. The analysis was based on specifically modified TaqMan probe system (Iinno-train Diagnostik GmbH). The PCR was performed according to the manufacturer's instructions and the subsequent pre- and postreads are generated on a FluoVista and finally was the HLA-DRB1 type concluded by the FluoGene Software.

Indirect immunofluorescence assay on HEp-2 cells was used to detect numerous of autoantibodies against cytoplasmatic or nuclear antigens, among which are i.e., autoantibodies against double-stranded DNA (ds-DNA), SS-A/Ro, SS-B/La, Sm, Scl-70, Jo-1, U1RNP (RNP70, A, C), Ribosomal P, PCNA, fibrillarin, RNA polymerase III, PM-Scl100, Mi-2, centromere B, etc. The AESKUSLIDES® ANA-HEp-2 instruction manual was followed, and the laboratory used a screening titer of 1:160 and the 51.100 ANA HEp-2 standard kit. The samples were screened for ANAs using the Helios reader (AESKU, Mikroforum Ring 2, 55234 Wendelsheim, Germany), a fully automated Indirect immunofluorescence processor, and when one in three images were positive, the sample was confirmed ANA positive and the specific fluorescence patterns was identified by the laboratory technologist.

Anti-TPO antibodies were detected using the Kryptor immunoflourecent assay, BRAHMS (Hennigdorf, Germany), following the manufacturer's manual. A concentration ≥ 60 kU/l was considered positive.

LA was detected by a simplified dilute Russell's viper venom test (Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany). The assay investigates the ratio of the coagulation time of patient plasma mixed with the LA1 screening reagent and the coagulation time after mixing plasma with the LA2 confirmatory reagent. If the ratio was > 1.2, LA was considered to be present.

aCL and β2GPI antibody of IgM and IgG isotypes were detected by a chemiluminescence 2-step immunoassay with magnetic particle separation (Bio-Flash, Werfen, Bruxelles, Belgium). A clinically relevant cut-off value for both antibodies was set to 35 kU/l.

2.4 Subgroups

The patients were divided into subgroups based on whether or not they had a low p-MBL level, the HLA-DRB1*03 phenotype and/or the HLA-DRB1*07 phenotype, respectively.

2.5 Statistical analysis

Data was collected from the RPL Center's database (Data Protection Agency of The North Denmark Region, Approval Number 2018-5) and Stata ® (MP 15.0 for Mac, revision 19, June 2017) was used for the statistical analyses. Categorical variables were compared using χ^2-test, while when less than five observations were expected in a group, Fisher's exact test was used. OR and 95% confidence intervals (CI) were calculated using univariate logistic regression. No attempt was made to adjust for potential confounding due to the sample size. A p-value < 0.05 was considered significant.

3 Results

In total, 644 RPL patients were included of whom 88.7% had three or more pregnancy losses. The majority had pregnancy losses after natural conception while 36.2% were supported with assisted reproductive technology (ART) treatment including *in-vitro* fertilization (IVF) and intra-cytoplasmatic sperm injection (ICSI).

The frequencies of the HLA-DRB1*03 and -DRB1*07 phenotypes in all RPL patients were 20.8.% and 19.6%, respectively, compared with 24.9% and 18.4%, respectively, in a Danish bone-marrow donor cohort (p=0.034; OR = 0.79, 95% CI 0.64-0.98 and p=0.532; OR 1.08, 95% CI 0.87-1.35 for comparisons between patients and controls with each of the two phenotypes) (22).

When comparing baseline characteristics between patients with low or normal p-MBL levels, the HLA-DRB1*03 and/or HLA-DRB1*07 phenotype, no clinically relevant or statistically significant differences were observed (Table 1).

The frequency of each autoantibody or autoantibody class did not vary between patients with low or normal p-MBL levels, and presence of the HLA-DRB1*03 or HLA-DRB1*07 phenotype, respectively. (Table 2). We observed non-significantly higher frequencies of ≥2

TABLE 1 Baseline characteristics in all RPL patients and when grouping the patients according to plasma-MBL level, HLA-DRB1*03 or HLA-DRB1*07 phenotype, respectively.

	All RPL	Mannose-binding lectin plasma level		HLA-DRB1*03		HLA-DRB1*07	
		≤500	>500	Pos	Neg	Pos	Neg
	(N= 644)	(N= 237)	(N= 407)	(N= 134)	(N= 510)	(N= 126)	(N= 518)
Age, mean SD	33 5.2	33.1 5.0	33.0 5.0	32.7 5.3	33.1 5.1	33.1 5.0	33.0 5.2
BMI, median 25th-75th percentile	25.0 22.3-29.0	24.5 22.0-28.5	25.3 22.5-29.0	26.0 22.0-29.0	24.9 22.5-28.5	25.1 23.0-28.5	24.9 22.0-20.0
Pregnancy losses, median 25th-75th percentile	3 3-4	3 3-4	3 3-4	3 3-4	3 3-4	3 3-4	3 3-4
sRPL, N %	321 49.8%	120 49.4%	201 49.4%	63 47.0%	258 50.6%	69 54.8%	252 48.7

RPL, recurrent pregnancy loss; sRPL, secondary recurrent pregnancy loss.

autoantibodies in patients with low p-MBL level compared to normal p-MBL level (p=0.14) and in patients with minimum one HLA-DRB1*03 allele compared with patients with no HLA-DRB1*03 allele (p=0.11). Also, a tendency was found for a lower frequency of APL antibodies among patients with minimum one HLA-DRB1*07 allele compared to patients with no HLA-DRB1*07 allele (p=0.08) (Table 2).

Among patients who who were positive for one or more autoantibodies and had low p-MBL level, the frequency of carrying an HLA-DRB1'03 allele was higher than among those with one or more autoantibodies and normal p-MBL level (OR = 2.4, 95% CI 1.2-5.0; p = 0.01) (Table 3). All other pairwise comparisons of HLA-DRB1 phenotype frequencies between subsets of patients divided according to presence/absence of autoantibodies and p-MBL levels showed no significant differences (Table 3). Moreover, when examining the HLA-DRB1*03/X genotypes, no differences between patients with low or normal p-MBL in women without autoantibodies were observed. However, among patients positive for ≥1 autoantibody, notable differences in HLA-DRB1*03/X genotypes were seen between patients with low or normal p-MBL levels; however, none of these differences were significant. (Table 4).

ANAs were found with increased prevalence in patients with the combination of HLA-DRB1*03 and low p-MBL level compared with those without this combination (OR 2.0; 95% CI 1.0 - 3.9; p <0.05). The presence of the all other autoantibodies was also increased in patients with HLA-DRB1*03 and low p-MBL (ORs between 1.5 and 1.7) but these associations were not statistically significant (Table 5).

4 Discussion

In this study, we found that the combination of carriage of HLA-DRB1*03 and p-MBL levels ≤500 µg/l is associated with presence of autoantibodies especially ANAs in patients with RPL.

In previous large case-control studies, we have reported that HLA-DRB1*03 and HLA-DRB1*07 are conferring susceptibility to RPL (21, 22). Only the studies published by the groups of Christiansen et al. found the HLA-DRB1*03 frequency significantly

TABLE 2 The frequencies of antibody positivity in all RPL patients and when grouping the patients according to plasma-MBL level, HLA-DRB1*03, or HLA-DRB1*07 phenotype, respectively.

	All RPL	Mannose-binding lectin plasma level			HLA-DRB1*03			HLA-DRB1*07		
	Total	≤500 µg/l	>500 µg/l		Pos	Neg		Pos	Neg	
AB	(N= 644) N, %	(N= 237) N, %	(N= 407) N, %	**P**	(N= 134) N, %	(N= 510) N, %	**P**	(N= 126) N, %	(N=518) N, %	**P**
ANA	82 12.7%	35 14.8%	47 11.6%	0.24	22 16.4%	60 11.8%	0.15	18 14.3%	64 12.4%	0.56
TPO	86 13.4%	36 15.2%	50 12.3%	0.30	18 13.4%	68 13.3%	0.98	14 11.1%	72 13.9%	0.41
APL	50 7.7%	19 8.0%	31 7.6%	0.86	12 9.0%	38 7.5%	0.56	5 4.0%	45 8.7%	0.08
1 AB	148 23.0%	55 23.2%	93 22.9%	0.76	28 20.9%	120 23.5%	0.66	30 23.8%	118 22.8%	0.84
≥1 AB	183 28.4%	72 30.4%	111 27.3%	0.39	39 29.1%	144 28.2%	0.84	36 28.6%	147 28.3%	0.97
≥2 AB	35 5.4%	17 7.2%	18 4.4%	0.14	11 8.2%	24 4.7%	0.11	6 4.8%	29 5.6%	0.71

AB, antibody; Pos, positive; Neg, Negative; ANA, antinuclear antibody; TPO, thyroid-peroxidase; APL, antiphospholipid.

TABLE 3 The frequency of carrying at least one of the HLA-DRB1 alleles in patients grouped according to presence or absence of at least one of the investigated autoantibody classes and low or normal plasma MBL levels.

HLA-DRB1 phenotype	0 AB			≥1 AB		
	MBL ≤500 µg/l N_{total}=165 N (%)	MBL >500 µg/l N_{total}=296 N (%)	P	MBL ≤500 µg/l N_{total}=72 N (%)	MBL >500 µg/l N_{total}=111 N (%)	P
01	31 (18.8)	66 (22.3)	0.38	13 (18.1)	23 (20.7)	0.66
03	35 (21.2)	60 (20.3)	0.81	22 (30.6)	17 (15.3)	**0.01**
04	53 (32.1)	92 (31.1)	0.82	24 (33.3)	43 (38.7)	0.46
07	31 (18.8)	59 (19.9)	0.77	12 (16.7)	24 (21.6)	0.41
08	14 (8.5)	17 (5.7)	0.26	8 (11.1)	9 (8.1)	0.49
09	5 (3.0)	10 (3.4)	1.0	1 (1.4)	4 (3.6)	0.65
10	3 (1.8)	4 (1.4)	0.71	1 (1.4)	2 (1.0)	1.0
11/12	32 (19.4)	48 (16.2)	0.39	17 (23.6)	23 (20.7)	0.54
13	49 (29.7)	77 (26.0)	0.40	15 (20.8)	26 (23.4)	0.68
14	7 (4.2)	22 (7.4)	0.18	7 (9.7)	6 (5.4)	0.27
15	46 (27.9)	88 (29.7)	0.68	14 (19.4)	30 (27.3)	0.24
16	2 (1.2)	5 (1.7)	1.0	3 (4.2)	3 (2.7)	0.68

AB, antibody; MBL, mannose binding lectin. The bold values denote statistically significant P-values.

increased in women with RPL compared with controls (21, 25). In a meta-analysis of 18 case-control studies investigating HLA-DR phenotype frequencies in RPL patients and controls, exclusion of patients positive for autoantibodies was done in 5 of the 7 studies finding HLA-DRB1*03 less frequent in RPL patients than in controls. In contrast, exclusion of autoantibody-positive patients was not done in any of the studies finding HLA-DRB1*03 increased in RPL patients (26). Thus, such selection bias may have distorted the observed HLA-DR distribution in the RPL cohort, and it is likely that the meta-analysis would have shown HLA-DRB1*03 significantly associated with RPL if a sensitivity analysis had been done excluding the 5 studies where autoantibody positive RPL patients were excluded.

TABLE 4 The frequency of carrying at least one of the HLA-DRB1*03/X genotypes in patients grouped according to presence or absence of at least one of the investigated autoantibody classes and low or normal plasma MBL levels.

HLA-DRB1 genotype	0 AB			≥1 AB		
	MBL ≤500 µg/l N_{total} = 35 N (%)	MBL >500 µg/l N_{total} = 60 N (%)	P^a	MBL ≤500 µg/l N_{total} = 22 N (%)	MBL >500 µg/l N_{total} = 17 N (%)	P^b
01/03	5 (14.3)	8 (13.3)	1.0	4 (18.2)	1 (5.9)	0.36
03/03	3 (8.6)	4 (6.7)	1.0	1 (4.5)	1 (5.9)	1.0
03/04	7 (20.0)	12 (20.0)	1.0	1 (4.5)	4 (23.5)	0.15
03/07	5 (14.3)	8 (13.3)	1.0	3 (13.6)	0 (0.0)	0.24
03/08	1 (2.9)	0 (0.0)	0.37	2 (9.1)	3 (17.6)	0.64
03/09	1 (2.9)	3 (5.0)	1.0	0 (0.0)	0 (0.0)	–
03/10	0 (0.0)	0 (0.0)	–	0 (0.0)	0 (0.0)	–
03/11 or 03/12	3 (8.6)	5 (8.3)	1.0	2 (9.1)	2 (11.8)	1.0
03/13	5 (14.3)	9 (15.0)	1.0	2 (9.1)	1 (5.9)	1.0
03/14	0 (0.0)	2 (3.3)	0.53	2 (9.1)	1 (5.9)	1.0
03/15	4 (11.4)*	8 (13.3)*	1.0	5 (22.7)*	4 (23.5)*	1.0
03/16	1 (2.9)	1 (1.7)	1.0	0 (0.0)	0 (0.0)	–

AB, antibody; MBL, mannose-binding lectin [a,b] Comparing patients with low or normal p-MBL; *, comparing patients without autoantibodies with genotype HLA-DRB1*03/15 versus patients with autoantibodies without the genotype, p = 0.13.

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TABLE 5 The frequency of each of the three autoantibody classes in patients with or without the HLA-DRB1*03/low plasma MBL combination.

HLA-DRB1 phenotype and p-MBL level combination								
AB	DRB1*03 pos N_{total} =134		DRB1*03 pos and MBL ≤500 µg/l N_{total}=57		DRB1*03 neg and/or MBL >500 µg/l N_{total}=587		OR (95% CI)	p
	B	%	N	%	N	%		
ANA	22	16.4	12	21.1	70	11.9	2.0 (1.0-3.9)	**0.05**
TPO	18	13.4	11	19.3	75	12.8	1.7 (0.8-3.2)	0.17
APL	12	9.0	6	10.5	44	7.5	1.5 (0.6-3.6)	0.41

AB, antibody; OR, odds ratio; CI, confidence interval; ANA: antinuclear antibody; TPO, thyroid-peroxidase; APL, antiphospholipid; MBL, mannose-binding lectin; [a]Two patients had missing ANA measurement and one had missing TPO measurement. The bold values denote statistically significant P-values.

The overall frequency of the HLA-DRB1*03 phenotype in RPL women in this study was lower than the frequency in a large previously published cohort of Danish bone marrow donors (22). We have previously reported an increasing population incidence of RPL (27) and a declining frequency of HLA-DRB1*03 among RPL patients (22). The declining frequency of the allele in RPL patients was confirmed in the present study. This pattern of temporal weakened effect of some susceptibility HLA alleles in diseases has also been described for type I diabetes mellitus (28, 29). It has been speculated whether the declining frequency of susceptibility HLA alleles in patients together with the increasing population incidence of type I diabetes are due to an increasing proportion of diabetes type I cases being caused by environmental and lifestyle changes (28, 29). In analogy with the studies in diabetes type I, we speculate whether HLA susceptibility alleles (and especially HLA-DRB1*03) with time play a declining role whereas environmental or lifestyle factors could play an increasing role in the RPL pathogenesis. However, a deeper insight into the complex and probably multifactorial pathophysiology causing RPL is needed before we can make a realistic estimate of the contribution of immunogenetic, environmental, and lifestyle factors to RPL.

More simple explanations for the failure to confirm the association previously found between HLA-DRB1*03 and RPL (21) are possible. A small statistical power may explain why the present study did not detect the association, however, now two large, independent studies of non-overlapping cohorts including 1078 (22) and 663 (this study) RPL patients, respectively, have not been able to confirm the association. Therefore, the lack of statistical power is a less likely explanation. Another possible explanation is a changed referral practice. The vast majority of the RPL patients in the prior study finding an association (21) had conceived spontaneously and had a history of predominantly clinical miscarriages confirmed by ultrasound scans whereas approximately 1/3 of RPL patients in this study had conceived by ART (IVF or ICSI) and an increased proportion of their pregnancy losses were biochemical pregnancies due to the change in the definition of RPL by European Society of Human Reproduction and Embryology's Guideline group in the meantime between the two studies. The immunological component in the pathogenesis of the losses in our current RPL patients may have been weakened by such changes in patient characteristics.

We have in repeated studies in different populations of RPL patients and controls found that low levels of p-MBL are associated with increased risk of RPL with ORs between 1.46 and 1.79 (23, 24). Moreover, the most recent study high p-MBL levels (≥3000µg/l) seemed to protect against RPL (23). In the present study, most blood samples were taken prior to pregnancy whereas 10.2% were taken in the first trimester. A previous study found that only a minor elevation of p-MBL levels could be detected from before pregnancy to the first trimester (30). Since the huge majority of our patients' pregnancy losses happen in the first trimester, we believe that the measurements made before or in early pregnancy reflect the p-MBL levels in the high-risk period for pregnancy loss and therefore, the MBL measurement from both timepoints are relevant. Although being considered an acute-phase protein (31), p-MBL only seems to increase in response to infections in individuals with MBL-2 genotypes predisposing to high p-MBL levels (31) so low p-MBL levels <500 µg/l are considered to be stable also during infection/inflammation.

In patients with unexplained RPL, various autoantibodies can be detected with higher prevalence than in controls, and they are thought to have a negative prognostic impact. In the European Society of Human Reproduction and Embryology's Guideline for RPL (12), it is recommended to screen RPL patients for LA, anti-TPO antibodies, ANAs, and APL antibodies – and among APL antibodies especially aCL antibodies since plenty of studies have documented the association between aCL antibodies and RPL whereas only few studies have documented the association between β2GPI antibodies and RPL (4). Although these autoantibodies seem to affect the prognosis in RPL patients negatively, it is unclear whether they act directly to the fetus and trophoblast and cause miscarriage, whether they are markers for an overall dysregulated immune response towards pregnancy, or whether they are confounding factors associated with more basic risk factors such as genetic factors of importance for immune regulation.

As HLA-DRB1 polymorphism and MBL deficiency are both associated with autoimmunity and with RPL separately, the natural question arises whether autoantibody development is an intermediate or causal factor between the immunogenetic profile and occurrence of RPL. We tried to answer this question in a former study: In a case-control study of Danish and Czech patients with RPL, Christiansen et al. (32) found a significant difference between the prevalence of

patients being HLA-DRB1*03 positive among ANA positive and ANA negative patients (55% vs 28%, p <0.05) and a significant difference in the prevalence of the allele between aCL antibody-positive patients and the background population (35% vs 21%, p < 0.05). It was concluded that HLA-DRB1*03 is a susceptibility gene for production of the two autoantibodies in Caucasian RPL women.

We performed an updated study on the association between relevant immunogenetic biomarkers and autoantibody production in a larger population of RPL patients and tested more associations than previously: between 3 genetically determined biomarkers - two HLA class II gene variants and MBL deficiency against 3 classes of autoantibodies. The study illustrates that several genetic variants interact in the pathogenesis of autoimmunity in RPL patients. Essentially, we confirmed the previously reported statistically significant association between ANA and HLA-DRB1*03 but now only in the subset of patients with MBL deficiency. This disparity may reflect the previously mentioned weakened effect of HLA class II susceptibility alleles over time seen in some autoimmune disorders.

Although we have recently reported that HLA-DRB1*07 is significantly associated with RPL (22), it is evident that this allele is not associated with production of the tested autoantibodies (Table 2). The pathophysiological mechanisms causing RPL in HLA-DRB1*07 positive patients seem not to involve autoantibody production but may instead be mediated by HLA-DRB1*07 restricted T-cells directed against male-specific minor histocompatibility antigens on the fetus or trophoblast (33).

No study has so far clarified which mechanisms underly the association between RPL and low plasma MBL. There could be several mechanisms. One mechanism may be related to MBL's ability to bind to and promote clearance of necrotic and apoptotic cells and debris probably through opsonizing these cells and particles and promoting phagocytosis by mainly macrophages (34). If apoptotic trophoblast cells or debris shed from the placenta are not rapidly cleared by this mechanism, they may instead be phagocytosed by endometrial endothelial cells, which may result in increased release of proinflammatory cytokines (35). The increased proinflammatory response may injure the endothelial cells in placenta and consequently cause vascular clotting and trophoblast/fetal ischemia. Thus, pregnant women with low p-MBL may be at a higher risk for harmful proinflammatory responses at the feto-maternal interface due to delayed clearance of apoptotic trophoblast cells/debris.

