Understanding human immunity in idiopathic Recurrent Pregnancy Loss

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Abstract

Miscarriage, defined as the loss of a pregnancy before a viable gestation, affects 1 in 6 couples. Recurrent pregnancy loss (RPL), defined as two or more miscarriages, affects up to 1.9% of couples. The physical, psychological, and financial impact of miscarriage can be substantial. However, despite its multifactorial etiology, for up to 50% of couples a reason behind this condition cannot be identified, termed ‘idiopathic RPL’. Much recent research has strived to understand this, with immune dysregulation being a source of particular interest. In this short review we summarize the current evidence on the complex role of the immune system both pre- and early post-conception in RPL. A key question is whether systemic peripheral blood markers, in particular natural killer cell and T cells, may be utilized to accurately predict and/ or diagnose those pregnancies at high risk of loss. Given the invasive nature of endometrial testing, identification of reliable peripheral immune biomarkers is particularly appealing. Clinical trials using potent immunomodulatory agents, including intravenous immunoglobulin, donor leukocyte immunization, and tumor necrosis factor (TNF)-α inhibitors, have been undertaken with the primary objective of preventing miscarriage in women with RPL. Standardisation of both diagnostic and prognostic immune cell testing assays is required to permit accurate identification of those women who may benefit from immunomodulation. Prompt clarification is required to meet the increasing expectation from couples and clinicians, as without these advancements women are at risk of exposure to potent immune-therapies and subsequent studies are at risk of failure, generating further controversy regarding the role of immune dysregulation in women with RPL. Through this review we highlight clear gaps in our current knowledge on immune activity in RPL.

Keywords: Recurrent pregnancy loss, miscarriage, pregnancy, immune system, natural killer cells

Introduction

Miscarriage is defined in the UK as the spontaneous loss of a fetus prior to the 24th gestational week(1, 2). Recurrent pregnancy loss (RPL), also termed recurrent miscarriage (RM), defined by the European Society of Human Reproduction and Embryology as two previous losses, affects 0.7%-1.9% of women(2, 3). Importantly, women with are are at increased risk of serious obstetric complications, including fetal growth restriction, preterm birth, and stillbirth(2). The potentially serious psychological impact of this condition is also highly significant, with significant risk of post-traumatic stress disorder, anxiety, and depression following RPL(2, 4, 5).

RPL is multi-factorial, with a range of contributory factors now understood as summarized in Table 1. For ~50% of women, the underlying pathogenesis of RPL is however unknown and the condition is termed 'idiopathic' (6, 7). Furthermore, even though 'idiopathic' RPL is defined by the lack of an identifiable underlining reason, it remains unclear whether idiopathic and non-idiopathic cases are exclusively underpinned by discrete pathogenic mechanisms. For the purpose of this review, 'idiopathic RPL' describes couples for which underlying maternal comorbidities and/or genetic aberration has been excluded prior to, or alongside immunological investigation. We discuss current revised concepts regarding idiopathic RPL, with immune dysregulation at the forefront of much recent research activity.

Table 1. Factors contributing to the etiology of recurrent pregnancy loss (RPL)

Factors contributing to RPL etiology
Maternal demographics (age>35 years, higher order loss, and obesity)(8, 9)
Environmental (smoking, alcohol consumption, high caffeine intake, cocaine use, organic solvent exposure, vitamin D insufficiency)(10-13)
Maternal comorbidities (e.g. hereditary and acquired thrombophilia, including antiphospholipid syndrome, thyroid disease, diabetes mellitus, and infections, such as *chlamydia trachomatis*, *ureaplasma*, and *mycoplasma*, which can potentially induce chronic endometritis(14-20)
Genetic abnormalities (parental chromosomal abnormalities in 5.7% of couples with RPL and fetal aneuploidy in cases of early spontaneous miscarriages)(21, 22)
Male factors (microdeletions of the Y chromosome and sperm DNA fragmentation may be involved in the etiology of RPL, although routine analysis is not currently recommended)(1, 3, 23)

In human pregnancy, immune cell activity appears highly dynamic with a plethora of pro- and anti-inflammatory responses contributing to many critical reproductive processes including implantation, trophoblast invasion, spiral artery remodeling, fetal growth, and parturition(24, 25). The "implantation checkpoint hypothesis' suggests that multiple immune checkpoints exist at distinct menstrual cycle stages, highlighting the need for an endometrium that is both receptive and selective for embryo implantation(7). Aberrant immune activity in RPL may result in an excessively receptive, but poorly selective, endometrium, thereby permitting implantation of "low-quality" embryos with impaired growth and survival potential(7, 26). On the other hand, improper decidualization, potentially due to aberrant progesterone signalling, could also result to an immune environment unable to support "high-quality" embryo implantation and development(7, 27).