MBL may also, in theory, affect antibody/autoantibody production through more direct ways since it can bind to and influence B lymphocytes (36) and since mice studies have found increased proliferation and IgG production in MBL null mice compared to wild type in response to Group B Streptococcus vaccination (37).

Another mechanism may be related to the results from previous reports suggesting that blocking asymmetric IgG antibodies possessing mannose-rich oligosaccharide residues in the Fab residue may play a role in RPL since the levels of such antibodies increase during normal pregnancy but not in pregnancies of women with RPL (38). MBL may bind to the mannose-rich residues of the asymmetrical antibodies enhancing their blocking effect (also on autoantibody production) whereas in women with low p-MBL, the blocking effect of asymmetrical antibodies may be reduced.

More experimental studies are needed to test these hypotheses. In our Center, we are currently performing studies in order to rule out whether the clearance rate of fetal and trophoblast antigens after a delivery is depending on the p-MBL level.

The finding in this study that an interaction of HLA-DRB1*03 and MBL is associated with the presence of autoantibodies in RPL is new. Only a weak association was found between HLA-DRB1*03 and ANA, and no overall association between this allele and the other investigated autoantibodies. These findings may be surprising since previous studies have found HLA-DRB1*03 being associated with the presence of a series of autoimmune diseases. However, almost all previous studies have focused on patients with clinical autoimmune disease (39–42) where the autoimmune reactions causing their clinical symptoms are more pronounced than in otherwise healthy RPL patients who typically is less or not at all affected in terms of clinical symptoms when not pregnant potentially because of lower autoantibody titers. This may explain the absent/weak association to solitary HLA-DRB1*03 found this study (Table 2). The finding that no other HLA-DRB1 allele than HLA-DRB1*03 seems to interact with low MBL is a new but negative finding, however, it does contribute to our knowledge about the pathogenesis of RPL. New independent studies are, however, needed to confirm these results.

It is recommended that a panel of relevant immunogenetic variants are investigated in patients with RPL and that models are developed for the seemingly complex interaction between the variants and the risk of RPL.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

Ethics statement

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study.

Author contributions

CN-P collected data and performed statistical analyses. CN-P and OC analyzed the results and wrote the article. RS was responsible for the HLA genotyping and p-MBL measurements. UK critically reviewed the manuscript and the statistics. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Towards reproducible research in recurrent pregnancy loss immunology: Learning from cancer microenvironment deconvolution

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The most recent international guidelines regarding recurrent pregnancy loss (RPL) exclude most of the immunological tests recommended for RPL since they do not reach an evidence-based level. Comparisons for metanalysis and systematic reviews are limited by the ambiguity in terms of RPL definition, etiological and risk factors, diagnostic work-up, and treatments applied. Therefore, cohort heterogeneity, the inadequacy of numerosity, and the quality of data confirm a not standardized research quality in the RPL field, especially for immunological background, for which potential research application remains confined in a separate single biological layer. Innovative sequencing technologies and databases have proved to play a significant role in the exploration and validation of cancer research in the context of dataset quality and bioinformatics tools. In this article, we will investigate how bioinformatics tools born for large-scale cancer immunological research could revolutionize RPL immunological research but are limited by the nature of current RPL datasets.

KEYWORDS

RPL, reproductive immunology, transcriptomics, bioinformatics, cancer microenvironment

Abbreviations: EC, endometrial cancer; ESHRE, European Society of Human Reproduction and Embryology; M, Macrophages; RM, recurrent miscarriage; RMSE, root mean square error; RPL, recurrent pregnancy loss; TME, tumor microenvironment; Treg, regulatory T cell; uRPL, unexplained recurrent pregnancy loss.

Introduction

Pathology definition

RPL is defined as two or more pregnancy losses before the 24th week of gestation, according to the most recent international guidelines of the ESHRE (European Society of Human Reproduction and Embryology) in 2017 (1), and its prevalence ranges from 1% to 5% within fertile couples (2). Known etiological factors for RPL include infections, parental chromosomal abnormalities, endocrinological and metabolic dysfunction, autoimmune diseases, antiphospholipid syndrome, major thrombophilia, and uterine anatomical abnormalities. Furthermore, several risk factors have been proposed, such as medical and family history, age, stress, lifestyle, smoking, obesity, chronic endometritis, endometriosis (3, 4), and abnormal decidualization. Nevertheless, more than 50% of RPL cases remain unexplained (uRPL) (5).

Immune system in pregnancy

The immune system plays a key role in each pregnancy step: initiation, propagation, and termination of pregnancy (6). At a very early phase in pregnancy, the invasion of the trophoblast and the implantation process requires a pro-inflammatory environment that must switch to immune tolerance towards the semi-allogenic fetus during the later stages of pregnancy. However, the break of this tolerance is necessary at term to induce labor. The decidualization process, which is abnormal *in vitro* in women affected by RPL (7), is crucial for pregnancy outcome since it controls not only trophoblast invasion but also confers immune tolerance towards the fetus (8). In order to regulate trophoblast invasion and to promote fetal tolerance and homeostasis, the decidua holds a unique composition of immune cells with specialized properties (6). For fetal tolerance, decidual macrophages and regulatory T cells (Tregs) are of relevance; both cell types are enriched in the decidua and with an immune regulatory profile (9). During trophoblast invasion, decidual macrophages polarization shifts from a M1-skewed phenotype to a mixed M1/M2 profile, until reaching a major proportion of regulatory M2-like phenotype at the immune-tolerance establishment (10, 11). Furthermore, Tregs show an augmented suppressive profile in the decidua (12). Accordingly, aberrant activation and polarization of both cell-types may be involved in pregnancy complications including RPL.

RPL immunology

In uRPL, an aberrant immune response at the fetal-maternal interface has been hypothesized to be responsible for its onset. Briefly, an increased pro-inflammatory response has been observed in RPL, by dysregulation of M1/M2, Th1/Th2, and Th17/Treg balance. An increasing pro-inflammatory cytokine response (e.g., high levels of TNF, IL-6, IL-17, and IFN-γ), associated with M1, Th1 and Th17 dominance, along with a diminished Th2 responses inhibiting IL-10 and G-CSF production, could confer a lack of immunosuppression due to misregulation of Treg amounts (13). In addition, a vast range of dNK functions that are involved in the regulation of vascular remodeling, fetal growth, and immune responses in normal pregnancy may exhibit altered function in uRPL (12). Furthermore, a predisposition to break auto-tolerance has been associated with RPL *via* anti-cardiolipin antibodies (ACA), anti-nuclear antibodies (ANA), anti-ds DNA and anti-thyroid-peroxidase (anti-TPO) antibody activation. Dysregulation of the maternal immune response to fetal/trophoblast antigens has been demonstrated, including through killer immunoglobulin-like receptor (KIR), human leukocyte antigen (HLA), mannose-binding lectin (MBL) and H-Y antigen regulation (14, 15).

Immunity in ESHRE guidelines

ESHRE guidelines exclude most of the immunological tests recommended for RPL, likely because they do not reach an evidence-based level. This is due to the lack of a broader consensus in the RPL scientific research setting, for which most of the studies cannot be compared for meta-analysis and systematic review since their data are not homogeneous in terms of definition, etiological and risk factors, diagnostic work-up, and treatments. Yet, not all the members of the consensus beyond ESHRE guidelines 2017 perfectly agreed with this document, as claimed in the document itself (1). Indeed, a review by Li J (16)., testifies how the definition of RPL remains heterogeneous also in studies posterior to 2017. Moreover, tests proposed by ESHRE (Table 1) are not sufficient to predict patient prognosis, stratify them into risk categories, or provide referrals for appropriate treatments and follow-up during subsequent pregnancies (5).

Immunity in RPL and endometrial cancer

Immune tolerance at fetal-maternal interface shares common pathways with immune escape in endometrial cancer (EC), which leads to cancer progression. Nevertheless, what is finely regulated and limited in time and space at the maternal-fetal interface in terms of immune tolerance, is conversely aberrant and irreversible in EC immune escape (17). To investigate fetal-maternal immune tolerance and its disruption in pregnancy complications, such as uRPL, could represent a model system through which immune escape molecular pathways could be unraveled in EC to better stratify patients beyond current guidelines classification, which is still not able to accurately predict prognosis and recurrences (18). Therefore, the EC immunological background could be valuable for expanding our knowledge of immune tolerance disruption in pregnancy complications.

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TABLE 1 ESHRE Guidelines on immunological biomarkers.

	ASSOCIATION	CONTRIBUTING FACTORS	TREATMENT	Prognosis
Anti-HY immunity	moderate	yes	no	negative impact on future live birth
Cytokines	yes	unclear		unknown
Cytokines (polymorphism)	no			
Antinuclear Antibodies	most studies	probably not		unclear
NK in PB	weak	no	no	unclear/no
NK cell cytotoxicity in PB	unclear		no	no
NK in endometrium/uterus	weak		no	unclear

RPL research methodology

From a biotechnological context, the vast majority of the available RPL experimental data is based on either signal-based-omics (e.g., microarrays) or targeted phenotypic profiles (e.g., flow cytometry). These approaches, as shown by many meta-analyses, are suffering from a lack of reproducibility or are limited by a mere phenotypic description (such as the cytokinome) without the mechanistic and complete reconstruction of the tumor-host microenvironment (19, 20) (Figure 1), and their level of aggregability and comparability is hindered by experiment-specific confounding and batch effects.

Scope

In this article, we will investigate how biotechnological approaches and bioinformatics tools born for large-scale cancer immunological research could revolutionize RPL research but are limited by the nature of current RPL data.

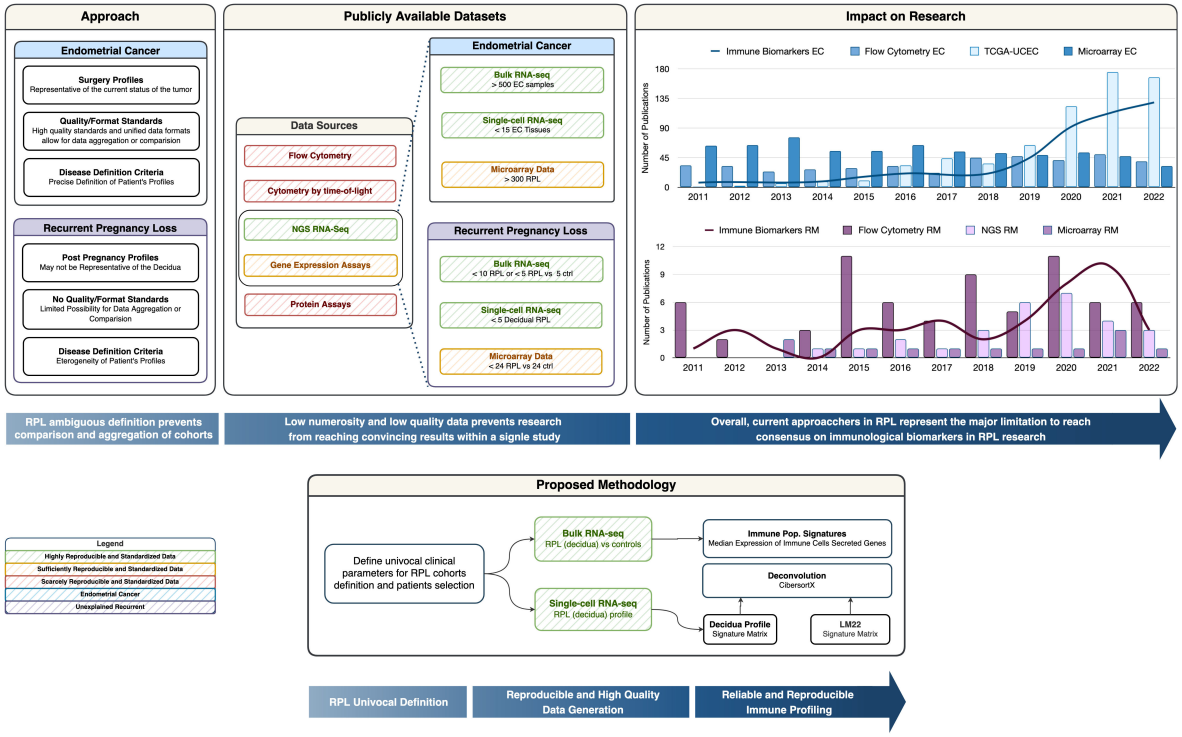

FIGURE 1
Graphical Abstract. The *Approach* box provides a summary of Recurrent Pregnancy Loss definition pitfalls compared to Endometrial Cancer Research. The *Publicly available datasets* box provides insight into the reproducible and non-reproducible technologies in the immunological field, along with a representation of the numerosity for the cohorts in RPL *vs*. EC. The *Impact on Research* box represents the trend of publication on PubMed with the keywords listed in the legend and we can observe how the number of publications on biomarkers is correlated with the biotechnological revolution. The *Proposed methodology* box represents a simplified schema of in-silico immune profiling.

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Methods

RPL patient cohorts

We first sought public datasets available in the literature containing endometrial tissue RNA expression in recurrent pregnancy losses and relative controls. A review by Li J (16). et al. listed a summary of -omics RPL cohorts: although an effort is being made to reach higher numerosity and data quality, in most cases data is not publicly released. To our knowledge, only two studies have published datasets that satisfied a 10-patient-per-group constraint: RNA-seq on 10 RPL and 10 Infertile (FASTQ available at SRP052612) and Microarray Gene Expression from 24 RPL and 24 controls (series matrix GSE165004). Additionally, we searched for RPL datasets of single cell-RNA seq and found one (21) which provides the gene expression levels in the decidua of 3 RPL samples *vs*. 3 control samples. For each patient, 8 immunological populations were considered which are listed in Table 2. Cohort profiles and definition criteria are described in Table 3.

Bioinformatic analysis

The proportion of immune cells can be faithfully inferred from bulk RNA-seq profiles through the employment of deconvolution algorithms. CIBERSORTx is a support vector regression-based approach for characterizing cell subsets proportions which relies either on well-known immune cell expression profiles (LM22 signature matrix) or on a custom signature matrix built on single-cell data. For each sample, immune cell sub-population proportions have been estimated with the default setup (perm: 500, rmbatchBmode: TRUE, Quantile Normalized: TRUE).

Microarray data was already quantile normalized. Single-cell data normalization is carried out by CIBERSORTx along with the generation of the signature matrix. CIBERSORTx and Seurat packages were used respectively for deconvolution and single-cell data preprocessing. Comparisons between RPL and controls were computed *via* t-test. Immune cell signatures have been computed through a weighted geometric mean.

Results

Differential deconvolution on endometrial cell population expression signatures

We implemented an immune-cell activity analysis based on the expression level of genes secreted by immunological cellular subtypes according to the most recent data available from Vallvé-Juanico et. Al (22). We generated an expression signature for each immunological cellular subtype, by considering the geometric mean of expression level genes secreted by immunological cells. According to cell population expression signatures, neutrophils-specific gene expression was significantly higher in the RPL population compared to controls (Figure 2). Although the role of neutrophils in RPL is currently being evaluated (23), the limited abundance of these cells in the endometrium led us to interpret these findings as not representative of the immunological background in RPL. In addition, NK cell gene expression values appear to be significantly lower in comparison to other cell populations.

CIBERSORTx deconvolution on microarray data

We then sought more finely tuned methodologies to estimate immune cell proportions. We performed microenvironment deconvolution through the CIBERSORTx (24) software based on the LM22 signature matrix (median RMSE: 0.73) and obtained the results described in Table 2. A significantly higher proportion of T follicular helper cells (10.7% *vs*. 10.1% p-value: 0.002), and significantly lower proportion of M2 macrophages (5.8% *vs*. 6.0%,

TABLE 2 List of populations considered and relative abundances in RPL.

Populations	Single Cell	Proportions (SC sig. mat.)	Proportions (LM22 sig. mat.)	Expected proportions
Natural Killer	**dNKe**	18%	6%	50-70%
	dNKb	15%		
	dNKa	26%		
	dNKc	0%		
	dNKd	0%		
Macrophages	**dM**	10%	24%	20%
T-Cells	**dTreg**	18%	19%	10-20%
	dCD8T	2%		
	dCD4T	12%		
B-Cells			10%	< 5%

TABLE 3 Cohort parameters summary.

	Microarray	RNA-Seq	Single Cell RNA-Seq
Numerosity	24 RPL *vs.* 24 ctrl	10 RPL	3 RPL *vs.* 3 ctrl
Phase	19-21 of the menstrual cycle	Mid-Luteal phase	Therapeutic termination of pregnancy
Parity	0	0	0
Age	< 35	< 40	< 37
Comorbidities	no	unspecified	unspecified
Events	3 +	3 to 8	2 +
Consecutive events	2 (within < 20 weeks)	unspecified	unspecified
Normal levels/status of	FSH, LH, E2, PRL, TSH, Leiden, prothrombin, antithrombin III, protein C and S activity, lupus anticoagulant, cardiolipin antibody, beta2-glycoprotein antibody, karyotype	unspecified	Endocrine status, chromosomal status, uterine anatomy, renal status

p-value: 0.02) were observed. These results are in line with previous reports on aberrant immune responses in RPL (25, 26). However, no evident clustering is obtained to stratify the two populations (Figure 3A), which is also coherent with the results obtained in other studies based on the same microarray dataset (27). We further reasoned that the limitations in describing the immune profile alteration of RPL are not related to the choice of the deconvolution method: indeed, another tool, *ImmuCellAI* (28), has revealed similar results in other studies (27).

Since the LM22 signature matrix has been validated only on TCGA solid tumor datasets we also attempted to design an RPL-specific signature matrix based on 3 decidual single-cell profiles. For this purpose, we extracted decidual cell expression levels and calculated the mean for each immune cellular subtype for each patient. Out of the 8 original populations, only 5 were sufficiently profiled (NK cells, Tregs and other $CD4^+$ T-cells, $CD8^+$ T-cells, macrophages). Then, we structured the resulting data frame according to CIBERSORTx input constraints and completed the

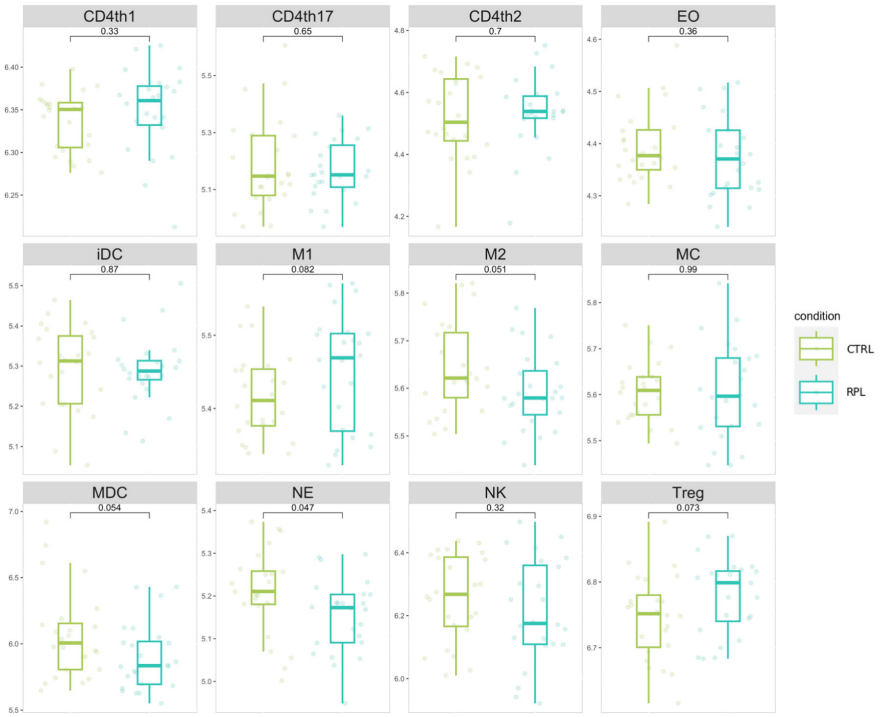

FIGURE 2
Immune cell populations signature distribution comparison. Values represent the gene weighted geometric mean of microarray expression levels, transformed to a log2 scale. The indicated p-values refer to the t-test comparison between RPL and control group.

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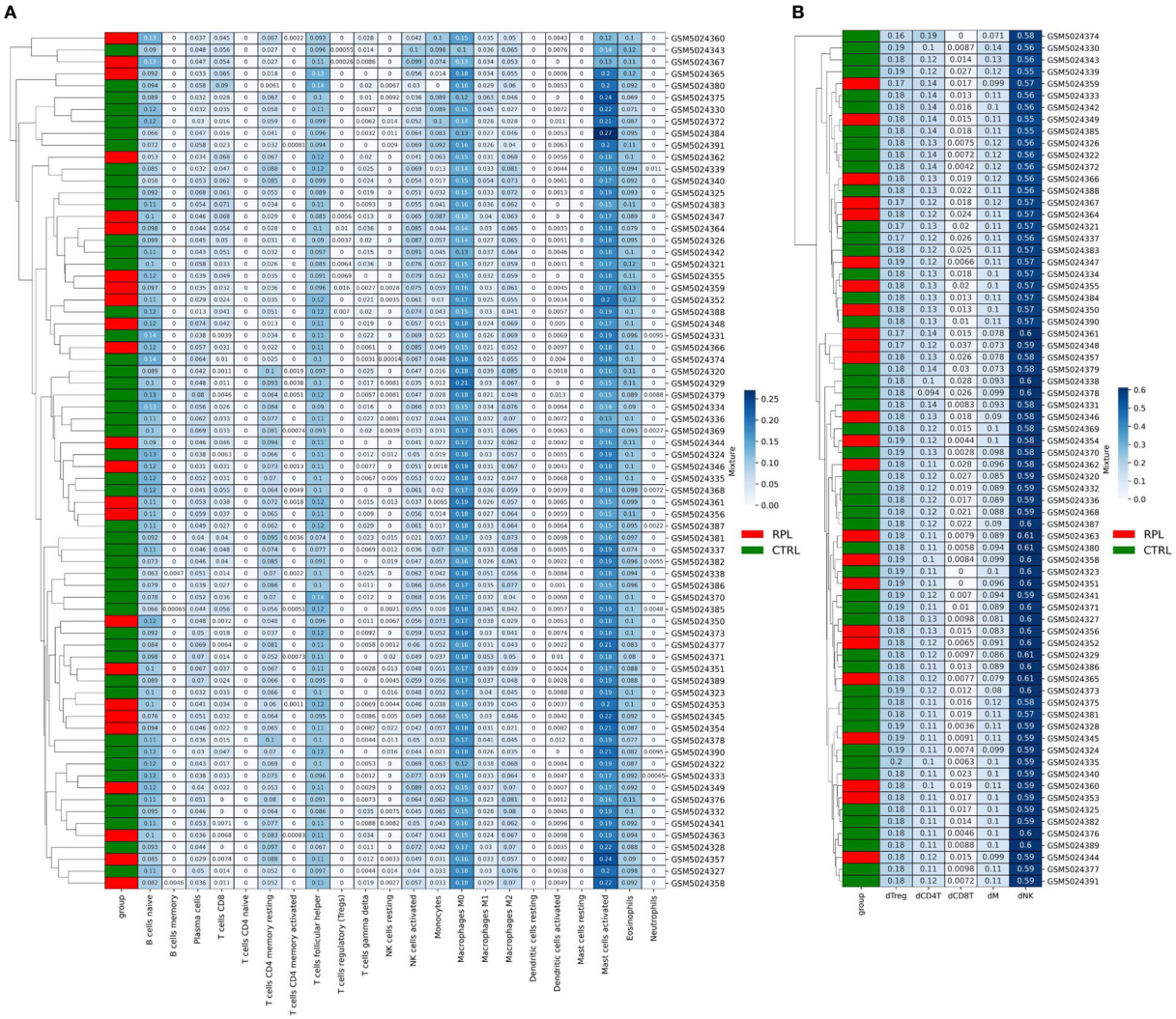

FIGURE 3
(A) CibersortX LM22-based Deconvolution of Microarray Expression Data. Samples do not cluster according to the RPL condition. **(B)** CibersortX cecidual immune cell-based deconvolution of microarray expression data.

procedure as indicated in the CIBERSORTx manual. The new RPL signature matrix was used to perform the deconvolution (median RMSE: 0.91). Once again, no clustering and/or statistically significant differences in immunological populations proportions were found (Figure 3B), however, we observed that the immune profile described with the second approach produced more consistent results with the immune expected cell proportions (Table 2). Although such results could be biased by the presence of fewer immune cell populations, we believe that the peculiar secretion profile of decidual immune cells should be taken into consideration when performing deconvolution.

Discussion

Traditional experimental assays are time-consuming, expensive, and laborious in the heterogeneity of cancer characterization. Thus, more efficient approaches have been introduced in cancer research to improve the traditional research settings to reach a precision/personalized medicine level. The availability of high-dimensional data has significantly improved cancer research and led to the employment of advanced modeling techniques, such as machine learning and deep learning. Such methodological improvements driven by cancer research have strong potential for discovery of new and complex unknown features (29), but will require meticulous standardization of quality and quantity, as for TCGA (The Cancer Genome Atlas) datasets.

Publicly available data of RPL cohorts showed quite a few limitations for data accessibility and reanalysis, for either format incompatibility with standard bioinformatics tools or quantitative/qualitative limitations of the cohorts. While the Lucas et al. NGS (30) cohort provides high quality data, no healthy control population is provided. Likewise, the microarray dataset cannot be considered satisfactory as this technology is often irreproducible (19, 20), especially due to lower abundance transcripts that have a strong overlap with genes related to the microenvironment. Further,

non-solid tumor datasets may not be entirely compatible with the CIBERSORTx signature matrix (LM22) (31). As an RPL-specific signature matrix would be required for this scope, the numerosity of the RPL single-cell RNA-seq cohort was likely too scarce to represent the immune cell landscape of all patients, not to mention that the immune populations considered consist of only 5 immune cell subtypes (NK, Treg, CD4T, CD8T, M), which are rather insufficient to fully characterize the RPL immune profile.

Along with data availability limitations, one major limitation in RPL research is that separate cohorts cannot be compared nor aggregated due to the prominent level of heterogeneity in RPL definition criteria, diagnostic work-up to perform, etiological factors to investigate and their own definition, as described in Li J (16). et al. Nevertheless, it should be considered that RPL incidence is quite high in couples and has a significant impact on health systems since it affects not only the natality and psychological aspects of these women, but also their future health, since RPL women show a higher risk for obstetrical complications, cardiovascular diseases, and venous thromboembolic events later in life (32–34).

Our attempt reinforces the limitations already expressed by ESHRE 2017 guidelines on the impossibility to reach an immunological evidenced-based level, which is imputable both to the inadequacy of the *single gene* approach and the scarcity in terms of numerosity and quality of RPL studies. Research, development, and the definition of standardized procedures should be considered to produce adequate RPL databases, so to enable the application of new methodological approaches. For instance, extensive transcriptomics studies on the decidua will enable to deconvolute and pinpoint the specific cell populations that are dysregulated in RPL and compare their behavior to the best-known mechanisms of the EC immunological microenvironment.

Data availability statement

Publicly available datasets were analyzed in this study. This data can be found here: GEO Series GSE165004 https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE165004 SRA SRP052612 https://www.ncbi.nlm.nih.gov/sra?term=SRP052612 https://ngdc.cncb.ac.cn/gsa-human/browse/HRA000237.

Author contributions

VB and MB contributed to conception and design of the study. VB, MB, and MP explored the available datasets. EP, AP, and EV contributed to the clinical interpretation of the results. MB and MP performed the bioinformatics and statistical analysis. VB wrote the first draft of the manuscript. MB, and MP wrote sections of the manuscript. VB, MP, and MB reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Dysregulated low-density granulocyte contributes to early spontaneous abortion

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Spontaneous abortion (SA) is a common adverse pregnancy event with unclarified pathogenesis and limited therapeutic efficiency. Although most SA cases with the euploid embryo(s) are associated with immunological factors, the contribution of low-density granulocyte (LDG) in SA pathogenesis is rarely reported. This study aimed to investigate the serial characteristics and possible contribution of LDG and their subpopulations in early pregnancy, especially in early SA. Unpregnant (UP), normally pregnant (NP), and SA women were recruited, and the peripheral blood and endometrium/decidua were collected for LDG isolation and histological observation. The percentage, phenotype, and subpopulations of LDG were analyzed *via* flow cytometric analysis, and the ability of Nets formation was assessed by immunofluorescent and immunohistochemical assays. As a result, 43 participants were enrolled, including 10 UP, 15 NP, and 18 SA women. Compared with the UP group, the LDG percentage in peripheral blood mononuclear cells (PBMCs) and decidual immune cells (DICs) increased in the NP group, while the loss of this increase was observed in the SA group. Meanwhile, $CD16^{int/-}$ cell percentage in peripheral blood LDG (PB-LDG) increased in the NP and SA groups, and insufficient activation of $CD16^{hi}$ PB-LDG characterized by reduced CD11b expression was discovered in the SA group. Moreover, the LDG percentage in DICs was higher than that in PBMCs, and the decidual LDG (D-LDG) showed a surface marker expression profile that is easier to be activated in the pregnant cohort (NP + SA women). Finally, increased decidual Nets formation was observed in the SA group compared with the NP group, and more Nets formation was detected in D-LDG of NP and SA women following PMA stimulation. Overall, LDG participates in the maintenance of early pregnancy, while dysregulated LDG is responsible for early SA, providing novel potential targets for further exploration of SA pathogenesis and therapeutics.

KEYWORDS

low-density granulocytes, normal-density granulocytes, peripheral blood mononuclear cells, maternal-fetal immunity, neutrophil extracellular traps, early-pregnancy, spontaneous abortion

Introduction

From the perspective of immunology, the embryo can be considered a semi-homograft, carrying half of the genes of both parents and interacting with the maternal immune system without being rejected (1). Spontaneous abortion (SA) is a common adverse pregnancy event, but the etiology is not well described, and therapeutic efficiency is also far from satisfactory. Growing evidence suggests that most SA cases in the euploid embryo(s) are associated with immune factors. Except for the known autoimmune disorders, such as antiphospholipid syndrome and positive anti-thyroid antibodies, the immunological mechanisms of a considerable part of SA are still unclear (2). Meanwhile, the known endocrine, metabolic, and infectious factors, such as polycystic ovarian syndrome, hypothyroidism, and chronic endometritis, could perturb the decidualization process. However, a defect in decidualization can result from changes in immune cells, at least partially (2, 3). Therefore, expanding the understanding of pregnancy-related immune changes and potential mechanisms is urgently needed to explore SA pathogenesis and therapeutic strategies.

Various immune cells infiltrate the maternal-fetal interface in early pregnancy and regulate immune balance. As a critical part of innate immunity, neutrophils are involved in pregnancy and delivery, especially in fertilized egg formation and embryo implantation (4). Neutrophils kill pathogens *via* multiple antimicrobial mechanisms, such as phagocytosis, reactive oxygen species (ROS), and releasing bactericidal enzymes and neutrophil extracellular traps (Nets) (5). Nets are extracellular web-like DNA decorated with histones and antimicrobial proteins released from activated neutrophils and form a powerful antimicrobial mechanism. Nets contain various damage-associated molecular patterns, including DNA, so uncontrolled Nets formation can sustain inflammation and cause tissue damage or dysfunction in the host (6). In addition to immune response, NETs are also involved in angiogenesis, thrombosis, and tissue remodeling (7, 8).

Although the underlying mechanisms are debated, abnormal changes in neutrophil activity are associated with pregnancy complications, including spontaneous abortion (SA) (9, 10). Previous studies have confirmed that neutrophils can be isolated from decidua in the first three months of pregnancy, and decidual neutrophils show an activated phenotype and increased anti-apoptotic ability (9, 11). However, the phenotypes, functional characteristics, and interrelationships among the subtypes of neutrophils in SA, both in peripheral blood (PB) and decidua, have not been clarified. Low-density granulocyte (LDG) were first reported in systemic lupus erythematosus (SLE), and their subsets coexisted with monocytes after gradient centrifugation. Most autoimmune or infectious diseases studies showed that LDG is a group of pro-inflammatory cells. Meanwhile, compared with normal-density neutrophils (NDG), LDG is easier to be activated and exhibits stronger NETosis (12, 13), which reflects the state of neutrophils with NETs formation and contributes to the host defense against pathogens.

LDG is mainly reported in peripheral blood of autoimmune diseases, tumors, infections, thrombosis, and other conditions. Croxatto et al. reported that leukocytes isolated from decidua contained, in the purified mononuclear cell frequency, a population of LDG (11), indicating that LDG may play a role in pregnancy. However, the role and potential mechanisms of LDG in pregnancy and adverse pregnancy events (such as SA) are rarely reported. Therefore, this study aimed to investigate the serial characteristics of LDG, including the percentage, phenotype and subpopulations, and ability to form Nets (Netosis), in peripheral blood and endometrium/decidua of unpregnant, normally pregnant, and SA women and analyze the possible contribution of LDG in early pregnancy and SA, which have not been reported yet. As a result, the findings contributed new evidence of the involvement of LDG in pregnancy and highlighted the participation of dysregulated LDG and its subpopulations in SA pathogenesis, laying a foundation for further study of SA pathogenesis and therapeutics.

Materials and methods

Participants

This study was approved by the Reproductive Medicine Ethics Committee of Chengdu Jinjiang Hospital for Maternal & Child Health Care (approval number: 2019003) and followed the Helsinki Declaration. All participants were informed of the nature of the study and signed a written informed consent before participation.

The participants were recruited between Oct 2020 and Mar 2022, including unpregnant (UP), normally pregnant (NP), and SA women. Intrauterine pregnancy was diagnosed through serum and urine β-human chorionic gonadotropin (β-hCG) tests and Doppler ultrasound, and the SA was diagnosed as the unintentional end of pregnancy. The inclusion criteria were: (1) less than 35 years old, body mass index (BMI) < 28 kg/m^2, and no history of smoking and drinking in the three months before endometrial collection (UP women) or during this pregnancy (NP and SA women); (2) for participants in UP and NP groups, no history of adverse pregnancy (such as SA, premature delivery, pregnancy-induced hypertension, placental abruption), hypertension, cryptorrhea (such as diabetes), and immune and infectious diseases; for participants in SA group, experienced at least one SA before this pregnancy, no normal childbearing history, and no use of immunomodulators and anticoagulants during this pregnancy; (3) no anatomical abnormalities of uterine, such as septate, unicornate, bicornate and didelphis uteri, were observed in the ultrasound examination before participation; (4) the UP volunteers agreed to donate endometrial tissue one week after ovulation, the NP women chose to terminate the healthy pregnancies voluntarily, and the SA women selected induced abortion after the demised fetus was confirmed; (5) no chromosomal abnormalities were identified in the aborted tissues; (6) the days of pregnancy (DOP) in NP and SA women were 42 to 70 days. As a result, 43 participants were

recruited, including 10, 15, and 18 in the UP, NP, and SA groups, respectively, and the general characteristics are shown in Table S1.

Collection of peripheral blood and decidual/endometrial tissues

The peripheral blood in all groups was collected. A part was used for blood routine and D-dimer tests, a part was used for plasma separation and dsDNA level determination, and the rest was used to separate peripheral blood mononuclear cells (PBMCs) and normal-density granulocyte (NDG).

For NP and SA participants, fetal heartbeat was confirmed again with a Doppler ultrasound before the induced abortion. After vacuum aspiration, the endometrial/decidual tissues were picked out from the aspirated tissues and repeatedly washed with normal saline to minimize blood contamination. The non-decidual tissue was carefully examined and removed. Part of the endometrial/decidual tissues was fixed and paraffin-embedded for immunofluorescent and immunohistochemical staining, and the remainder was stored in a culture medium for isolation of endometrial/decidual immune cells (EICs/DICs).

Isolation of PBMCs and EICs/DICs

PBMCs were isolated with Histopaque-1119 and Histopaque-1077 (Sigma, USA) gradient centrifugation procedure. Briefly, Histopaque-1119, Histopaque-1077, and whole blood samples were sequentially added into a 50ml centrifuge tube and centrifuged at 700×g for 30 mins at room temperature. The stratified liquid from top to bottom is plasma, PBMCs (including LDG), Histopaque-1077, peripheral blood NDG (PB-NDG), Histopaque-1119, and red blood cells. The PBMCs and PB-NDG were collected, and the former was used for subsequent peripheral blood LDG (PB-LDG) screening and isolation.

Endometrial and decidual tissues were cut into about 1 mm^3 pieces and digested with 1 mg/mL (0.1%) collagenase type IV (Sigma, USA) and 150 U/ml DNase I (Sigma) at 37°C for about 60 mins with gentle agitation. The cell suspension was passed through 100μm, 70μm, and 40μm cell strainer and centrifuged at 700×g in a discontinuous Percoll gradient (20%, 40%, and 60%) for 30 mins. The decidual immune cells (DICs) between 40% and 60% Percoll solution (densities of 1.056 to 1.077 g/mL) were collected and used for subsequent endometrial LDG (E-LDG) and decidual LDG (D-LDG) isolation.

Phenotypic analysis of PBMCs, PB-NDG, and EICs/DICs by flow cytometry

The isolated PBMCs and E/DICs were washed with stain buffer and subsequently stained with the antibody-conjugates, including APC-Cy7 CD45, PerCP-Cy5.5 CD15, BV510 CD14, BV711 CD62L, BV605 CD11b/MAC-1, FITC CD16, and Alexa 647 CD66b (BD, USA), for 30 minutes at 4°C in the dark. PB-NDG were stained with the above antibodies except for APC-Cy7 CD45, PerCP-Cy5.5 CD15, and BV510 CD14 because the concentration of PB-DNG with $CD45^+CD15^+CD14^-$ we separated was more than 97% (Figure S1A). Then, the stained cells were washed with stain buffer and acquired *via* an FCM with CellQuest software. More than 1×10^4 cells in each sample were detected. The results were analyzed using Flowjo software and expressed as a percentage of positive cells.

LDG isolation and identification

LDG were isolated from the PBMCs and E/DICs by magnetic bead selection and identified by FCM. Briefly, the PBMCs and E/DICs were incubated with anti-CD14 mAbs (Milteny, Germany) for 15 mins, and the CD14- cells were isolated by negative selection using anti-CD14 magnetic beads and MACS dissociator (Milteny). The CD14- cells were further incubated with anti-CD15 mAbs (Milteny) for 15 mins, and the CD15+/CD14- cells (LDG) were isolated by positive selection using anti-CD15 magnetic beads and MACS dissociator. The purity of the isolated LDG was identified by staining the cells for 30 min at 4°C with monoclonal antibodies specific for CD14 and CD15 and evaluating them by FCM. The percentage of CD14-/CD15+ cells was identified as more than 95%.

Quantitation of NTEs *via* dsDNA detection

dsDNA in NETs of plasma was quantified by Picogreen dsDNA Assay kit (Invitrogen, USA) according to the manufacturer's instructions.

Immunofluorescent and immunohistochemical staining

For immunofluorescent staining, paraffin slides of endometrial/decidual tissues were processed and incubated with primary antibodies, including anti-CD15 (Abcam, USA; 1:1000) and anti-H3Cit (ab5103, USA; 1:1000), before being stained with fluorescent secondary antibodies and DAPI. The images were acquired with a fluorescence microscope (Olympus, Japan), and the number of endometrial/decidual samples with Nets positive was counted. Meanwhile, the paraffin slides were processed and stained with PAD4 antibody (Affinity, USA; 1:200) before being stained with secondary antibodies and chromogen substrate for immunohistochemical staining.