The immunological dialogue occurring at the fetal-maternal interface within the endometrium and later the maternal decidua is fascinatingly complex involving a diverse array of immune cells and mediators. Immune cell recruitment appears important, with the maternal decidua enriched with a unique innate and adaptive leukocyte infiltrate; comprising 70% uterine Natural Killer cells (uNKs), 20%-30% macrophages, and 3%-10% T Cells(28-30). Decidual immune cells actively interact with invasive fetal-derived extravillous trophoblast cells (EVTs)(28), which are negative for Human Leukocyte Antigen (HLA)-A, -B, and -D and HLA class II, which likely permits protection from T cell-mediated cytotoxicity. Positive expression of HLA-C, HLA-E, and HLA-G, thereby also facilitates protection from NK cell-mediated attack through inhibitory Killer-cell Immunoglobulin-like (KIR), Natural Killer Group (NKG)2, and Immunoglobulin-Like Transcript (ILT) receptor-mediated interactions(28, 31-37). Importantly, fetal-maternal interactions are measurable in peripheral maternal blood, with HLA-negative fetal syncytiotrophoblast cells shown to interact with circulating maternal immune subsets during hemochorial placentation(38-40). Although these cells are possibly subject to NK cell killing, defensive mechanisms such as soluble HLA-G secretion have been postulated to control peripheral NK (pNK) responses(41, 42).

Given the difficulties of invasive uterine sampling, a key question is whether dysregulated uterine immune cell activity is reflected by peripheral immune cell frequencies and function pre-conception and/or during early pregnancy. The relationship between endometrial and decidual uNK subsets and their peripheral counterparts remains of great interest to those seeking reliable biomarkers/prognostic tests. Different theories regarding uNK origin, including pNK recruitment to the decidua and de novo development of decidual NK (dNK) cells from endometrium-resident progenitors, exist(43). This is similarly true for adaptive subsets, with modifications in $CD4^+$ and $CD8^+$ T cell populations measured in the uterus compared to peripheral blood(44). Whether cytokine secretion and activity differ between the uterus and periphery has again generated much interest, with significant efforts to characterise differences in both healthy and high-risk reproductive cohorts(45, 46). As shall be discussed, high variation in the methodology and eligibility criteria in previous reports has almost certainly contributed to conflicting data, thereby generating questions regarding the significance of immunological alterations observed RPL. In this review, we aim to present current emerging concepts regarding the phenotype and function of specific innate and adaptive immune cell subsets in normal pregnancy and RPL, as summarised in text and tabular forms. This includes evidence regarding more novel immune subsets which may generate future interest. We also present a current overview of immunological tests and potential therapeutic strategies.

Innate immunity in RPL

NK cells

NK cell numbers

Due to their high prevalence in the uterine lymphocyte population, reaching up to 90%, NK cells have been classically associated with reproductive success(47), with key roles in early uterine artery remodeling, trophoblast invasion and placentation identified(28). It is however postulated that uncontrolled NK cell endometrial recruitment and/or failed $CD56^{dim}$ cell conversion to less cytotoxic $CD56^{bright}$ cells may occur in women with RPL. Increased anti-fetal-placental cytotoxicity and more intense induction of angiogenesis may confer a deleterious, high oxidative stress environment(48). Although an increase in both pNK and uNK cells, in particular cytotoxic $CD56^{dim}$ ones, has been linked to RPL, the predictive accuracy of measuring NK cell titers remains unclear with significantly heterogenic findings obtained (Table 2). These differences could simply reflect cohort or methodological inconsistencies, but it is also possible that NK cell elevation is only present in some women with idiopathic RPL. A systematic review on the prognostic value of pNK and uNK cell testing in regards to pregnancy outcome in women with infertility and RPL concluded that lack of a consensus on the definition of "normal"/"abnormal" NK cell test results is a primary factor contributing to these heterogenic findings(49).

NK cell cytotoxicity

NK cell activity also appears dysregulated in women with RPL, although current evidence is conflicting with both reduced and increased cytotoxicity reported(50, 51)(Table 2). Elevated cytotoxicity could reflect a local anti-fetal immune activity but also a systemic response to increasing circulating volumes of fetal-derived trophoblast microparticles. Conversly, reduced pNK and uNK cytotoxicity could indicate a possible association between early pregnancy loss and defective decidual $CD56^{+}$ leukocyte activity(52). The cytotoxic activity of uNK cells in women with RPL has not been thoroughly examined, possibly due to difficulties in isolating these cells without affecting their activation status. Additional experiments and alternative markers to more conclusively define NK cell cytotoxicity are required to establish the validity of these heterogenic findings.

NK cell cytokine production

Although most studies have utilized NK cell cytotoxicity as a marker of NK cell activity, cytokine production may also be important. During pregnancy, a local and peripheral anti-inflammatory, pro-regulatory shift in NK cell cytokine production is described and likely contributes to pregnancy maintenance. In women with RPL, even prior to conception, NK cytokine secretion appears dysregulated (Table 2). Of particular interest are IFN-γ and TNF-α, both of which may exert key roles in human pregnancy. IFN-γ is considered important for pregnancy establishment, being involved in the decidual vessel remodeling, while a protective role of TNF-α has also been displayed(53), with control of trophoblast invasion described(54). Albeit important during initial implantation, excessive or imbalanced cytokine production may be deleterious, with dysregulated levels postulated as important within the context of RPL. Overall, evidence supporting the use of cytokine profiling in women with RPL is highly heterogenic and is not routinely recommended in current clinical practice(55).

Table 2. Evidence of the role of Natural Killer (NK) cells in human pregnancy and recurrent pregnancy loss (RPL). Theories regarding the alterations in NK cell numbers, cytotoxicity, and cytokine production in normal pregnancy and RPL are displayed separately in the respective sections of the table. CD: cluster of differentiation, EVTs: extravillous trophoblast cells, IFN-γ: interferon-gamma, IL: interleukin, pNK: peripheral NK, TGF-β: transforming growth factor-beta, TNF-α: tumor necrosis factor-alpha, uNK: uterine NK