Phorbol-12-myristate-13-acetate stimulation and Nets formation detection

The isolated PB-LDG, PB-NDG, and E/D-LDG were seeded on a poly-lysine-pretreated glass cover in a 48-well plate and stimulated with PMA (100 ng/mL, Sigma) for 4 hours. Then, the cells were fixed with 4% paraformaldehyde (Sigma) and incubated with primary antibodies, including anti-MPO (Abcam, USA;

1:1000) and anti-H3cit (Abcam; 1:1000), followed by staining with fluorescent secondary antibodies and DAPI. Images were obtained using a FluoView FV1000 confocal microscope (Olympus, Japan) and analyzed with Olympus FV10-ASW software (Olympus). The percentage of positively stained cells was calculated, and all sections were assessed independently by three blinded investigators (Ye Hx, Lan Li, and Jia Y), and the quantitative results were determined *via* consensus.

Statistical analysis

Statistical analyses and data graphs were generated with GraphPad Prism software (version 9.4, GraphPad Inc., USA). Continuous data were expressed as median with interquartile range (IQR) and assessed for normality using the Kolmogorov-Smirnov and D'Agostino & Pearson tests. Student's *t*-test was used to analyze the differences between the two groups. When the variances of the two groups differed in the *F* test, the Mann-Whitney *U*-test was used to compare the two groups. One-way analysis of variance with Tukey's *post hoc* analysis (for normally distributed data) or Kruskal-Wallis *H* with Student-Newman-Keuls analysis (for non-normally distributed data) was performed for comparisons among three or more groups. A $P < 0.05$ was considered statistically significant.

Results

General characteristics of the enrolled participants

Forty-three participants were enrolled in this study, including 10 unpregnant (UP), 15 normally pregnant (NP), and 18 SA women (Table S1 and S2). There was no significant difference in age and body mass index (BMI) among the three groups and in days of pregnancy (DOP) between the NP and SA groups. Meanwhile, all participants in the SA group experienced at least one SA before this pregnancy (at least two SA if this SA was included) (Table S1). Meanwhile, there was no significant difference in the peripheral blood neutrophils/lymphocytes ratio (PB-NLR), platelet/lymphocytes ratio (PB-PLR), and D-dimer levels among the three groups (Table S3).

LDG percentage in PBMCs increases after eight weeks of normal pregnancy, while the loss of this increase is associated with SA

In PBMCs, the LDG percentage was quantified *via* FCM analysis (Figure 1A), and the difference among the groups was investigated. As shown in Figure 1B, the LDG percentage was significantly higher in the NP group than in the UP and SA groups, indicating that the LDG percentage in PBMCs increases in normal pregnancy, and the loss of this increase may be associated with SA. Meanwhile, no significant difference in the MFI of CD16 and CD62L was observed among the three groups, but the MFI of CD11b was significantly higher in the NP group than in the SA group, suggesting an insufficient activation of PB-LDG in the SA group (Figure 1C).

Interestingly, correlation analysis revealed that the LDG percentage in PBMCs was positively correlated with DOP in the NP group but was not in the SA group (Figure 1D). Then, subgroup analysis was performed according to whether the DOP was more than 56d (when the placenta began to form and take over the function of the corpus luteum at about eight weeks). The LDG percentage in the NP subgroup with DOP > 56d (57-70 days) was significantly higher than that in the subgroup with DOP ≤ 56d (42-56 days). However, subgroup difference was not observed in the SA group (Figure 1E). Meanwhile, in women with DOP ≤ 56d, the LDG percentage showed no significant difference among the three groups. However, in women with DOP > 56d, the LDG percentage in the NP group was significantly higher than in the UP and SA groups (Figure 1F). These findings indicate that the LDG percentage in PBMCs increases mainly after eight weeks of normal pregnancy, and the loss of this increase may also be related to SA.

Increased $CD16^{int/-}$ PB-LDG correlates with pregnancy, and insufficient activation of the $CD16^{hi}$ PB-LDG may be associated with SA

According to the CD16 expression, peripheral blood NDG (PB-NDG) and PB-LDG can be classified as $CD16^{int/-}$ and $CD16^{hi}$ subpopulations and representative CD16 plots in FCM analysis are shown in Figure 2A. In PB-NDG, no significant difference was observed in the percentage of CD16hi and CD16int/- cells and in the mean fluorescence intensity (MFI) of CD62L and CD11b among the three groups (Table S4 and Figure S1). The percentage of $CD16^{int/-}$ cells was significantly higher, while the percentage of $CD16^{hi}$ cells was significantly lower in LDG than in NDG (Figure 2B). In PB-LDG, the percentage of $CD16^{int/-}$ cells in the NP and SA groups was significantly higher, while $CD16^{hi}$ cells in the SA group were significantly lower than those in the UP group. However, there was no significant difference in the $CD16^{int/-}$ and $CD16^{hi}$ cell percentage between the NP and SA groups (Figures 2C, D). These results suggest that increased $CD16^{int/-}$ PB-LDG is related to pregnancy, while a decreased $CD16^{hi}$ PB-LDG may be associated with SA.

The immune cell surface markers were further analyzed to explore the potential link between PB-LDG and SA. As shown in Figure 2E, the MFI of CD11b in $CD16^{hi}$ LDG was significantly higher than that in $CD16^{int/-}$ LDG, suggesting that $CD16^{hi}$ LDG is more easily activated than $CD16^{int/-}$ subsets. In $CD16^{hi}$ LDG, the MFI of CD11b in the SA group was significantly lower than that in the NP group (Figure 2F), while this difference was not observed in $CD16^{int/-}$ LDG (Figure 2G). These results suggest an insufficient activation of $CD16^{hi}$ LDG in the SA group, and this was further supported by the findings that the MFI of CD11b of $CD16^{hi}$ LDG in

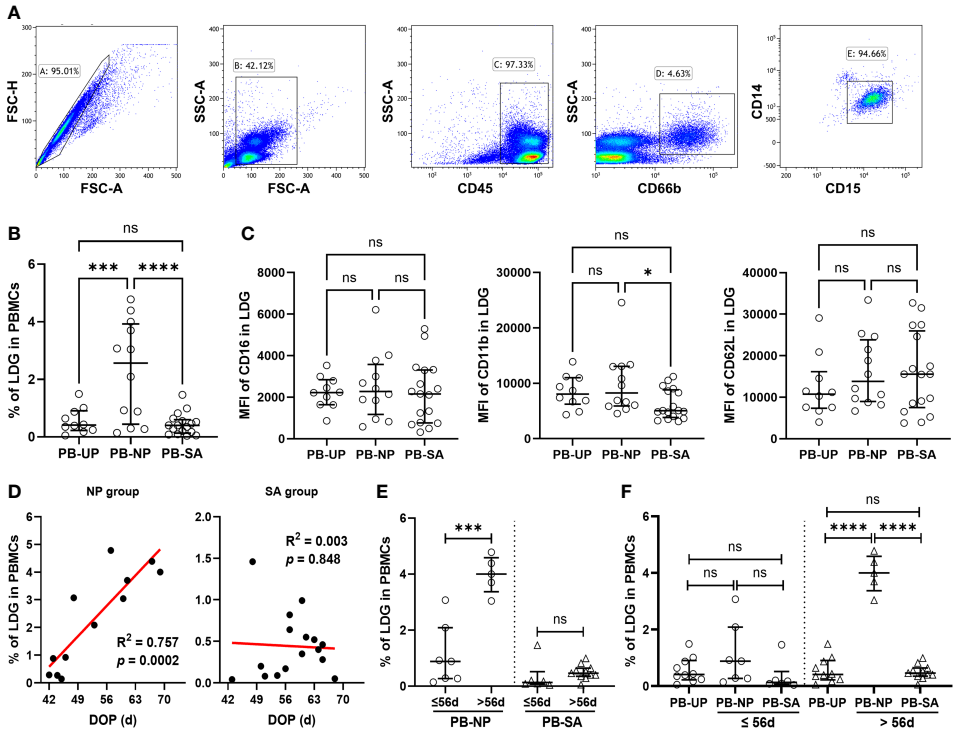

FIGURE 1
Loss of increase and insufficient activation in PB-LDG is associated with SA. PBMCs were isolated from the peripheral blood of UP, NP, and SA women by density gradient centrifugation. **(A)** PB-LDG was identified as $SSC^{hi}CD45^{+}CD15^{+}CD14^{-}$ singlets according to the gating strategy shown in the five panels. **(B)** Comparison of the LDG percentage in PBMCs of 10 UP, 12 NP, and 17 SA women. **(C)** Comparison of the fluorescence intensity (MFI) of CD16, CD11b, and CD62L in PB-LDG among UP, NP, and SA groups. **(D)** Correlation analysis of the LDG percentage in PBMCs and DOP in pregnant women (NP + SA). Data are presented as dots and median with interquartile range, and each symbol represents an individual donor. **(E)** Comparison of the LDG percentage in PBMCs between the subgroup with DOP > 56d or ≤ 56d in NP and SA groups. **(F)** Comparison of the LDG percentage in PBMCs among UP, NP, and SA groups with DOP ≤ 56d or > 56d. T-test or Mann-Whitney U test was used to identify the differences between the two groups, and one-way analysis of variance (ANOVA) with Tukey's *post hoc* analysis or Kruskal-Wallis H with Student-Newman-Keuls *post hoc* analysis was performed for comparisons among the three groups. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$. ns, not significant.

UP and NP groups was more than twice that of $CD16^{int/-}$ LDG (mean: 8762.18 vs. 3555.96 and 12942.11 vs. 6196.477, respectively) but less than 50% higher in the SA group (mean: 5051.374 vs. 7005.47) (Table S5). Therefore, the above results indicate that the insufficient activation of the $CD16^{hi}$ PB-LDG may be related to SA.

LDG percentage in DICs increases in normal pregnancy, and the loss of this increase is associated with SA

EICs/DICs with a density of less than 1.077 mg/ml were isolated, and the LDG percentage in EICs/DICs was quantified. The results were consistent with that in PBMCs, i.e., the NP group was significantly higher than SA and UP groups (Figure 3A), indicating the LDG percentage in DICs increases in normal pregnancy, and the loss of this increase is associated with SA. Meanwhile, the expressions of CD16, CD11b, and CD62L in E/D-LDG were also analyzed. No significant difference in the MFI of CD16 and CD62L among the three groups was observed, but the MFI of CD11b in the NP and SA groups was significantly higher than that in the UP group, suggesting increased activation of D-LDG in early pregnancy (Figure 3B). Although the MFI of CD11b in the SA group was higher than in the NP group, the difference was not statistically significant.

LDG percentage is higher in DICs than in PBMCs, and D-LDG is more easily activated than PB-LDG in early pregnancy

The LDG percentage in PBMCs and EICs/DICs and the surface markers in PB-LDG and E/D-LDG were compared. There was no statistical difference in the LDG percentage between PBMCs and EICs in the UP group (Figure 4A), but a significantly higher LDG percentage in DICs than in PBMCs was observed in the NP and SA groups (Figures 4B, C). Compared with PB-LDG, the MFI of CD16 in UP and NP groups and CD11b in the UP group was significantly lower, while the MFI of CD11b in the SA group was significantly higher in E/D-LDG (Figures 4A-C). Moreover, in the pregnant women (NP + SA), there were significantly higher LDG percentage in DICs and MFI of CD11b in D-LDG and significantly lower MFI of CD16 and CD62L in D-LDG than in PBMCs and PB-LDG, respectively (Figure 4D). These results indicate that LDG percentage is higher in DICs than in PBMCs, and D-LDG is more easily activated than PB-LDG in early pregnancy.

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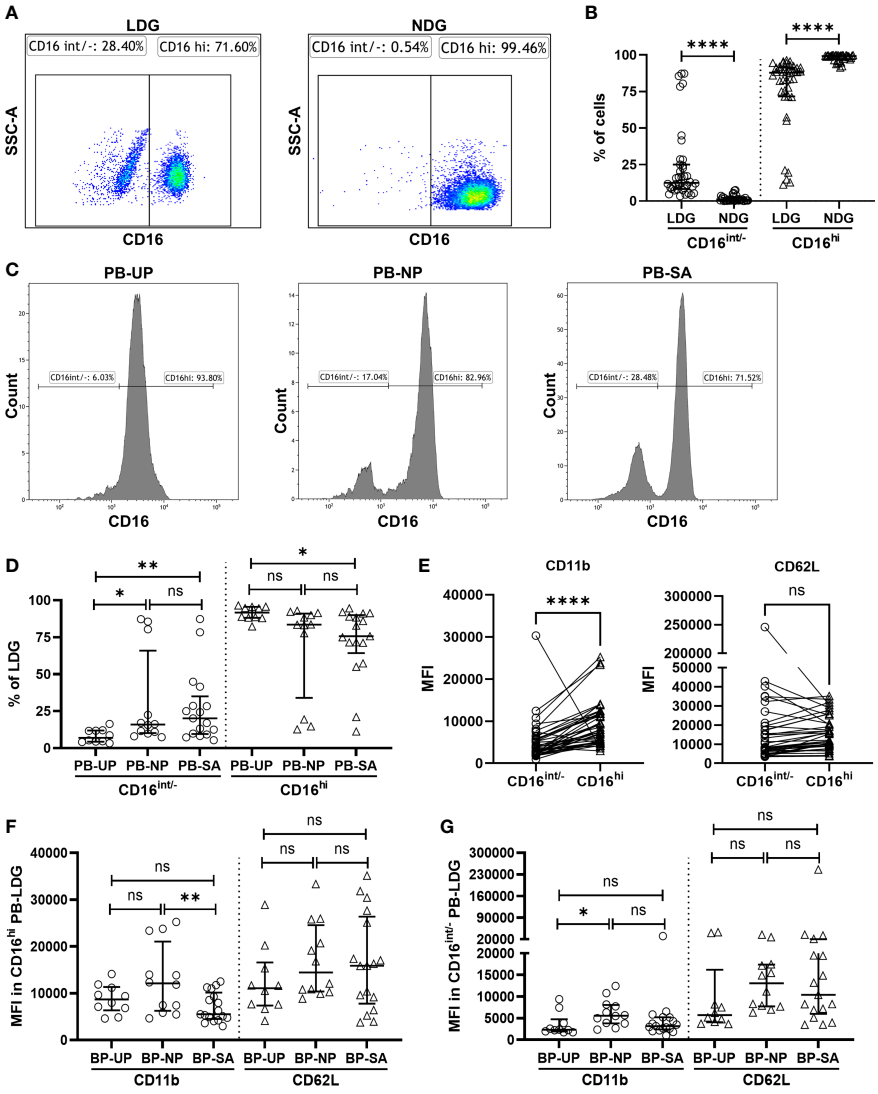

FIGURE 2
Insufficient activation of decreased CD16^{hi} PB-LDG correlates with SA. **(A)** PB-LDG and PB-NDG were classified as CD16$^{int/-}$ and CD16^{hi} subpopulations and data were shown from representative CD16 plots in FCM analysis. **(B)** Comparison of the percentage of CD16$^{int/-}$ and CD16^{hi} cells between PB-LDG and PB-NDG. **(C)** Representative FCM analysis of the percentage of CD16$^{int/-}$ and CD16^{hi} cells in PB-LDG. **(D)** Comparison of the percentage of CD16$^{int/-}$ and CD16^{hi} cells in PB-LDG. **(E)** Comparison of the MFI of CD11b and CD62L between CD16$^{int/-}$ and CD16^{hi} LDG. **(F, G)** Comparison of the MFI of CD11b and CD62L in CD16^{hi} LDG **(F)** and CD16 $^{int/-}$ LDG **(G)**. Data are presented as dots and median with interquartile range, and each symbol represents an individual donor. T-test or Mann-Whitney U test was used to identify the differences between the two groups, and one-way ANOVA with Tukey's *post hoc* analysis or Kruskal-Wallis H with Student-Newman-Keuls *post hoc* analysis was performed for comparisons among the three groups. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$. ns, not significant.

Increased decidual Nets formation is associated with SA

Peripheral blood plasma was collected, and the dsDNA concentration was detected to determine the formation of Nets. The results showed that the dsDNA concentration in UP, NP, and SA groups increased gradually, and the highest value was observed in the SA group and was significantly higher than in the UP group (Figure 5A). Meanwhile, the Nets formation in the endometrial/decidual tissues was investigated with double-label immunofluorescence, in which neutrophils were labeled with CD15 (red) and Nets were labeled with H3cit (green). As shown in Figure 5B, CD15-positive neutrophils in the UP group were morphologically intact with little H3cit expression. The CD15-positive neutrophils were also intact in the NP group, but H3cit increased without prominent reticular structure, suggesting a restrained Nets formation. In the SA group, the CD15-positive neutrophils were fragmentary, and H3cit appeared in a reticular pattern, indicating a large amount of depolymerized chromatin and NETs formation. Quantitatively, the number of Nets-positive decidual/endometrial samples (women) was significantly higher in the SA group (7, 58.33%) than in the NP (2, 20%) and UP (0) groups, while there was no significant difference between the NP and UP groups (Figure 5C), indicating an increased decidual Nets formation is associated with SA. Because Nets formation was associated with PAD4, immunohistochemical staining also

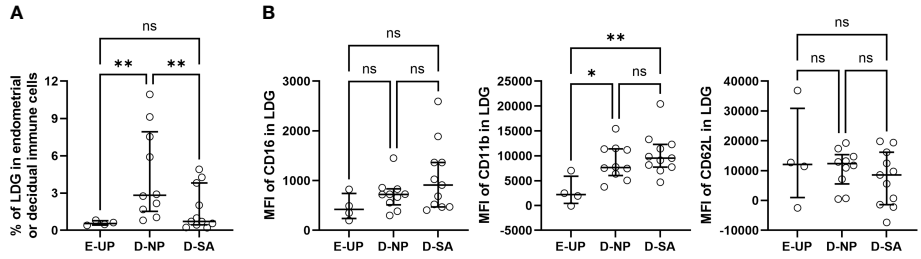

FIGURE 3
Loss of increase in decidual LDG (D-LDG) is associated with SA. Endometrial/decidual immune cells (EICs/DICs) were isolated, and the LDG percentage in EICs/DICs was quantified and identified. **(A)** Comparison of the LDG percentage in EICs/DICs of 4 UP, 10 NP, and 11 SA women. **(B)** Comparison of the MFI of CD16, CD11b, and CD62L in E/D-LDG among UP, NP, and SA groups. Data are presented as dots and median with interquartile range, and each symbol represents an individual donor. One-way ANOVA with Tukey's *post hoc* analysis or Kruskal-Wallis H with Student-Newman-Keuls *post hoc* analysis was performed for comparisons among the three groups. * $p < 0.05$, ** $p < 0.01$. ns, not significant.

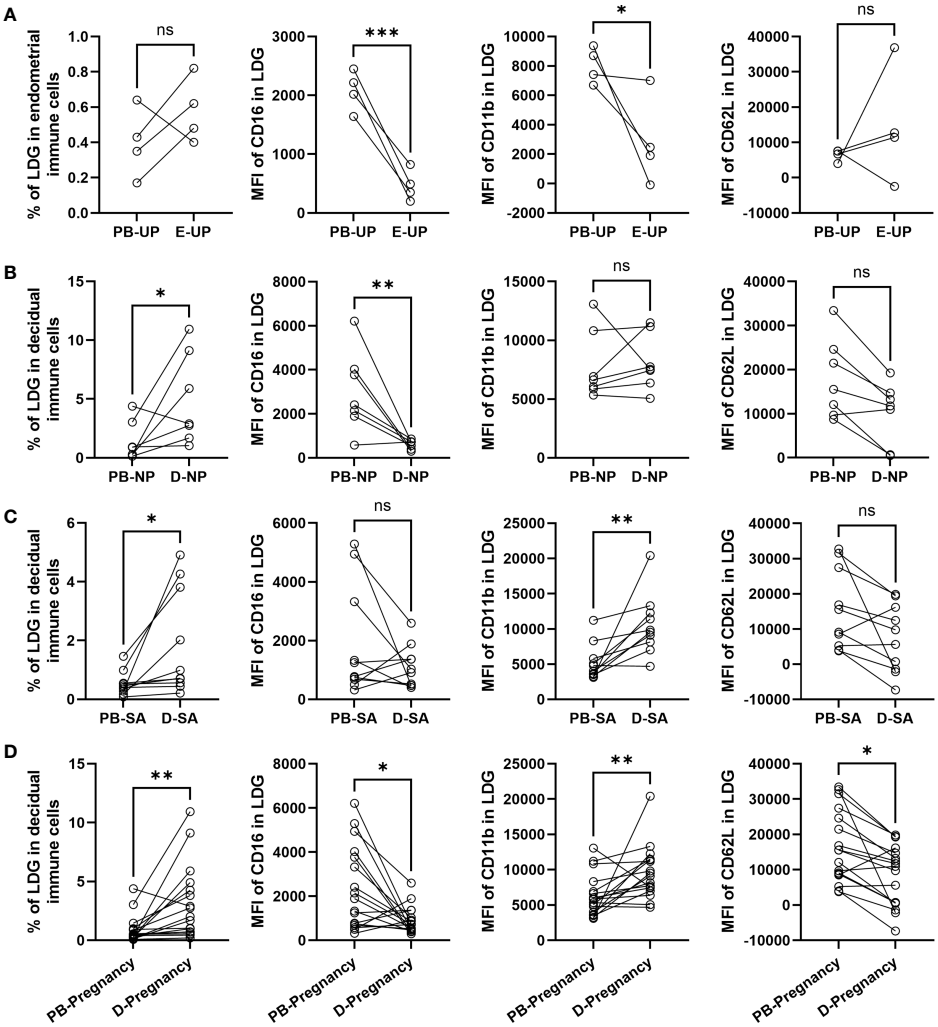

FIGURE 4
LDG percentage is higher in DICs than in PBMCs, and D-LDG is more easily activated than PB-LDG in early pregnancy. **(A-D)** The LDG percentage in PBMCs and EICs/DICs and the MFI of CD16, CD11b, and CD62L between PB-LDG and E/D-LDG in the UP **(A)**, NP **(B)**, and SA **(C)**, and pregnant (NP +SA) **(D)** women were quantified and compared. Data are presented as dots, and each symbol represents an individual donor. T-test or Mann-Whitney U test was used to identify the differences between the two groups. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. ns, not significant.