<table>
<tr><th colspan="2">Natural Killer (NK) cells</th></tr>
<tr><td colspan="2">NK cells accumulate in the luteal phase endometrium and early-pregnancy decidua, progressively disappearing from mid-gestation onwards, being absent at term(28, 56)
→ potential role in preparation for and during early pregnancy</td></tr>
<tr><td colspan="2">Two main subpopulations present in both peripheral blood and uterus(57-60):
• $CD56^{dim}CD16^{+}$: Major circulatory subset; primarily cytolytic activity
• $CD56^{bright}CD16^{-}$: Most prevalent in uterus; lower cytotoxic ability and a higher capacity for cytokine production</td></tr>
<tr><td colspan="2">Normal pregnancy:</td></tr>
<tr><td colspan="2">• Dense uNK cell infiltrate surrounds fetal trophoblasts, coinciding with trophoblast invasion(28, 56, 61)
• uNK cells do not kill EVTs(62, 63)
• NK cells' activating receptor interaction with their ligands on trophoblasts results in the production of growth and angiogenic factors, cytokines, chemokines, and matrix metalloproteinases(64-67)</td></tr>
<tr><td colspan="2">Key roles in: A) angiogenesis and spiral artery remodeling, B) trophoblast growth, differentiation, and invasion, C) placental tissue building and remodeling
<u>But</u> NK cell function in pregnancy remains unclear</td></tr>
<tr><td colspan="2">NK cell numbers</td></tr>
<tr><td>Normal pregnancy:</td><td>Women with RPL:</td></tr>
<tr><td rowspan="2">• 1^{st} and 2^{nd} trimester compared to non-pregnant: No difference in pNK cell numbers(68)</td><td>• Pre-conception and during pregnancy: ↑ pNK cells(69-73)
• In pregnant and non-pregnant (incl. idiopathic): ↑ $CD56^{dim}$ and thus ↓ $CD56^{bright}/CD56^{dim}$ pNK cell ratio(71, 72)
• <u>But</u> unaltered pNK cell numbers and $CD56^{bright}/CD56^{dim}$ NK ratio(74)</td></tr>
<tr><td>↑ Peripheral blood $CD56^{+}$, $CD56^{dim}$, and $CD56^{bright}$ cell levels measured as predictive biomarker for subsequent loss(71, 75-77)</td></tr>
<tr><td>• Early pregnancy: significant NK cell infiltration in the uterus(28, 61)</td><td>• In pregnant and non-pregnant (incl. idiopathic): ↑ uNK cell infiltration(78, 79)
• In non-pregnant women with idiopathic miscarriage: ↑ $CD56^{dim}$ and ↓ $CD56^{bright}$ uNK cell frequencies(78, 80)
• <u>But</u> meta-analysis found no significant change in uNK cell frequencies(69)</td></tr>
<tr><td colspan="2">NK cell cytotoxicity</td></tr>
<tr><td>Normal pregnancy:</td><td>In women with RPL:</td></tr>
<tr><td rowspan="3">1^{st} to 3^{rd} trimester: ↑ activated $CD56^{bright}$ pNK cell percentage(81)</td><td>• Pregnant and non-pregnant: ↑ pNK cell cytotoxicity(50, 70, 73)
• ↑ Activated pNK cell levels(72, 82, 83)
• <u>But</u> in pregnant and non-pregnant: unaltered(84) or ↓(85) pNK cell cytotoxicity</td></tr>
<tr><td>↑ Pre-conception pNK cell activity suggested as a prognostic miscarriage marker(86)</td></tr>
<tr><td>• In pregnant: ↑ activated $CD56^{+}$ uterine cell levels(87)</td></tr>
<tr><td colspan="2">NK cell cytokine production</td></tr>
<tr><td colspan="2">NK cells secrete Type-1 (TNF-α and IFN-γ), Type-2 (IL-5 and IL-13), and immunosuppressive (IL-10 and TGF-β) cytokines(88):
• Type-1 cytokine-secreting pNK cells are the major subpopulation at the non-pregnant state</td></tr>
<tr><td>Normal pregnancy:</td><td>In women with RPL:</td></tr>
<tr><td>• ↓ IFN-γ production by pNK cells(89)
• ↑ $IL\text{-}10^{+}$ $CD56^{dim}$ and $CD56^{bright}$ pNK cell levels(88)</td><td>• In non-pregnant women with idiopathic RPL: ↑ $IFN\text{-}\gamma^{+}TNF\text{-}\alpha^{+}$ and ↓ $IL\text{-}4^{+}IL\text{-}10^{+}$ $CD56^{bright}$ pNK cells(74)</td></tr>
<tr><td>• High levels of $TGF\text{-}\beta^{+}$ $CD56^{dim}$ and $CD56^{bright}$ uNK cells(88)</td><td>• <u>But</u> in non-pregnant women with idiopathic RPL: ↓ prevalence of $TNF\text{-}\alpha^{+}$ and/or $IFN\text{-}\gamma^{+}$ uNK cells(90)</td></tr>
</table>

NK cell memory

Even though NK cells are considered an innate immune subset, an ability to form immunological memory (mNK) has been displayed [Reviewed in (91)]. mNK cells have been most extensively studied in the context of cytomegalovirus (CMV) infection, where they persist after initial infection and display an enhanced antigen-specific response upon secondary challenge(92-94). Antigen-specific memory responses have been exhibited by liver-resident NK cells(95), while cytokine-induced mNK cells were also described(96).

It is now understood that the risk of a subsequent miscarriage increases with each subsequent loss(2). It has been hypothesised that during an index pregnancy, NK cell “priming” occurs in the decidua, resulting in a more effective response in any subsequent pregnancy(97). Pregnancy-specific mNK cell generation independent of CMV-induced alterations has been suggested(98). Even though the origin and role of these cells remains to be elucidated, pregnancy-trained mNKs may be generated in response to signals in the uterine microenvironment during the first pregnancy, with more effective production of VEGFα and IFN-γ to support local spiral artery remodelling and angiogenesis in subsequent pregnancies(65, 99, 100). Whether aberrant mNK responses are linked to pregnancy loss and RPL is yet to be elicited but of certain interest.

Antigen-presenting cells

Dendritic Cells (DCs) and macrophages, have a key role in antigen recognition and presentation and are involved in the orchestration of lymphocyte-mediated immunity. There is now evidence to suggest peripheral and decidual DCs may support pregnancy success (Table 3). For instance, prior to trophoblast invasion peripheral DCs may promote the overall inflammatory stage required for pregnancy establishment without inducing anti-fetal immunity. Due to their very low frequency in the uterus, the role of DCs in reproductive success has not been extensively studied and thus any potential role in RPL is not known.

Monocytes and macrophages are also gaining increased research interest. Macrophages, which mainly arise from circulating monocytes upon tissue migration(101), represent one of the major effector immune populations. Using a three-dimensional *in vitro* system, monocyte/macrophage recruitment in response to trophoblast signals has now been displayed, indicating a possible mechanism for monocyte migration from the peripheral blood to the uterus during pregnancy, enhancing the local macrophage population(24). Characteristically, macrophages are recognised for their ability to phagocytose dead and potentially harmful cells, bacteria, and debris(101). During menstruation, key roles in tissue degradation, clearance of local debris, and possibly endometrial regeneration and angiogenesis have been shown(102). Although the exact function of macrophages in pregnancy appears complex, with gestation-specific pro- and anti-inflammatory functions reported, establishment of early blastocyst implantation is amongst those actions described(27, 103)(Table 3). Dysregulated macrophage acitivty has been reported in other malplacentation disorders, like preeclampsia and preterm birth(104). Whether alterations in monocyte or macrophage numbers or activity contribute to immune dysregulation in idiopathic RPL remains to be explored.

Table 3. Evidence of the role of antigen-presenting cells (APCs) in human pregnancy and recurrent pregnancy loss (RPL), including information on the numbers and activity of dendritic cells (DCs) and macrophages (Mφs), displayed in the respective sections of the table. CCR2: C-C chemokine receptor type 2, CD: cluster of differentiation, EVTs: extravillous trophoblast cells, FasL: Fas ligand, HLA: human leukocyte antigen, IL: interleukin, MMPs: matrix metalloproteinases, uNK cells: uterine Natural Killer cells

<table>
<tr><th colspan="2">Antigen-presenting cells (APCs)</th></tr>
<tr><td colspan="2">Dendritic cells (DCs)</td></tr>
<tr><td colspan="2">Low frequency in endometrium and early pregnancy decidua (~1.7% of decidual leukocytes)(105, 106)</td></tr>
<tr><td>Normal pregnancy:</td><td>In the peripheral blood of women with RPL:</td></tr>
<tr><td>1st and 2nd trimester: ↑ peripheral blood DCs compared to the non-pregnant state(107, 108)</td><td>In women with miscarriage(107):
• ↓ DC capacity for cytokine production
• ↑ Numbers of mature DCs</td></tr>
<tr><td>Peripheral blood DCs(107, 108):
• ↑ Mature and activated DCs
• <u>But</u> a state of incomplete DC activation has been suggested, potentially leading to defects in T cell activation through antigen presentation</td><td>During the later stage of fetal growth DC activity could promote anti-fetal T cell-mediated responses, through antigen presentation, and thus pregnancy loss(109)</td></tr>
<tr><td rowspan="3">Decidual DCs possibly adopt an immature tolerogenic profile, possessing a decreased T cell stimulatory capacity(106, 110)</td><td>In the decidua of women with RPL:</td></tr>
<tr><td>1st trimester: mature DC decrease observed during the 7th-11th week of healthy pregnancy not detected, suggesting a potential association between RPL and mature DC activity(111)</td></tr>
<tr><td>Defective senescent DC clearance by uNK cells during decidualization could be associated with RPL(112)</td></tr>
<tr><td colspan="2">Macrophages (Mφs)</td></tr>
<tr><td>Normal pregnancy:</td><td>In the uterus of women with RPL:</td></tr>
<tr><td>Second largest immune cell population in early pregnancy decidua (20%-30% of leukocytes)(30)</td><td>In idiopathic spontaneous miscarriage(113) but not RPL(114): ↑ decidual Mφs</td></tr>
<tr><td>Prior to EVT invasion: Mφs may cooperate with uNK cells, mediating the disruption and disorganization of endothelial and vascular smooth muscle cells, and thus contributing to vascular remodeling through the secretion of MMPs and the phagocytosis of apoptotic cells(115)</td><td>Endometrial Mφs ↑ in women with idiopathic RPL and subsequent miscarriage(78)</td></tr>
<tr><td>• M2 phenotype is considered as essential during pregnancy(104, 116), with immunomodulatory agents like IL-10 being expressed by decidual Mφs(97).
• <u>But</u> M1/M2 dichotomy appears an oversimplification, with the balance of different pro- and anti-inflammatory Mφs likely dependent upon the stage of pregnancy(117).</td><td rowspan="3">Polarization towards the pro-inflammatory M1 phenotype may promote pregnancy complications(104, 116)</td></tr>
<tr><td>Shifts in Mφ activity during pregnancy(104, 117):
a) Peri-implantation period: pro-inflammatory M1 activity
b) Placental development: anti-inflammatory M2 predominance
c) Prior to parturition: pro-inflammatory M1 predominance</td></tr>
<tr><td>Tolerogenic M2 Mφ recruitment and polarization is thought to be influenced by hormones like estrogen and progesterone(116)</td></tr>
<tr><td>FasL expression by Mφs possibly mediates apoptosis of Fas+ activated T cells, eliminating T cell-mediated anti-fetal immunity and controlling trophoblast invasion(113)</td><td>↑ FasL expression by Mφs could promote excessive trophoblast apoptosis, leading to pregnancy loss(113, 118)</td></tr>
</table>