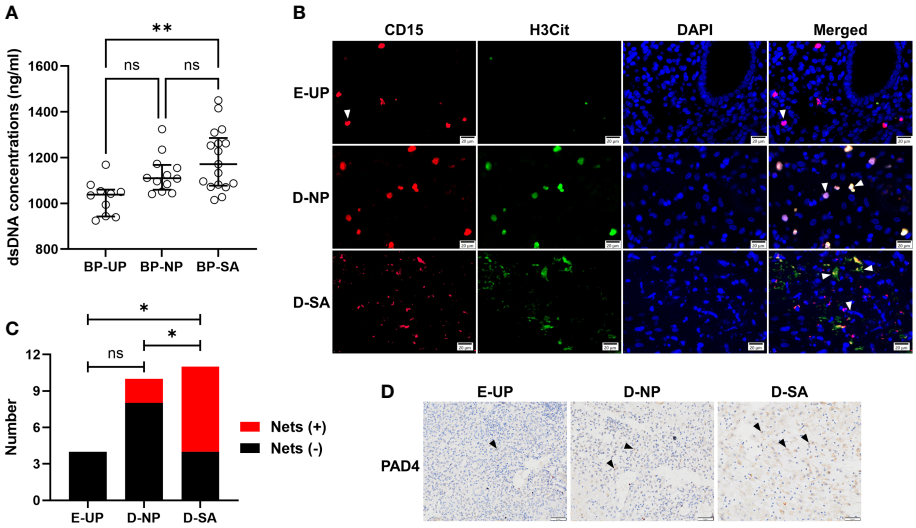

FIGURE 5
Increased decidual Nets formation is associated with SA. **(A)** Comparison of the dsDNA concentration in peripheral blood plasma among UP, NP, and SA groups. **(B, C)** The Nets formation in the endometrial/decidual tissues was double-labeled, in which neutrophils were labeled with CD15 (red) and Nets were labeled with H3cit (green) **(B)**, and the number of Nets-positive decidual/endometrial samples was counted and compared among UP, NP, and SA groups **(C)**. The white arrows indicated typical neutrophil changes in different groups. **(D)** Representative image of the immunohistochemical staining of PAD4 (black arrows), which was associated with Nets formation, in endometrial/decidual tissues. Data are presented as dots and median with interquartile range (**A**, each symbol represents an individual donor) or case number **(C)**. One-way ANOVA with Tukey's *post hoc* analysis or Kruskal-Wallis H with Student-Newman-Keuls *post hoc* analysis was performed for comparisons among the three groups. * $p < 0.05$, ** $p < 0.01$. ns, not significant.

revealed that the PAD4-positive cells in the SA group were overwhelmingly higher than those in the NP and UP groups (Figure 5D).

Increased Nets formation in D-LDG is correlated with early pregnancy

To further clarify whether the Nets formation in PB-LDG and D-LDG was related to pregnancy or SA, the PB-NDG, PB-LDG, and E/D-LDG were isolated and stimulated by PMA, and the Nets formation was quantified. As a result, Nets formation was detected in all cells following PMA stimulation. There was no statistical difference in the percentage of Nets-positive cells in PB-LDG and PB-NDG among the three groups (Figures 6A, B and S2A, S2B) and in the percentage of Nets-positive cells between PB-LDG and PB-NDG (Figure S2C). However, the Nets formation in D-LDG of the SA and NP groups was significantly higher than that in E-LDG of the UP group (Figures 6C, D). Meanwhile, the percentage of Nets-positive cells in the SA and NP groups was significantly higher in D-LDG than in PB-LDG, while no significant difference in the UP group (Figure 6E). These results indicate that both PB-NDG, PB-LDG, and E/D-LDG can form Nets, and increased Nets formation in D-LDG is correlated with early pregnancy.

Discussion

The excessive maternal immune responses must be strictly controlled to ensure a successful pregnancy (14). Although many studies reported the roles of immune cells in maternal-fetal immunity (15), the contribution of neutrophils is often overlooked. In recent years, the involvement of neutrophils and their subpopulations, especially the LDG, in pregnancy is being discovered, but it is still rarely reported in SA (16–18). In this study, we first observed that LDG percentage in PBMCs and DICs increases in normal pregnancy, while the loss of this increase is associated with SA. Meanwhile, we found that the increased $CD16^{int/-}$ cell percentage in PB-LDG correlates with pregnancy, and insufficient activation of the $CD16^{hi}$ PB-LDG may be related to SA. Moreover, the LDG percentage was higher in DICs than in PBMCs, and D-LDG was more easily activated than PB-LDG in early pregnancy. Finally, the histological analysis discovered that increased decidual Nets formation is associated with SA, and the PMA-stimulative assay verified that increased Nets formation in D-LDG correlates with early pregnancy. These findings demonstrated the serial characteristics of LDG and their subpopulations in peripheral blood and endometrium/decidua of unpregnant, normally pregnant, and SA women and highlighted the involvement of LDG in early pregnancy and SA, providing promising targets for further exploration of SA pathogenesis and therapeutics.

The neutrophil is a homogeneous and terminally differentiated cell population with higher density than mononuclear cells because of the enrichment of granular proteins (19). Recent studies identified several neutrophil subpopulations, of which LDG attracts increasing attention (20–22). However, the definition of granulocyte with lower density is not unified. Except for LDG, many other terms are also commonly used, such as low-density neutrophils (LDN) (6, 23), granulocytic myeloid-derived

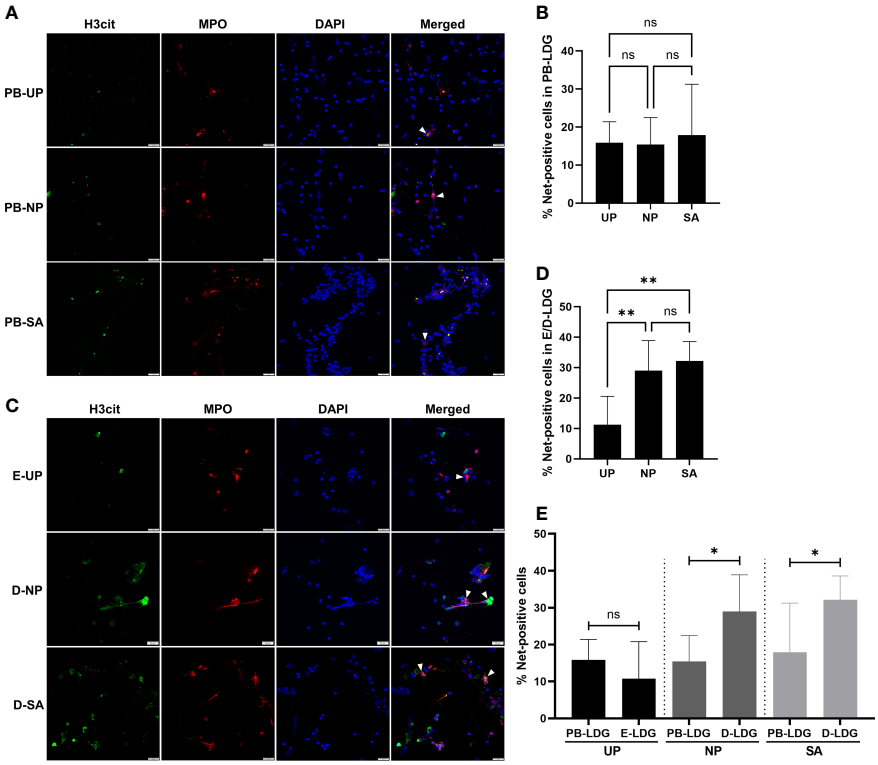

FIGURE 6
Increased Nets formation in D-LDG is correlated with early pregnancy. The PB-LDG and E/D-LDG were isolated and stimulated by PMA, and the Nets formation was detected and quantified. **(A)** Representative image of Nets formation labeled with H3cit (green) and MPO expression (red) in PB-LDG. **(B)** Comparison of the percentage of Nets-positive cells in PB-LDG. **(C)** Representative image of Nets formation labeled with H3cit (green) and MPO expression (red) in E/D-LDG. **(D)** Comparison of the percentage of Nets-positive cells in E/D-LDG. **(E)** Comparison of the percentage of Nets-positive cells between PB-LDG and E/D-LDG. Data are presented as median with interquartile range. T-test or Mann-Whitney U test was used to identify the differences between the two groups, and one-way ANOVA with Tukey's *post hoc* analysis or Kruskal-Wallis H with Student-Newman-Keuls *post hoc* analysis was performed for comparisons among the three groups. * $p < 0.05$, ** $p < 0.01$. ns, not significant.

suppressor cells (G-MDSC) (24, 25), polymorphonuclear myeloid-derived suppressor cells (PMN-MDSC) (26–28). Of note, the definitions and functions are reported differently for these cells. For example, PMN-MDSC and LDN are enriched in the low-density PBMCs fraction following density centrifugation and express similar surface markers, including $CD11b^{+}CD14^{-}CD15^{+}CD66b^{+}$ (23). Unlike these phenotypes, Li et al. defined the PMN-MDSC as $HLA\text{-}DR^{-}/^{low}CD11b^{+}CD33^{+}CD15^{+}CD14^{-}$ (26). Meanwhile, the ability of PMN-MDSC to suppress T cell function was contrary to that of LDN in SLE (23, 29). Moreover, these cells have been reported in other autoimmune, cancer, and infectious diseases with either pro-inflammatory (LDG) or suppressive effects (PMN-MDSC) (22). Overall, these cells may be at least partially identical according to the isolation protocol and phenotype identification. Therefore, it is necessary to conduct more studies on this specific subpopulation of neutrophils to achieve unification of definition and identification.

Compared with the UP women, we found that the LDG percentage in the NP group increased in both peripheral blood and decidua. However, this increase was not observed in SA women. These results are similar to those reported by Li et al. on the changes in PMN-MDSC during early pregnancy (26). In addition, they reported that PMN-MDSC was the main subset of MDSC in human decidua and exhibited an immunosuppressive effect. Our study also confirmed that the LDG percentage in decidua was higher than in PBMCs during early pregnancy. Similar changes in PMN-MDSC were also reported previously (30). Meanwhile, we found that the LDG percentage in PBMCs is associated with gestational age and significantly increased after eight weeks in a normal pregnancy but exhibited inconspicuous changes in SA. However, verifying whether a similar phenomenon exists in D-LDG requires a more extensive sample size. Moreover, the placenta begins to form and take over the function of the corpus luteum in a process termed luteoplacental shift at about eight weeks of pregnancy (2, 31), whether the increase of LDG percentage in PBMCs is a prerequisite for the normal placental formation and function and the maintenance of normal pregnancy is interesting and worth further exploration.

CD16 expresses on many immune cell surfaces and appears late during neutrophilic maturation (32–34). LDG is a mixed population of mature and immature neutrophils and can be classified into different subsets according to the CD16 expression.

In adult anti-neutrophil cytoplasm autoantibody vasculitis (AAV) patients and healthy controls, Ui Mhaonaigh et al. revealed that the $CD16^{+}$ cell percentage was significantly lower in LDG than in NDG, and there was a significant increase in $CD16^{int/-}$ cells in the LDG compared to NDG fraction. Our study revealed similar changes in $CD16^{hi}$ and $CD16^{int/-}$ LDG from UP, NP, and SA women compared to NDG. However, the changes of CD16 expression in PB-LDG were like that observed in the LDG in umbilical cord blood and granulocyte of humanized mice treated with granulocyte-colony stimulating factor (G-CSF) (33). Considering that the increase of $CD16^{int/-}$ in LDG may be the result of the acute granulopoiesis-induced increase in the number of immature neutrophils (35), whether the changes of LDG and their subpopulations were a non-specific feature of acute illness need to be verified in more disease models. Nonetheless, we revealed that the percentage of $CD16^{hi}$ LDG in SA women was significantly lower than in UP women, but no significant difference was observed between NP and UP women. Therefore, whether a decreased $CD16^{hi}$ PB-LDG is associated with SA needs more clarification.

Integrin CD11b is a receptor expressed on various leukocytes, such as monocytes, neutrophils, dendritic cells, and NK cells (36, 37). After granulocyte activation, CD11b transfers from an intracellular pool to the external surface of the neutrophil plasma and plays various biological functions, such as host defense, cellular inflammatory responses, and signal transduction (38, 39). CD11b exhibits anti-inflammatory effects, and recent studies have shown that CD11b activation inhibits TLR-dependent inflammation and autoimmunity, thereby reducing inflammatory damage (40). Meanwhile, CD11b deficient mice showed susceptibility to inflammatory and autoimmune diseases (41). In sepsis and SLE models, CD11b deficiency led to increased levels of pro-inflammatory cytokines (42). Sacks et al. reported that the expression of CD11b on the surface of leukocytes in NP women was higher than that in UP women (43). Our study revealed similar results in neutrophils, but CD11b expression in SA women was significantly lower than in NP women, indicating that the decreased CD11b expression might affect the immune response in SA pathogenesis.

Nets are reticular structures mainly composed of citrullinated histones, myeloperoxidase, neutrophil elastase, and other components and are involved in multifarious physical and pathological conditions (44–46). This study discovered that the Nets formation during pregnancy increased in peripheral blood and decidua, and this is consistent with the view that human pregnancy is associated with a mild pro-inflammatory state characterized by circulatory neutrophil activation (47). Nets formation during pregnancy was regulated in a multi-mode manner, such as HCG and CSF could promote NETosis, while progesterone restrained the NETotic process (48, 49). In the mouse model of early pregnancy, abundant Nets formation at the maternal-fetal interface could lead to fetal death (14). This study also found that the Nets formation in SA decidua was higher than that in NP decidua, indicating that excessive Nets formation may be responsible for fetal death in early SA. Moreover, PAD4 deficiency could lead to a failure of Nets formation and a significant reduction of pregnancy loss (50). We also found that the PAD4-positive cells in the SA decidua were overwhelmingly higher than those in the NP and UP decidua, indicating that PAD4 may be a crucial intersection between the Nets formation and SA occurrence and represents a promising therapeutic target.

This study also has several limitations. Firstly, this study was carried out in a small-sized human sample, and studies with a large ample are still needed. Secondly, SA is a highly heterogeneous disease with complex and diverse etiology. Some maternal risk factors, such as chronic endometritis, endocrine abnormalities, and antiphospholipid syndrome, were not considered in the inclusion criteria, and the relevant subgroup analysis was also not conducted due to the small sample size. Thirdly, DOP-based subgroup analysis was not performed in E/D-LDG because not enough decidual tissues with DOP > 56d were collected. Finally, endometrial and decidual NDG was not isolated for study, and CD16-based subgroup analysis in E/D-LDG was not conducted because the subpopulations could not be distinguished *via* FCM analysis.

In conclusion, LDG in peripheral blood and decidua participates in the maintenance of early pregnancy, while dysregulated LDG, involving the percentage, phenotype and subpopulations, and ability to form Nets, is responsible for early SA. Of course, more studies with larger sample sizes, optimized functional and rescue assays, and animal experiments, even human trials, are still required in the subsequent exploration of LDG-based immunological mechanisms and therapeutic strategies of SA.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving human participants were reviewed and approved by Chengdu Xi'nan Gynecology Hospital. The patients/participants provided their written informed consent to participate in this study.

Author contributions

HY conceived the project and designed experiments. HY, YD, LiL, and YJ screened and collected human tissues. HY, LaL, QZ, YS, and PY performed the experiments and acquired, analyzed, and interpreted the data. HY wrote the manuscript. YJ and JG supervised the project. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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Identification of MARK2, CCDC71, GATA2, and KLRC3 as candidate diagnostic genes and potential therapeutic targets for repeated implantation failure with antiphospholipid syndrome by integrated bioinformatics analysis and machine learning

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Background: Antiphospholipid syndrome (APS) is a group of clinical syndromes of thrombosis or adverse pregnancy outcomes caused by antiphospholipid antibodies, which increase the incidence of *in vitro* fertilization failure in patients with infertility. However, the common mechanism of repeated implantation failure (RIF) with APS is unclear. This study aimed to search for potential diagnostic genes and potential therapeutic targets for RIF with APS.

Methods: To obtain differentially expressed genes (DEGs), we downloaded the APS and RIF datasets separately from the public Gene Expression Omnibus database and performed differential expression analysis. We then identified the common DEGs of APS and RIF. Gene Ontology and Kyoto Encyclopedia of Genes and Genomes pathway enrichment analyses were performed, and we then generated protein-protein interaction. Furthermore, immune infiltration was investigated by using the CIBERSORT algorithm on the APS and RIF datasets. LASSO regression analysis was used to screen for candidate diagnostic genes. To evaluate the diagnostic value, we developed a nomogram and validated it with receiver operating characteristic curves, then analyzed these genes in the Comparative Toxicogenomics Database. Finally, the Drug Gene Interaction Database was searched for potential therapeutic drugs, and the interactions between drugs, genes, and immune cells were depicted with a Sankey diagram.

Results: There were 11 common DEGs identified: four downregulated and seven upregulated. The common DEG analysis suggested that an imbalance of immune system-related cells and molecules may be a common feature in the pathophysiology of APS and RIF. Following validation, MARK2, CCDC71, GATA2, and KLRC3 were identified as candidate diagnostic genes. Finally, Acetaminophen and Fasudil were predicted as two candidate drugs.

Conclusion: Four immune-associated candidate diagnostic genes (MARK2, CCDC71, GATA2, and KLRC3) were identified, and a nomogram for RIF with APS diagnosis was developed. Our findings may aid in the investigation of potential biological mechanisms linking APS and RIF, as well as potential targets for diagnosis and treatment.

KEYWORDS

repeated implantation failure, anti-phospholipid syndrome, bioinformatics analyses, nomogram, immune infiltration, machine learning

1 Introduction

Repeated implantation failure (RIF), which accounts for 5% to 10% of assisted reproductive technology (ART) failures (1), is a syndrome where patients with infertility continue to fail to achieve a clinical pregnancy despite several *in vitro* fertilization-embryo transfers (IVF-ET), a process with a cumulative live birth rate reaching 34.7% (2). Although the diagnostic criteria for RIF have not yet been standardized, clinical studies have defined RIF as the inability of women under the age of 40 to achieve clinical pregnancy following the transfer of at least four high-quality embryos in at least three fresh or frozen cycles (3). RIF has a complicated etiology that includes anatomical factors of the reproductive organs, chromosomal abnormalities of the couple or embryos, endocrine abnormalities, and infections (4). Although the etiology of some RIF patients has not yet been identified, it is believed to be connected to immunological factors (5). It has become evident that RIF is one of the most important issues in the assisted reproduction field because of its complicated etiology and the significant psychological burden it places on patients and their partners.