Adaptive immunity in RPL

T cells

T cells constitute a central component of adaptive immunity with both cytotoxic and immunomodulatory potential. In the peripheral blood and decidua, T cells are thought to acquire an important role in fetal antigen recognition and modulation of local immunity (Table 4). As shall be discussed, enhanced activity of these cells and a shift in the balance between T cell subsets may be involved in miscarriage pathogenesis.

Cytotoxic T cells

Cytotoxic T (Tc) cells are central in the defense against intracellular pathogens. They are capable of killing infected and malignant cells through multiple mechanisms, including cytokine secretion, cytotoxic granule release, and death receptor interactions(119). The exact role of Tc cells in pregnancy is unclear. Although the absence of HLA-A, -B, and -D expression on EVTs highlights a potential mechanism to evade Tc-mediated cytotoxicity, HLA-C expression possibly permits trophoblast recognition by Tc cells(120). Tc cells at the fetal-maternal interface could be less cytotoxic, facilitating local inflammatory processes such as implantation, without attacking the fetus. Heterogenic data has to date been collected in regards to Tc cell involvement in RPL (Table 4), hence, the value of measuring T cell frequencies and activity in RPL currently remains limited with further research required.

Table 4. Evidence of the role of total and CD8⁺ cytotoxic T (Tc) cells in human pregnancy and recurrent pregnancy loss (RPL). Information about total and cytotoxic T cells are displayed separately, in the respective table sections. APCs: antigen-presenting cells, CD: cluster of differentiation, EVTs: extravillous trophoblast cells, GM-CSF: granulocyte-macrophage colony-stimulating factor, HLA: human leukocyte antigen, NK cell: Natural Killer cell, PD-1: programmed cell death protein 1, Tim-3: T-cell immunoglobulin and mucin-domain containing-3

T cells	
Abundant in endometrium(46) with constant numbers throughout menstruation(121)	
Normal pregnancy:	**In women with RPL:**
Unaltered prevalence in peripheral blood(122)	• ↑ Peripheral T cell activation(123, 124) • <u>But</u> no significant alterations in T cell levels in the peripheral blood, endometrium, or decidua(78, 114, 125)
In uterus during early pregnancy: constant absolute numbers but ↓ prevalence due to NK cell surge(121)	
↑ in uterus throughout pregnancy(126): • 1st trimester: 5%-20% of lymphocytes • Late pregnancy: 40%-80% of lymphocytes	Predictive value: • ↓ Peripheral T cell numbers in women with subsequent miscarriage(127) • ↑ Endometrial CD4⁺/CD8⁺ T cell ratio in women with idiopathic RPL and subsequent miscarriage(80)
CD8⁺ cytotoxic T (Tc) cells	
Major immune effector subset, being capable of killing infected and tumor cells through a variety of mechanisms, including cytolytic granule formation, cytokine secretion, and death receptor interactions	
Normal pregnancy:	**In women with RPL:**
• Activation marker expression by uterine Tc cells suggests fetal antigen recognition(128) • Fetal antigen-specific Tc cells detected in maternal periphery(129) • Recognition of the fetus directly through HLA-C expression on fetal EVTs and indirectly through maternal APCs(120)	• ↑ Peripheral Tc cell levels and ↓ CD4⁺/CD8⁺ T cell ratio(130) • <u>But</u> ↓ peripheral Tc cell levels and unchanged CD4⁺/CD8⁺ cell ratio(73)
Trophoblast antigen-specific tolerance may be induced via upregulation of death receptors on maternal Tc cells(120, 131): • ↑ Tim-3⁺PD-1⁺ Tc prevalence in decidua compared to peripheral blood implying partial local suppression of Tc cell activity	• Non-pregnant women with idiopathic RPL: ↑ activated Tc prevalence in peripheral blood(132) • <u>But</u> in non-pregnant women with idiopathic RPL: unaltered peripheral Tc activation status(133)
<u>But</u> Tc lymphocytes may contribute to implantation via the secretion of colony-stimulating factors (incl. GM-CSF)(134)	

T helper cell cytokine profiling

The balance between the T helper (Th)1 and Th2 cytokines is considered important in guiding the immune responses during pregnancy. Direct effects of Th cell-associated cytokines in fetal development and survival have been described. Most cytokines are growth and/or differentiation factors, and thus could encourage fetal growth(135). As summarized in Table 5, a “Type-1/Type-2” hypothesis in both healthy pregnancy and miscarriage has been suggested, with a dominant Type-2 anti-inflammatory cytokine production contributing to pregnancy success, and a pro-inflammatory Type-1 ‘anti-fetal’ shift contributing to pregnancy loss, with overproduction of IFN-γ and TNF-α shown to induce abberant trophoblast apoptosis(54, 136). This appears an over-simplification however, since Th1 cytokines, including IFN-γ and TNF-α, also have key roles in local spiral artery remodeling (53, 100), and fetal protection (137). Further large, well-designed studies will be required to understand the significance of this shift (Table 5). It is possible a dynamic cytokine balance may be important, with the effects of individual pro- and anti-inflammatory cytokines dependent upon their relative concentration and the gestation stage(54, 138, 139). Strategies to modulate cytokine balance, like progesterone, could represesnt a valuable option to protect against RPL(140), but mechanistic data is required to confirm this.

Regulatory T cells

Regulatory T cells (Tregs) comprise a Th cell subpopulation, considered as a central immunosuppressive component of the adaptive immune system. A vital role for Tregs in pregnancy, particularly in the second trimester, has been suggested(141)(Table 5). Tregs may regulate excessive inflammation and induce fetal antigen-specific tolerance from early pregnancy(120). Experimental evidence indicates that defects in peripheral and decidual Treg numbers or activity could be linked to the pathogenesis of RPL, although the role of individual Treg subtypes requires further examination(142-144).

Table 5. Evidence of the role of CD4⁺ T helper (Th) cells, including regulatory T cells (Tregs), in human pregnancy and recurrent pregnancy loss (RPL). Information about the different T cell subtypes are displayed in the respective table sections. CD: cluster of differentiation, GM-CSF: granulocyte-macrophage colony-stimulating factor, HLA: human leukocyte antigen, IFN-γ: interferon-γ, IL: interleukin, Mφ: macrophage, NK cell: Natural Killer cell, TGF-β: transforming growth factor-β, TNF: tumor necrosis factor