Antiphospholipid syndrome (APS) is a serious autoimmune disease that is characterized by persistent positivity for anti-phospholipid antibodies (APLs), including lupus anticoagulant (LA), anticardiolipin antibody (ACL), and anti-β2-glycoprotein 1 (β2-GP1) antibody. APS can cause microthrombosis of the chorionic plate at the maternal-fetal interface, which can lead to recurrent miscarriage, placental malfunction, preterm pre-eclampsia, fetal growth restriction, fetal distress, and even stillbirth (6). According to studies (7), 5% to 20% of women of childbearing age exhibit clinical symptoms of APS. If patients with positive APLs are not treated with the proper interventions or therapies, the pregnancy loss rate can reach 24% to 60%. APL positivity is linked to IVF-ET failure and correlates strongly with RIF (8). IVF failure and pregnancy complications were more common in APL-positive patients than in negative patients (9). Patients with RIF had higher rates of positive ACL antibodies, anti-β2-GP1 antibodies, and anti-phosphatidylethanolamine antibodies. The IVF-ET failure rate was significantly higher in triple-positive or more APL subtype-positive patients than in normal people (10). These findings imply that infertility patients with APS are at risk of RIF.

Multiple mechanisms can trigger T cell activation and the accompanying cytokine production in APS patients *in vivo*, which affect the immune system's normal regulation and disturb immunological homeostasis (11). In addition, APL can inhibit chorionic villous cells from migrating or invading, decrease the expression levels of complement regulatory proteins, activate complement on the surface of trophoblast cells, and trigger inflammatory responses (12). Furthermore, APS patients can develop microvascular thrombosis, substantially impairing endometrial metaplasia and early embryonic implantation, which ultimately results in female infertility, unsuccessful implantation, and spontaneous abortion (13, 14). These findings strongly support a connection between APS and RIF. However, the underlying mechanisms are still poorly understood, making it necessary to investigate their shared pathophysiology and genetic details. In this study, we sought to explore the mechanisms by which APS affects immune tolerance in pregnancy, leading to embryo implantation failure, and to further establish and investigate the networks involved in inflammation and immune regulation in order to improve the diagnosis and clinical management of such patients.

To examine the common pathogenesis of APS and RIF, we screened public databases for common differentially expressed genes (DEGs) between the two diseases and used them to build diagnostic models and identify potential therapeutic drugs. This is the first study to investigate common markers between APS and RIF using a systems biology approach. This work will provide new insights and direction for understanding the biological mechanisms of both diseases, which will aid in the design of dual-purpose treatment methods. Figure 1 depicts the study flow chart for this investigation.

2 Materials and methods

2.1 Data collection and preprocessing

Microarray datasets were downloaded from the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/), which has a significant amount of microarray, second-generation sequencing, and other high-throughput sequencing data (15). Using the keywords

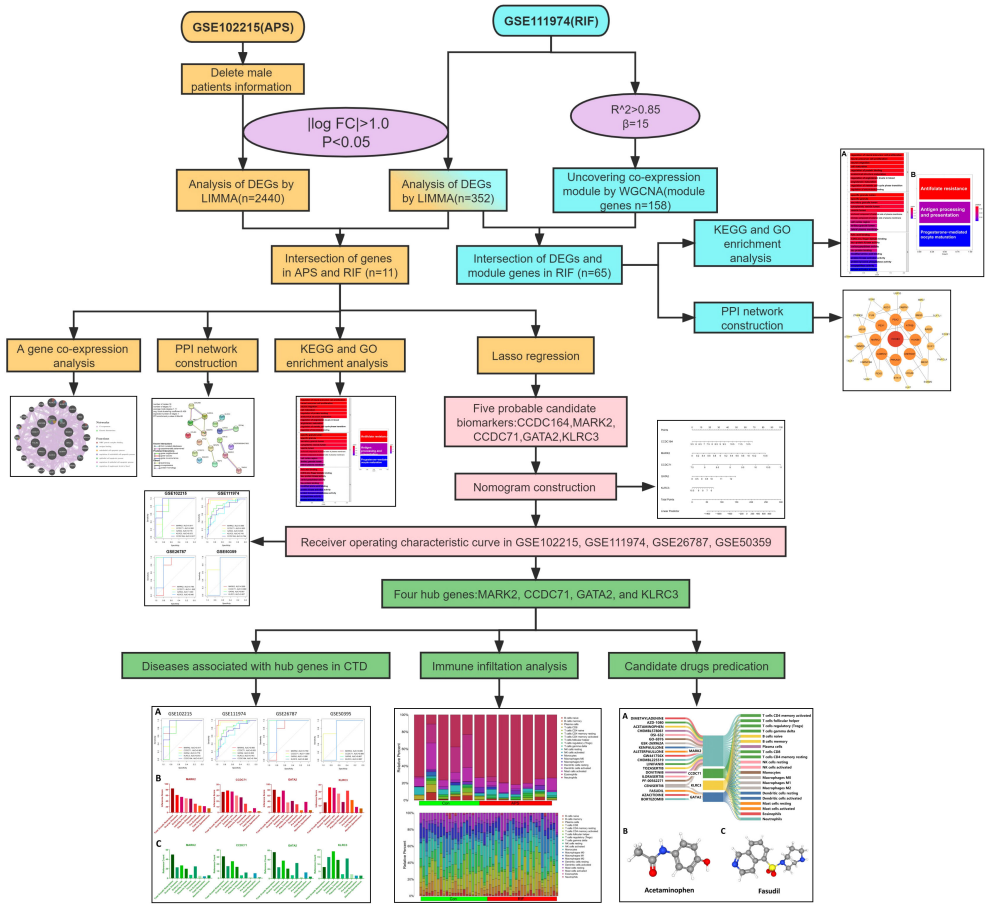

FIGURE 1
Flow chart of our research.

"antiphospholipid syndrome" and "implantation failure", we searched related gene expression datasets. The male specimens and non-human samples tested were eliminated. Finally, the GSE102215 (16) (based on GPL16791), GSE50395 (17) (based on GPL4133), GSE111974 (18) (based on GPL17077), and GSE26787 (19) (based on GPL570) datasets were downloaded from the GEO database.

2.2 Identification of common genes

For GSE102215, after excluding the male patients, the DEGs between healthy and APS samples were identified using the limma package (version 3.44.3) (20). DEGs were identified as genes that met the specific cutoff criteria of false discovery rate <0.05 and |log fold change|>1.0. For GSE111974, genes with false discovery rate <0.05 and |log fold change |>1.0 were defined as DEGs. DEG expression was visualized by volcano plots and heatmaps using the ggplot2 package (version 3.3.2) (21) and pheatmap package (version 1.0.12) (22). To screen for common DEGs associated with APS and RIF, the online Venn diagram platform (http://bioinformatics.psb.ugent.be/webtools/Venn/) was used to collect their overlapping DEGs.

2.3 Module gene selection of RIF by weighted gene co-expression network analysis

WGCNA is an algorithm that analyzes the gene expression patterns of multiple samples, clusters genes with similar expression patterns to form different modules, and analyzes the associations between modules and phenotypes or traits, as well as the hub genes in the network (23). To retrieve the RIF-related modules, WGCNA was employed to analyze the GSE111974 dataset. The data were reviewed to find any outliers in the samples, and all samples from the RIF dataset were properly clustered. Using the criterion of R^2>0.85, a suitable soft-thresholding power β was calculated to determine the scale-free topology. Next, co-expression modules were generated using hierarchical clustering, and the results were presented in a hierarchical clustering tree. Automatic module merging was performed for modules with highly related trait genes (minimum number of module genes set to 30, merge cutting height = 0.25). Finally, the expression profile of each module was obtained by calculating the module eigengene and the correlations between the clinical features and module eigengene. We selected the modules that had better correlation coefficients

with clinical characteristics(P-value<0.05), and the genes from those modules were then chosen for further analysis.

2.4 Gene ontology and kyoto encyclopedia of genes and genomes enrichment analysis

A dynamically updated set of controlled vocabulary is provided by GO, an internationally standardized classification system for gene functions, to comprehensively characterize the features of genes and gene products in living organisms (24). KEGG is a database that combines genomic and functional information and conducts systematic assessments of gene functions (25). The R package clusterProfiler (26) was used to perform a functional enrichment study, with a criterion of P-value<0.05. GO and KEGG analyses were performed twice in this work.

2.5 Construction of a protein-protein interaction network

Search Tool for the Retrieval of Interacting Genes (STRING; http://string-db.org) (version 11.5) is a comprehensive database for searching interactions between proteins, including direct physical interactions and indirect functional correlations between proteins (27). Using STRING with an interaction score > 0.4, the PPI networks of module genes and common DEGs were constructed. The PPI network was visualized by Cytoscape software (version 3.8.2) (28).

2.6 Co-expression analysis of common DEGs

To further explore the interrelation between common DEGs, the co-expression network was constructed using the GeneMANIA online tool. Using extensive genomic and proteomics data, the GeneMANIA (http://genemania.org/) (29) database allows for the construction of co-expression networks of identified common genes to recognize functionally related genes and weigh them based on expected values.

2.7 Machine learning

Candidate genes for RIF with APS were further filtered using the least absolute shrinkage and selection operator (LASSO) regression. While fitting a generalized linear model, LASSO regression is characterized by variable selection and complexity regularization, preventing overfitting and improving the predictive accuracy and comprehensibility of the clinical prediction models (30). LASSO regression was performed using the glmnet R packages (31). The genes screened by LASSO regression were considered candidate hub genes in RIF with APS diagnosis.

2.8 Nomogram construction and evaluation

The construction of nomograms is valuable in diagnosing clinical RIF with APS. Using the candidate hub genes, the nomogram was developed using the rms R package (32). "Points" denotes the score of candidate hub genes, and "Total Points" represents the sum of all the above gene scores. To evaluate the diagnostic value of candidate hub genes for RIF and APS, receiver operating characteristic (ROC) curves were subsequently constructed using the pROC R package, and the area under the ROC curve (AUC) was calculated separately (33). AUC>0.75 was considered an ideal diagnostic value. Genes with better diagnostic performance were screened for drug prediction.

2.9 Immune cell infiltration analysis

The CIBERSORT is a computational method that uses tissue gene expression profiles to identify the proportions of different immune cells (34). Immune cell infiltration analysis in controls versus APS and RIF samples was conducted with the Cibersort R package. Bar graphs were used to display the proportions of different immune cell types, and violin plots were used to compare these proportions between the APS and control groups, as well as the RIF and control groups. Correlation heatmaps were plotted with the corrplot R package (35) to show the correlations between the immune cells and candidate diagnostic genes.

2.10 Hub gene interactions with diseases and prediction of candidate drugs

The Comparative Toxicogenomics Database (CTD) (http://ctdbase.org/) (36) integrates data from a variable number of genes, chemical substances, functional phenotypes, and interactions between diseases. It greatly facilitates the study of disease-related environmental exposure factors and potential mechanisms of drug action. To investigate the relationships between candidate diagnostic genes and diseases, we analyzed the inference score and reference count for candidate diagnostic genes with associated diseases using CTD. Inference scores and reference counts were visualized by histograms.

Drugs that interact with candidate diagnostic genes were obtained from the Drug Gene Interaction Database (DGIdb) (https://dgidb.genome.wustl.edu/) (37) for predicting potential drugs for the treatment of APS and RIF, and the 3D structures are displayed by the PubChem website (https://pubchem.ncbi.nlm.nih.gov/) (38). The Sankey diagram was created using SankeyMATIC (https://sankeymatic.com/), which was used to characterize the interactions between potential drugs, candidate diagnostic genes, and immune cells.

2.11 Statistical analysis

We conducted statistical analyses with R software (version 4.2.1) and GraphPadPrism (version 8.0.1). The Wilcoxon test was used to measure the differences in expression between the two groups. Statistics were considered significant at *P*-value<0.05.

3 Results

3.1 GEO information

Four GEO datasets (GSE102215, GSE11974, GSE50395, and GSE26787) were selected for analysis. Table 1 summarizes the specific information of these four datasets, including the GSE number, platforms, disease, samples, source types, and groups. GSE102215 and GSE111974 were paired for the DEG analysis, and GSE50395 and GSE26787 were paired to confirm the hub genes' diagnostic efficacy.

3.2 Screening of common DEGs

The DEGs of RIF and APS were obtained from the GSE102215 and GSE111974 datasets separately, with 888 upregulated and 1,552 downregulated genes in APS and 206 upregulated and 146 downregulated genes in RIF (Figures 2A, B). Heatmaps of the DEGs in both datasets are shown in Figures 2C, D. We identified the common DEGs from the intersection of the Venn diagrams (Figure 2E). After excluding genes with opposite expression trends, 11 common DEGs (seven common upregulated and four common downregulated genes) with the same expression trends were found in GSE102215 and GSE111974 (Table 2).

3.3 PPI Network construction and enrichment analysis of common DEGs

With 99.92% co-expression and 0.08% genetic interactions, we created a sophisticated gene interaction network using the GeneMANIA database to understand the biological functions of these common DEGs. Twenty genes that were linked to the 10 common genes were identified, and the results revealed that they were mainly involved in MHC protein complex binding, antigen binding, endothelial cell apoptotic process, regulation of endothelial cell apoptotic process, epithelial cell apoptotic process, regulation of epithelial cell apoptotic process, and regulation of angiotensin levels in the blood (Figure 3A). According to the PPI network, AURKA, CENPH, and MARK2 had high degrees of number of connections to other points (Figure 3B).

GO and KEGG pathway enrichment analyses were performed to further explore the biological roles of these common DEGs. For biological processes, the common DEGs were mainly related to the regulation of neural precursor cell proliferation, neural precursor cell proliferation, neuron migration, cell maturation, regulation of protein binding, and anatomical structure maturation. For cellular components, the common DEGs were mainly related to specific granule lumen, specific granule, secretory granule lumen, cytoplasmic vesicle lumen, and vesicle lumen. Finally, for molecular functions, the common DEGs were mainly associated with folic acid binding, C2H2 zinc finger domain binding, and tau-protein kinase activity (Figure 4A). Furthermore, KEGG analysis revealed that the common DEGs were mainly enriched in antifolate resistance, antigen processing and presentation, and progesterone-mediated oocyte maturation (Figure 4B).

3.4 The co-expression modules in RIF

As an autoimmune disease, the pathophysiology of APS is likely strongly related to the imbalance of immunological homeostasis *in vivo*. To further clarify if RIF is associated with the immune environment *in vivo*, we analyzed the key RIF genes.

In the GSE111974 dataset, the gene that was significantly expressed ($P<0.05$) was selected as key genes of RIF for WGCNA analysis. All samples were clustered well and no sample was eliminated (Figure 5A). In our study, the optimal soft-power value of GSE111974 was $\beta=15$ (Figures 5B, C). A total of nine modules were identified. The correlations between modules and clinical diseases were then computed. The strongest positive association was seen in the cyan module (r = 0.89, P = 2e-17), while the light green module had the strongest negative correlation (r = -0.72, P = 1e-08) (Figures 5D, E). The list of genes in Co-expression Modules in RIF is given in Supplementary Table 2.

3.5 Screening and analysis of key genes in RIF

We selected the 65 overlapping genes in the DEG and module gene groups and labeled them as key genes Figure 6A,

TABLE 1 Summary of the four Gene Expression Omnibus (GEO) datasets involving antiphospholipid syndrome (APS) and repeated implantation failure (RIF) patients.

ID	GSE number	Platform	Disease	Samples	Source types	Grope
1	GSE102215	GPL16791	APS	6 patients and 6 controls	peripheral venous blood	Discovery cohort
2	GSE50395	GPL4133	APS	3 patients and 3 controls	peripheral venous blood	Validation cohort
3	GSE111974	GPL17077	RIF	24 patients and 24 controls	Endometrium	Discovery cohort
4	GSE26787	GPL570	RIF	5 patients and 5 controls	Endometrium	Validation cohort

APS, antiphospholipid syndrome. RIF, repeated implantation failure.

Supplementary Data Table 1). These genes were highly related to the pathogenesis of RIF. Moreover, the PPI network uncovered the close interactions between these common genes (Figure 6B). To investigate the potential functions of the key genes, we performed GO and KEGG pathway enrichment analyses. KEGG enrichment results showed that the mRNA surveillance pathway and longevity regulating pathway were significantly enriched (Figure 6C). As shown in the GO annotation results, the RIF key genes were markedly associated with embryonic development, pattern specification process, DNA-binding transcription activator activity, and RNA polymerase II-specific activator activity (Figure 6D).

3.6 Screening candidate diagnostic markers and developing a clinical predictive model

Candidate genes were screened using LASSO regression in preparation for nomogram construction and diagnostic value assessment. Five probable candidate biomarkers (CCDC164, MARK2, CCDC71, GATA2, and KLRC3) were found via the LASSO regression algorithm (Figures 7A, B). The nomogram was then developed using these five candidate biomarkers (Figure 7C).

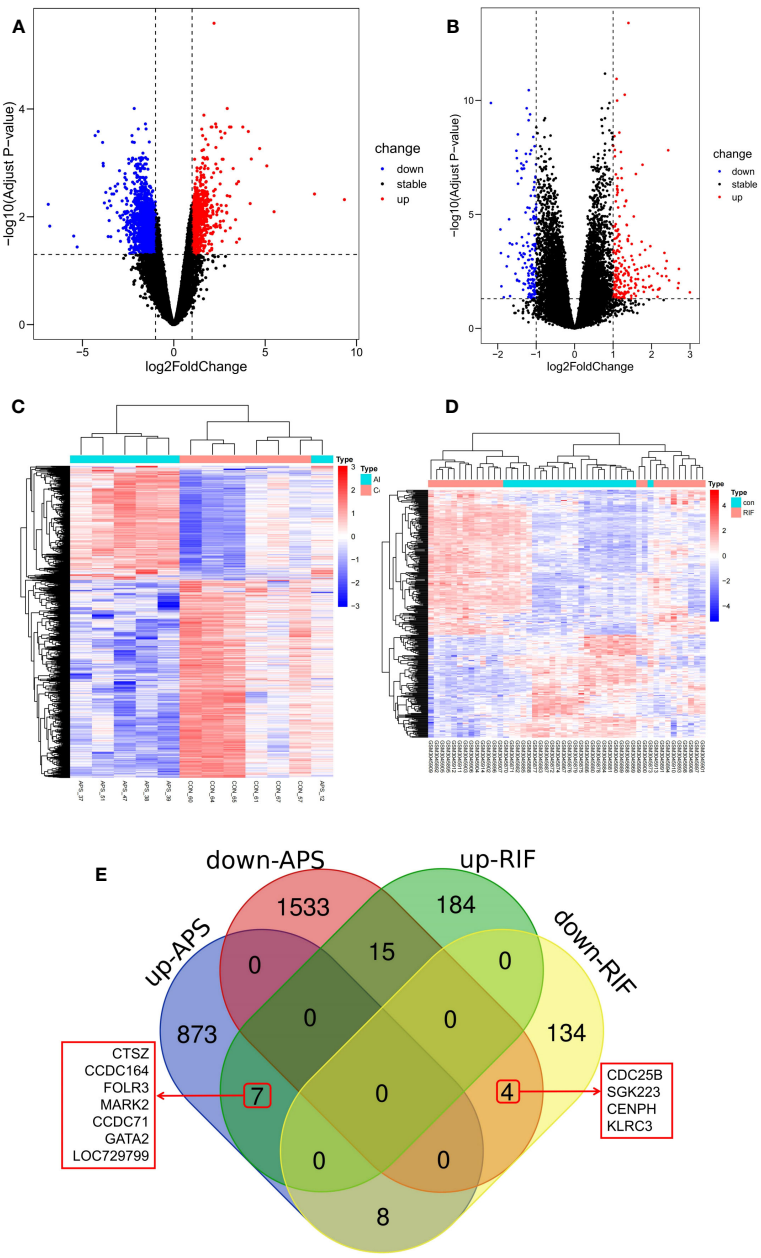

FIGURE 2
Screening of common differentially expressed genes (DEGs) between antiphospholipid syndrome (APS) and repeated implantation failure (RIF). **(A)** Volcano plots of APS DEGs. **(B)** Volcano plots of RIF DEGs. **(C)** Heatmap of the APS upregulated and downregulated genes. **(D)** Heatmap of the RIF upregulated and downregulated genes. **(E)** Venn diagram of the common DEGs in APS and RIF.

TABLE 2 The gene expression levels of 11 common differentially expressed genes (DEGs) in antiphospholipid syndrome (APS) and repeated implantation failure (RIF).

Gene samples	GSE 102215		GSE 111974		Up/Down
	logFC	P value	logFC	P value	
CTSZ	1.019004575	3.50E-06	1.0040625	1.07E-11	Up regulated
CCDC164	1.390055757	0.000210669	1.0521	0.001297453	Up regulated
FOLR3	1.259480744	0.042306007	1.412033333	0.000117374	Up regulated
MARK2	0.827205312	0.001059369	1.398616368	1.26E-18	Up regulated
CCDC71	1.001978252	0.000132575	1.2168125	2.48E-10	Up regulated
LOC729799	0.874322301	0.004536766	1.164225	1.49E-06	Up regulated
GATA2	1.51904247	0.00011236	1.150766667	6.79E-06	Up regulated
CDC25B	-1.254116899	0.000121245	-1.072204167	1.58E-07	Down regulated
SGK223	-1.119028202	0.000248632	-1.113441667	3.95E-05	Down regulated
CENPH	-1.186900689	0.007341801	-1.080745726	0.000316802	Down regulated
KLRC3	-1.937514717	1.60E-05	-1.093495833	0.000608419	Down regulated

3.7 Validation of candidate diagnostic genes

To verify the reliability of these candidate diagnostic genes, we selected the GSE102215 and GSE50395 datasets to analyze their diagnostic efficacy in APS and the GSE11974 and GSE26787 datasets to analyze their diagnostic efficacy in RIF. Unfortunately, we retrieved only four candidate diagnostic genes in the validation cohort. Collectively, all four diagnostic genes had good diagnostic efficacy (Figure 8A). Furthermore, the data suggest that these four candidate diagnostic genes were not only associated with poor pregnancy outcomes in women but also with thrombosis (Figures 8B, C).

3.8 Immune cell infiltration analysis

Because we found that the common genes appear to regulate RIF pathogenesis via the immune system, we hypothesized that these genes may be used as prospective diagnostic biomarkers for

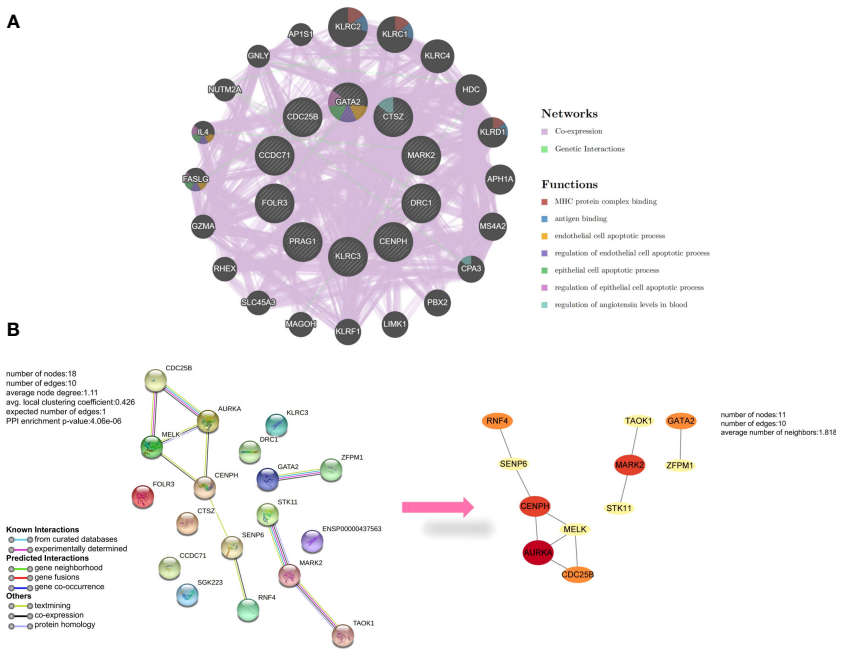

FIGURE 3
Analysis of common differentially expressed genes (DEGs) between antiphospholipid syndrome (APS) and repeated implantation failure (RIF). **(A)** Common DEGs and their co-expressed genes were analyzed using GeneMANIA. **(B)** The protein-protein interaction (PPI) network of the common DEGs.

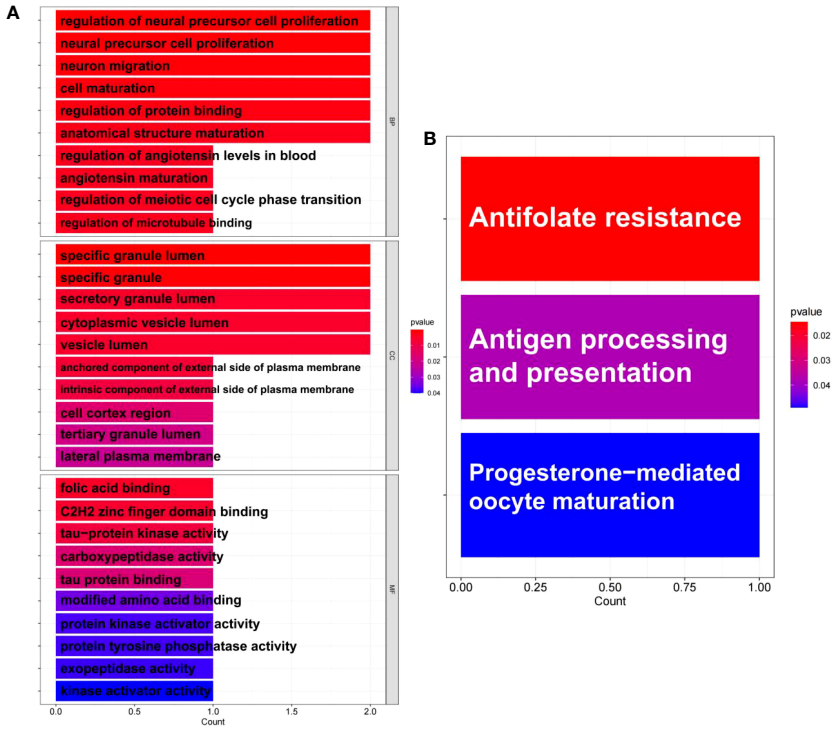

FIGURE 4
Enrichment analysis of common differentially expressed genes (DEGs) between antiphospholipid syndrome (APS) and repeated implantation failure RIF. **(A)** Gene Ontology (GO) enrichment analysis of the common targets. **(B)** Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of the common targets.

RIF with APS using the nomogram and ROC analysis. To better clarify the immune regulation of RIF with APS, we examined immune cell infiltration. The bar graphs show that the percentage of neutrophils and T cell populations varied significantly between the APS and RIF samples (Figures 9A, B). Compared with the normal samples, neutrophils and γδ T cells were significantly increased in the APS samples (Figure 9E). However, in RIF samples, the M0 macrophage population was increased, while γδ T cells, M1 macrophages, and M2 macrophages were decreased (Figure 9F). Figures 9C, D depict the association between individual immune cells, demonstrating that neutrophils had a significantly negative correlation with other immune cell types in APS.

Furthermore, we investigated the correlations between candidate diagnostic genes and the levels of various immune cells. In APS samples, MARK2, CCDC71, and GATA2 had strong negative connections with $CD8^+$ T cells and Treg populations. However, they had significant positive connections with both naive $CD4^+$ T cell and neutrophil populations. KLRC3 significantly negatively correlated with T cells and neutrophils, and significantly positively correlated with $CD8^+$ T cells (Figure 9G). In RIF samples, MARK2, CCDC71, and GATA2 had strong negative correlations with γδ T cells and M2 macrophages, but there was a positive correlation between KLRC3 and these two immune cells (Figure 9H).

3.9 Candidate drugs prediction

The DGIdb was analyzed to identify small-molecule drugs with potential therapeutic effects on RIF with APS. The Sankey diagram was then used to demonstrate the interactions between small-molecule drugs, genes, and immune cells (Figure 10A). In contrast to CCDC71 and KLRC3, MARK2 and GATA2 had a relative abundance of targeted drugs and were associated with a variety of immune cells, which were important potential therapeutic targets for APS and RIF. Acetaminophen and Fasudil were predicted to be potential drugs for the treatment of RIF with APS. The 3D structure tomography of Acetaminophen (Figure 10B) and Fasudil (Figure 10C) was found in PubChem.

4 Discussion

This study was conducted to investigate the connection between APS and RIF using bioinformatics and machine learning approaches. Three upregulated genes (MARK2, CCDC71, GATA2) and one downregulated gene (KLRC3) were identified as candidate diagnostic genes that connect APS and RIF. In addition, γδ T cells were an important cause of APS and RIF. Enrichment analysis further indicated that multiple immune responses,

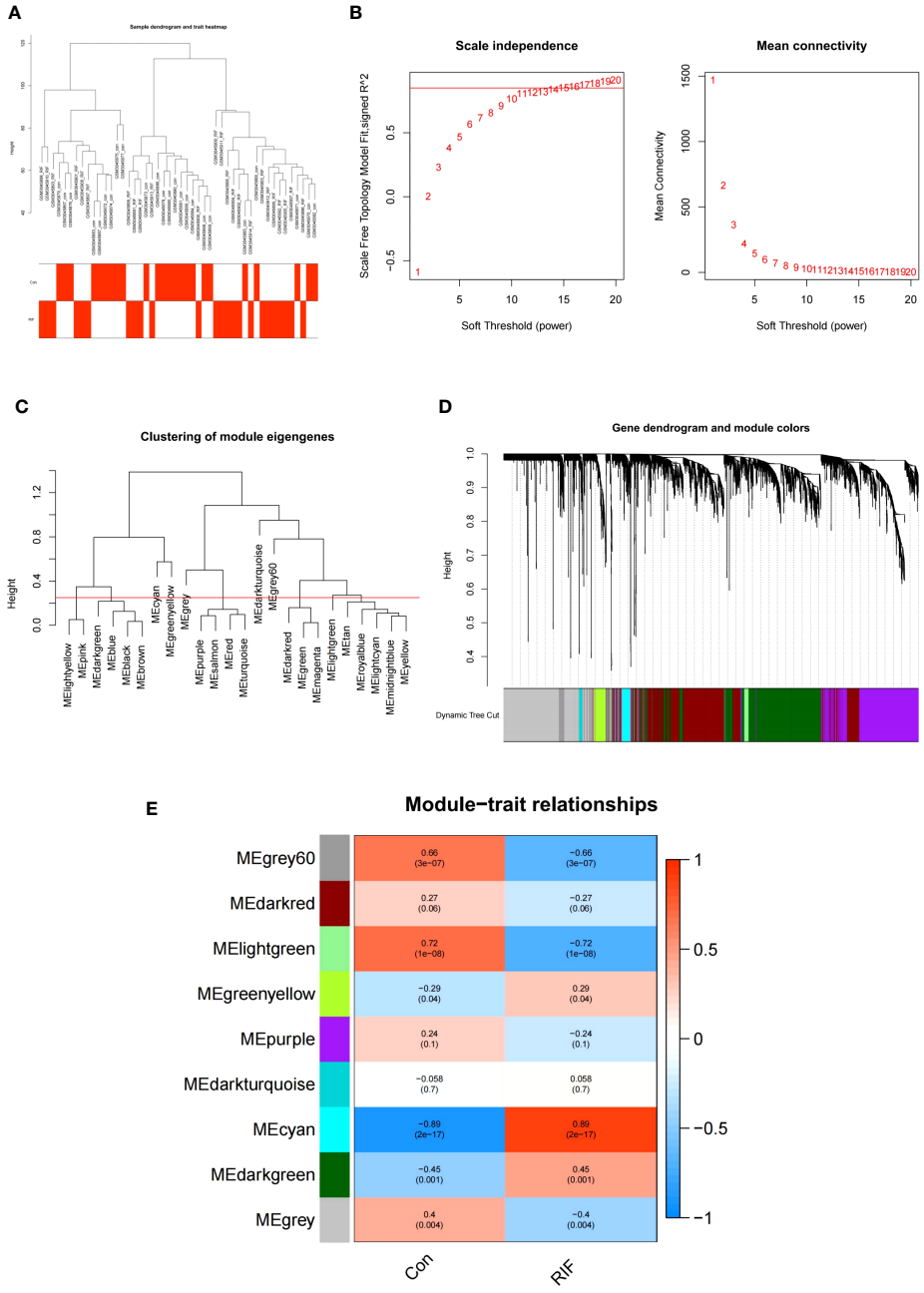

FIGURE 5
WGCNA of repeated implantation failure (RIF). **(A)** Sample clustering of RIF. The samples were classified into three significantly distinct clusters. All clusters were chosen for further analysis. **(B)** Selection of optimal thresholds. **(C)** The threshold was set to 0.25 to merge modules that are comparable in the cluster tree. **(D)** Different modules are produced and shown in different colors by aggregating genes with strong correlations into the same module. **(E)** Heatmap of the module-trait relationship in RIF. Each cell contains the corresponding correlation and *P*-value.

including antifolate resistance, antigen processing, and presentation and progesterone-mediated oocyte maturation, were closely correlated with higher expression levels of common genes. According to CTD, the four candidate diagnostic genes have strong associations with female fertility, thrombosis, and ovarian diseases. As a result, we concluded that MARK2, CCDC71, GATA2, and KLRC3 may be crucial in immunological mechanisms during the development of APS and RIF.

As two significant disorders impacting the reproductive health of women, APS and RIF are closely related and intricately intertwined. There is accumulating evidence that the two diseases share multiple common risk factors, and APS may contribute to the pathogenesis of RIF (39). Recently, the maternal-fetal immune response has become an important trend in RIF pathogenesis (40). Immune cells are crucial for embryo implantation, immune tolerance, and embryonic growth throughout a healthy pregnancy. Immune disorders in APS patients cause an imbalance of intercellular and cell-secreted cytokines, which can lead to maternal rejection of the fetus and ultimately pathological pregnancy. When Chao et al. (41) treated APS mice with a

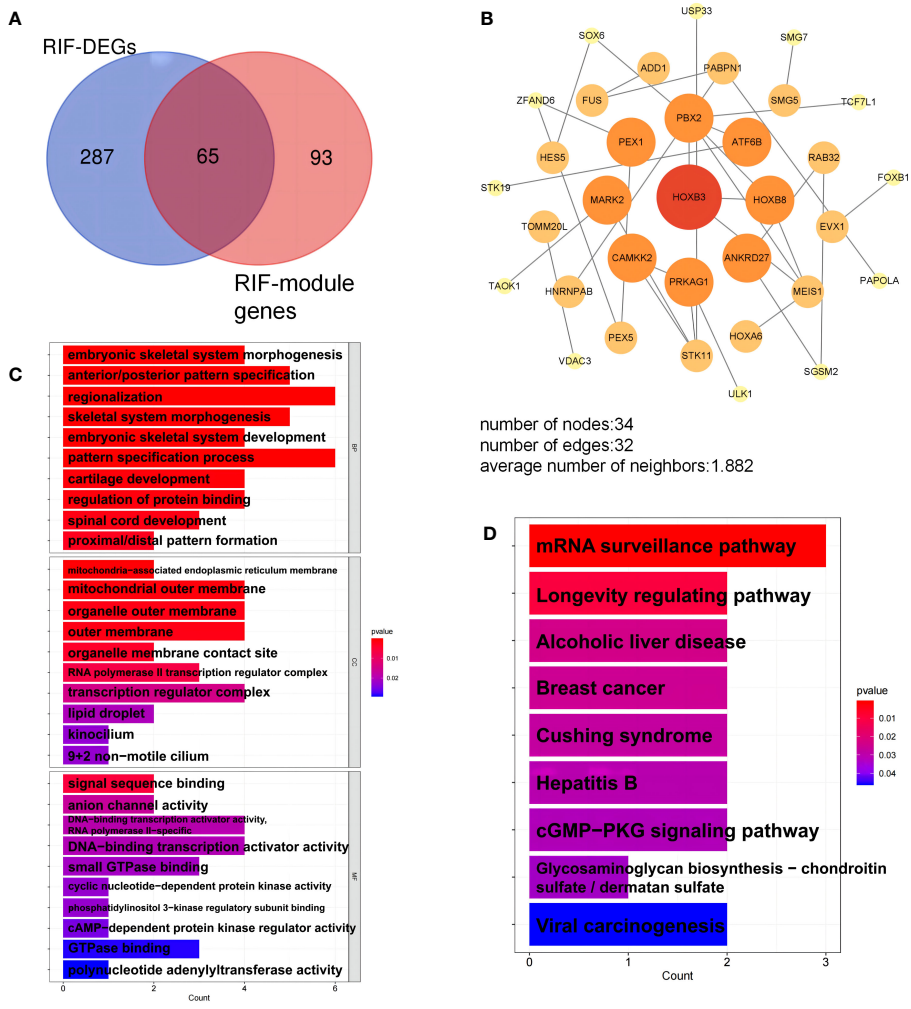

FIGURE 6
Analysis of key genes in repeated implantation failure (RIF). **(A)** The key genes are the overlapping differentially expressed genes (DEGs) and module genes. **(B)** The protein-protein interaction (PPI) network of the key genes. **(C)** Gene Ontology (GO) terms of biological process, cellular component, and molecular function were used for functional enrichment clustering analysis on key genes. **(D)** Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was performed on key genes.

mixture of DNA vaccine and FK506 adjuvant, they observed reduced Th1 and Th17 cell responsiveness and an increased frequency of $Foxp3^{+}CD4^{+}$Treg cells in splenic T cells after stimulation with β2-GP1. It is hypothesized that excessive Th1 and Th17 responses and reduced Treg levels are associated with the pathogenesis of APS. Excessive Th17 cell responses induce decidua natural killer (NK) cell activation and impair vascular reactivity of uterine arteries, leading to embryonic resorption (42). Wang et al. also suggested that IL-2 and TNF-α in recurrent miscarriage (RM) with APS peripheral circulation may be involved in the apoptosis of trophoblast cells and activation of NK cells, promoting the development of RPL with APS. Therefore, in RIF with APS, the imbalance of the maternal-fetal immunological microenvironment may be significant. These findings aid in illuminating the molecular mechanisms that connect APS and RIF. However, few researchers have explored the shared pathogenesis of APS and RIF at the genetic level. By combining data from multiple public databases, our study identified candidate diagnostic genes that can serve as biomarkers or potential therapeutic targets for APS and RIF, laying the foundation for determining common mechanisms of APS and RIF and possible clinical treatment methods.

In our study, we focused on four candidate genes to elucidate the relationship between APS and RIF. GATA2, a member of the GATA family of zinc-finger TFs, is classified with GATA1 and GATA3 as "hematopoietic" GATA factors that regulate the development of hematopoietic systems (43). GATA2 protein is crucial for controlling endometrial stromal cell decidualization (44), endothelial cell function and angiogenesis (45), adipocyte differentiation (46), and pituitary function (47). Rubel et al. (48) found that GATA2 is mainly expressed in the uterine luminal and glandular epithelium before embryo implantation and spatio-temporally co-localizes with the progesterone receptor. Mice with uterine-specific ablation of GATA2 displayed inadequate endometrial decidualization, implantation failure, and infertility. Furthermore, knockdown of GATA2 markedly decreased the expression of decidua markers in human endometrial stromal

cells (49). According to these results, normal expression of GATA2 is important for the maintenance of endometrial decidua and support of normal embryonic development. In our study, we found that both APS and RIF had increased GATA2 expression levels. Because endometrial decidualization is a critical step in the acquisition of endometrial tolerance and successful embryo implantation, this demonstrates the close association between GATA2 and female pregnancy and confirms its role in APS and RIF. In addition, GATA2 serves as a positive regulator of the cell cycle by inducing the proliferation of macrophages and endothelial cells (50), which may be a reason for the observed increase in endometrial macrophages in patients with RIF.

In mammals, the MARK family consists of four members: MARK1–MARK4. MARK2 is a serine/threonine protein kinase involved in the phosphorylation of microtubule-associated proteins, such as Tau proteins, cell cycle-regulated phosphatases, and class IIa histone deacetylases, such as HDAC7. MARK2 is localized to the cell membrane and controls microtubule stability by phosphorylating microtubule-associated proteins (51), and engages in the regulation of mammalian immune homeostasis (52), fertility (53), and growth and metabolism (54). Although B and T cells developed normally in MARK-null mice, $CD4^+$ T cells lacking MARK2 were expressed abnormally and produced more INF-γ and IL-4 upon stimulation through the T cell receptor *in vitro* (52). In addition, the response of B cells when attacked by T cell antigens was altered *in vivo*. In addition, MARK2 plays a crucial role in the regulation of prolactin (PRL) secretion in female mice. Because PRL is essential for maintaining luteal function and progesterone secretion, PRL deficiency caused by MARK2 dysfunction may lead to infertility in females (53). Our research revealed that MARK2 expression was elevated in both APS and RIF. Previous studies that have addressed MARK2 expression have only focused on its inhibition. According to our research, the mammalian immune system's stability depends on the MARK2 protein kinase, so aberrant expression could possibly result in autoimmune diseases and pregnancy complications.

CCDC71 and KLRC3 are also two important candidate genes identified in our study. KLRC3 is a member of the natural killer group (NKG) 2, which can specifically regulate humoral and cellular immunity. Although the role of KLRC3 in APS and RIF pathogenesis has not yet been investigated, it is associated with the development of other autoimmune diseases, metabolic diseases, and tumors (55–57). Downregulation of KLRC3 may lead to abnormal expression of the NKG2E receptor, which results in insufficient NK cell activation and defective NK cell function (56). In the mother, NK cells are involved in the invasion of trophoblast cells,

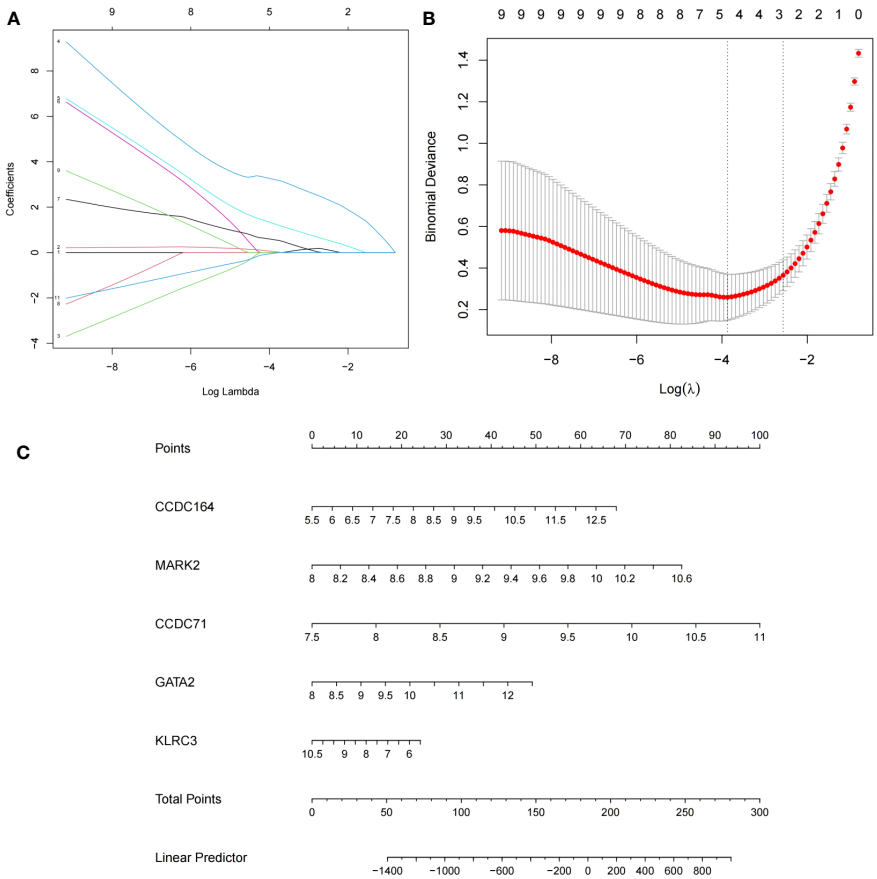

FIGURE 7
Establishment of the nomogram model of repeated implantation failure (RIF) with antiphospholipid syndrome (APS) and validation of these potential candidate biomarkers. **(A)** Tuning feature selection in the LASSO model. **(B)** LASSO regression coefficient profiles. **(C)** The nomogram for diagnosing RIF with APS.

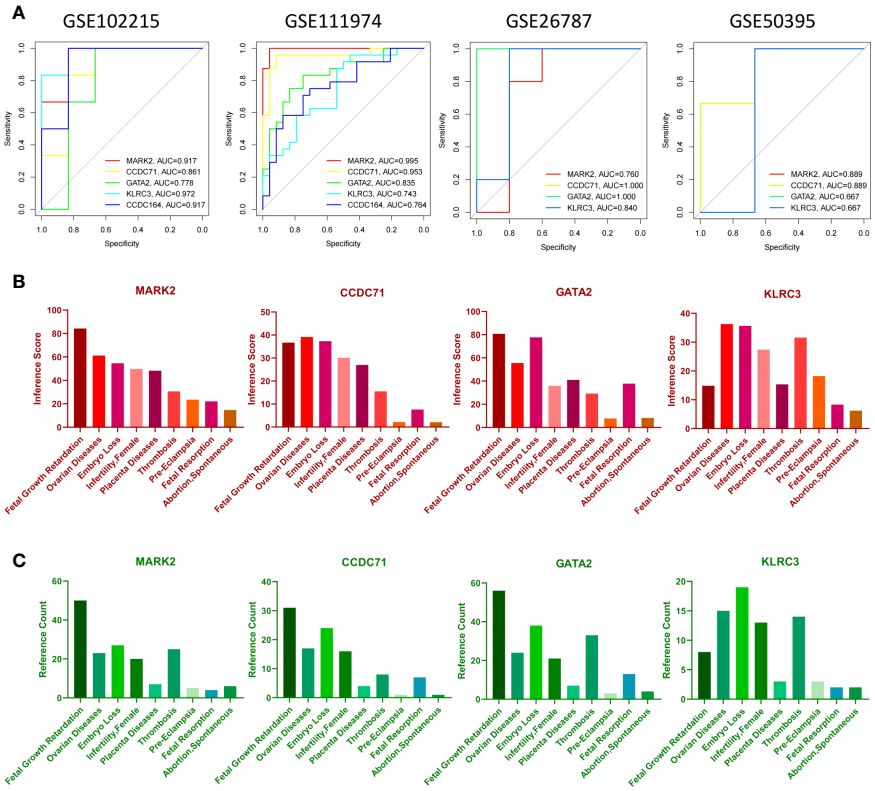

FIGURE 8
Verification of candidate diagnostic genes. **(A)** The receiver operating characteristic (ROC) curve of candidate diagnostic genes (MARK2, CCDC71, GATA2, and KLRC3) in GSE102215, GSE111974, GSE26787, and GSE50359. Inference score **(B)** and reference counts **(C)** between candidate diagnostic genes and fetal growth retardation, ovarian diseases, embryo loss, female infertility, placenta diseases, thrombosis, pre-eclampsia, fetal resorption, and spontaneous abortion in CTD.

remodeling of spiral arteries, secretion of cytokines, and regulation of the immunological balance of the maternal-fetal interface. Alterations in the quantity and function of NK cells, as well as imbalances between NK cells and other immune cells, may result in pathological pregnancies, including pre-eclampsia, RM, and RIF (58). Interestingly, a recent study suggests that insufficient rather than excessive uterine NK activation may contribute to the occurrence of RM and RIF (59), which further supports the impact of RIF with APS. CCDC71 protein, which has coiled-coil domains, is enriched in the nuclear periphery of HeLa and MCF7 cells and generates nuclear foci in U2OS cells (60). Currently, there are few studies on CCDC71 in APS and RIF. Therefore, the relationships between CCDC71, APS, and RIF deserve further exploration.

The results of KEGG analysis suggested that common DEGs play an important role in antifolate resistance, antigen processing and presentation, and progesterone-mediated oocyte maturation. Folic acid, as a one-carbon unit carrier, plays an important role in the prevention of birth defects and in preventing the development of adverse pregnancy outcomes (61). Folate deficiency causes an increase in DNA strand breaks and an increased incidence of errors during DNA replication (61), which in turn causes DNA hypomethylation and interferes with DNA synthesis and damage repair, resulting in DNA strand breaks, altered chromosomal recombination, and segregation abnormalities (62). Progesterone is closely related to the normal development of the embryo and has an important supportive role in early pregnancy. It has been proved by studies to be a specific marker of pregnancy and is of great value in the diagnosis of early pregnancy (63, 64). Our study found that common DEGs were strongly associated with antifolate resistance and progesterone-mediated oocyte maturation. However, further research is needed to determine whether and how much the common DEGs differ in expression levels among RIF patients.

The KEGG analysis results suggest that the immune disorder is the primary mechanism of APS and RIF. Immune cell changes are directly associated with immune homeostasis at the maternal-fetal interface. Abnormalities in immune cell quantity and function appear to significantly link APS and RIF. In our research, γδ T cells were lower in endometrial tissues in RIF than in normal tissues. These findings are consistent with the conclusions reached by Feng et al. (65) However, the number of γδ T cells was increased in the peripheral blood of APS patients. This may be a result of the different tissue types used to analyze the research participants (66), but our results still point to the significance of aberrant γδ T cells in the pathogenesis of APS and RIF. In subsequent work, the mechanisms by which γδ T cells affect endometrial tolerance also need to be further explored to provide more robust evidence for our current study.

Common DEGs are crucial and co-varied genes between APS and RIF, and their impacts on immune cells may be a potential

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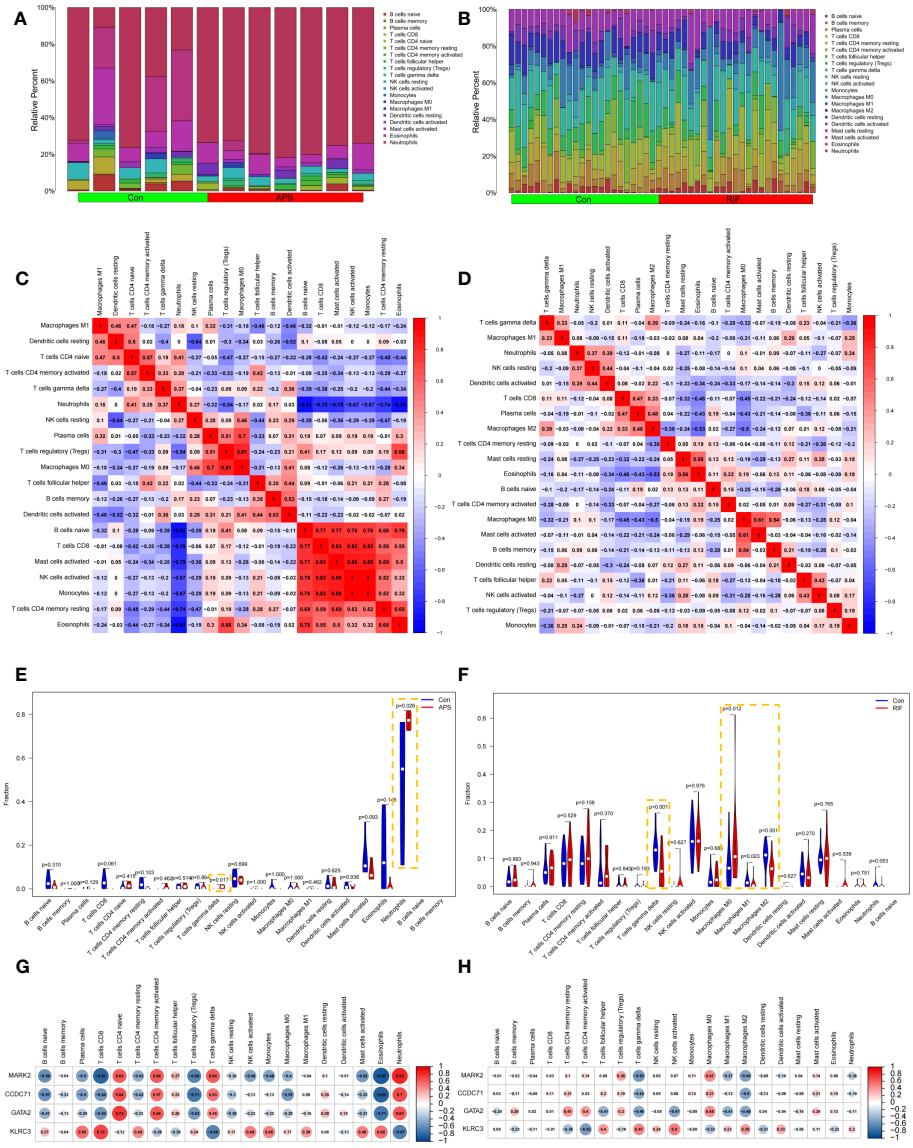

FIGURE 9
Immune infiltration analysis of antiphospholipid syndrome (APS) and repeated implantation failure (RIF). Bar graphs showing the immune infiltration of each sample in APS **(A)** and RIF **(B)**. Heatmaps showing the correlations between immune cells in APS **(C)** and RIF **(D)**. Violin plots showing comparisons of immune cells in APS **(E)** and RIF **(F)**. Correlations between immune cells and candidate diagnostic genes in APS **(G)** and RIF **(H)**.

mechanism for the link between APS and RIF. Our study demonstrated that MARK2, CCDC71, GATA2, and KLRC3 might affect the quantities of γδ T cells and macrophages. This implies that there are increased expression levels of candidate diagnostic genes in circulating immune cells in APS patients, with consequential changes in T cells and macrophages in the endometrium. This will disrupt maternal-fetal immune homeostasis and interfere with embryonic implantation (40), thereby increasing the risk of RIF or worsening RIF symptoms in patients with APS.

Finally, Acetaminophen and Fasudil were predicted as candidate drugs for the treatment of RIF with APS after reviewing the DGIdb database. Evidence suggested that Acetaminophen has negative immunomodulatory effects that can significantly increase the Treg cell population (67) and inhibit T cell-dependent antibody responses (68). Treg cells can assist with regulating maternal vascular function during pregnancy and support normal fetal and placental development, in addition to suppressing inflammation and preventing maternal over-immunity in response to the fetus (69). Through modifying the tolerance balance of T lymphocyte subsets at the maternal-fetal interface, Acetaminophen appears to contribute to the regulation of these maternal over-immune responses. Furthermore, Acetaminophen is less dangerous than other nonsteroidal antiinflammatory drugs because it does not produce severe gastrointestinal bleeding and has no impact on platelet function (70). However, the safe use of Acetaminophen during pregnancy remains a point of contention. Fasudil is another potential candidate drug identified in our study. It is a ras homolog-associated kinase inhibitor that participates in the regulation of T and B cells to achieve anti-inflammatory and

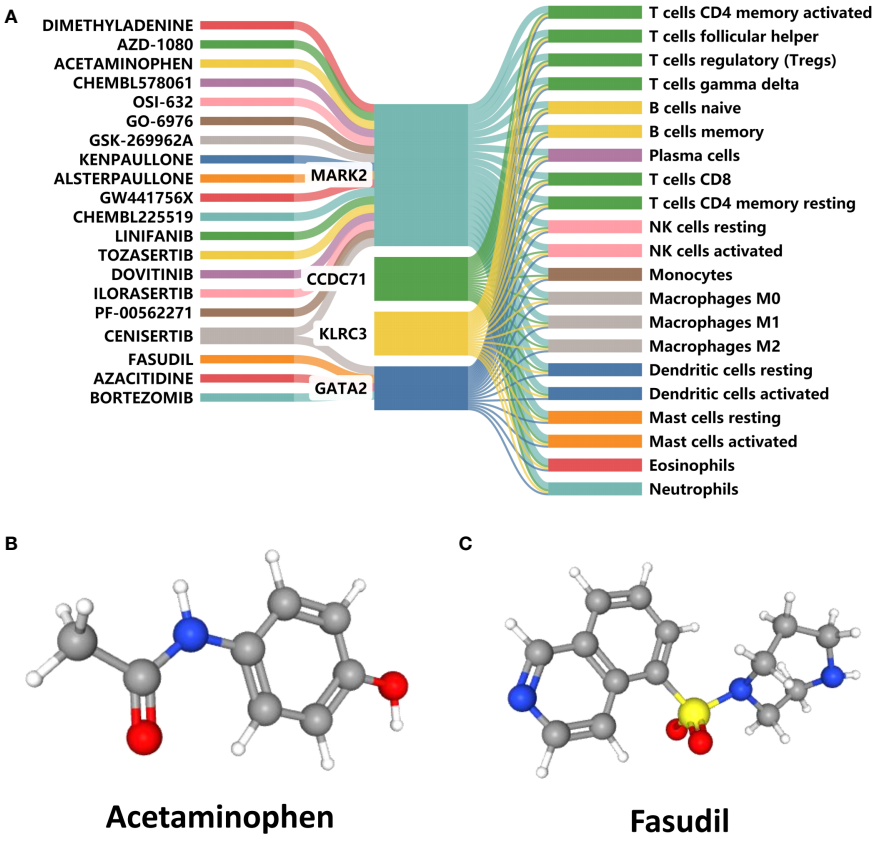

FIGURE 10
Prediction of candidate drugs. **(A)** Sankey diagram demonstrating the flow between candidate drugs, genes, and immune cells. **(B, C)** The 3D structure tomographs of the candidate drugs for repeated implantation failure (RIF) with antiphospholipid syndrome (APS).

immunomodulatory effects (71, 72). Excitingly, Fasudil has been shown to have beneficial effects in a range of cardiovascular and autoimmune diseases (73, 74). This suggests that perhaps Fasudil could play an unexpected role in the treatment of APS. These findings indicated that Acetaminophen and Fasudil might have effects on RIF with APS. In this study, maternal-fetal immune disorders are associated with the development of RIF, indicating that immunotherapy is a prospective treatment modality for RIF and APS. Although drug repurposing is an effective strategy to search for therapeutic candidates, due to these drugs and target genes are only experimental predictions, more evidence and data from extensive animal experiments and clinical trials need to be obtained.

4.1 Strengths and limitations

Our research addresses a gap in previous mechanistic studies and expands new ideas for future research. It is the first study to investigate the functions of common DEGs in APS and RIF and explore their link by bioinformatics. The application of new tools like machine learning and public databases makes our research more comprehensive and novel, and also makes the results more reliable. More importantly, our common DEG-based prediction model has demonstrated good predictive value. These genetic markers can provide higher specificity and sensitivity, especially when they are associated with specific disease subtypes or specific stages of disease progression.Certain genetic markers may be altered in the early or pre-disease stage, and can therefore be used as a tool for early diagnosis to avoid embryo implantation failures.Additionally, we visualized the prediction model, which can be used for clinical diagnosis and treatment. At the moment, the four candidate genes are only in the gene pool, there was no further analysis of the process of expression regulation of these genes, which is the focus of our next work. Because APS is a systemic disease that is limited by ethical and current experimental conditions, only datasets of peripheral blood samples can currently be retrieved, which may affect the results. As our research only used bioinformatics analysis with no animal or clinical experimental validation, further experimental research and clinical trials are needed to validate the biological functions of the candidate diagnostic genes. Furthermore, the safety and efficacy of candidate drugs *in vivo* also need to be verified.

5 Conclusion

In conclusion, through a series of bioinformatics analyses, we identified four key genes (MARK2, CCDC71, GATA2, and KLRC3) that could serve as biomarkers or potential therapeutic targets, and

then established an effective diagnosis model. Meanwhile, the candidate drugs were predicted. These findings may provide a new and powerful scientific foundation for the diagnosis and treatment of RIF with APS.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary materials. Further inquiries can be directed to the corresponding author.

Author contributions

XL and MZ conceived and designed the study. TG and MZ performed data analysis and interpretation. TG and YZ conducted bioinformatics analyses and created the figures and tables. MZ wrote the first draft, and XL and YZ conceptualized and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1126103/full#supplementary-material

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