T cells	
CD4⁺ T helper (Th) cell cytokine profiling	
Four major subsets(45, 135, 145, 146): • Th1: secrete pro-inflammatory cytokines (incl. IL-1, IL-2, IFN-γ, TNF-α, and TNF-β) and stimulate cellular immunity • Th2: secrete anti-inflammatory cytokines (incl. IL-4, IL-5, IL-10, IL-12, and IL-13) and activate humoral immunity • Th17: secrete pro-inflammatory cytokines (incl. IL-17, GM-CSF, IL-21, and IL-22) • Tregs: characterized by anti-inflammatory IL-10 secretion (discussed separately)	
Normal pregnancy:	**In women with RPL:**
Unaltered prevalence in peripheral blood (~40% of lymphocytes)(142)	Idiopathic RPL(132, 133): • ↑ Peripheral activated Th cell prevalence • <u>But</u> appeared anergic
	Th shift in the endometrium: ~11% of local cells in women with RPL, while ~3% in healthy women(78, 80)
• "Th1/Th2 hypothesis": Shift towards Th2-mediated responses, accompanied by ↓ Th1 cell function, reflected by a cytokine expression shift in the periphery and placental tissue(135, 147, 148) • Extended into the “Type-1/Type-2 hypothesis” representing the balance between pro-inflammatory Th1 and Th17 (Type-1) and anti-inflammatory Th2 and Treg (Type-2) activity	"Th1/Th2 hypothesis": shift towards Th1 responses, possibly directed against paternal trophoblast antigens(54, 147-151)
	• In peripheral blood of pregnant and non-pregnant: ↑ Type-1 cytokine production (incl. IL-1β, IL-12, IL-17, IL-18, IL-23, IFN-γ, TNF-β, and TNF-α)(147, 148, 150-153) • In the peripheral blood and decidua: ↓ Type-2 cytokine secretion, including low IL-10(148-152)
	Association with IL-10 promoter polymorphisms and polymorphisms linked to ↑ IFN-γ and TNF-α production(154)
Peripheral blood(153): • ↑ IL17⁺ Th cell prevalence in the 1st, compared to the 2nd trimester • <u>But</u> unaltered IFN-γ⁺ Th cell prevalence throughout pregnancy	• Pre-pregnancy: ↑ TNF-α⁺/IL-10⁺, TNF-α⁺/IL-4⁺, and IFN-γ⁺/IL-4⁺ ratios(155) • During the 1st trimester and at the time of miscarriage: pro-inflammatory shift in Type-1/Type-2 cytokines ratios(150, 151) • <u>But</u> in other similarly powered studies: no significant alterations in IL-12, IL-4, IL-13, TGF-β, and IFN-γ concentrations(122, 152)
Peripheral blood: ↓ IFN-γ and TNF-α and ↑ IL-10 level linked to pregnancy success(156)	↑ Type-1 cytokine production may promote T cell-, NK cell-, and Mφ-mediated cytotoxicity against fetal cells(45, 157, 158). This can be controlled by Type-2 cytokines(45, 54).
Regulatory T cells (Tregs)	
Normal pregnancy:	**In women with RPL:**
From the early pregnancy: Tregs may regulate excessive inflammation and induce fetal antigen-specific tolerance(120)	During miscarriage: ↓ decidual Treg concentrations(142-144)
Early pregnancy: ↑ peripheral blood Treg prevalence(141-143)	In healthy early pregnancy but not idiopathic RPL: Treg levels ↑ in decidua compared to peripheral blood(143, 144)
2nd compared to 1st trimester: ↑ peripheral blood Treg prevalence(141)	Pre-conception, 1st trimester, and during miscarriage: ↓ peripheral blood Treg levels(142-144)
Treg recruitment and local differentiation is promoted by trophoblasts(159)	Predictive value: low peripheral blood Treg levels during early pregnancy linked to miscarriage(160)
Post-partum: ↓ Treg levels, agreeing with withdrawal of fetal-induced stimulation(141)	In peripheral blood CD4⁺ cell cultures of women with idiopathic RPL: ↓ Treg-mediated suppression of IL-17 production(161)

T cell memory

Following the primary immunological response towards a pathogenic stimulus, a small percentage of T cells persists and differentiates into memory cells(162). As summarised in Table 6, central (T_{CM}) and effector (T_{EM}) T cell memory appears important in the establishment of tolerance to fetal-derived antigens(163). Further studies are required to understand the potential implication of T cell memory in healthy pregnancy and pregnancy-associated complications. It would be valuable to access the paternal antigen-specificity of these cells in first and subsequent pregnancies of the same and of different paternal backgrounds as well as examine their pro- or anti-inflammatory properties.

Table 6. Evidence of the role of T cell memory in human pregnancy and recurrent pregnancy loss (RPL). CCR7: C-C chemokine receptor type 7, CD: cluster of differentiation, CTLA-4: cytotoxic T-lymphocyte-associated protein 4, FoxP3: forkhead box protein P3, IFN-γ: interferon-γ, IL: interleukin, LAG-3: lymphocyte-activation gene 3, PD-1: programmed cell death protein 1, Th cell: T helper cell, Tim-3: T-cell immunoglobulin and mucin-domain containing-3

<table>
<tr><th colspan="2">T cells</th></tr>
<tr><td colspan="2">T cell memory</td></tr>
<tr><td colspan="2">• Central memory T (T_{CM}) cells: reside to the lymph nodes, exhibit limited effector function, and upon secondary antigen exposure they proliferate and differentiate into Th1 or Th2 cells under local cytokine influence(44, 162, 164)
• Effector memory T (T_{EM}) cells: reside in peripheral tissues and upon secondary antigen exposure they rapidly respond through the secretion of perforin, IFN-γ, IL-4, and IL-5, displaying limited proliferative ability(44, 162, 164)</td></tr>
<tr><td>Normal pregnancy:</td><td>In women with RPL:</td></tr>
<tr><td>Paternal antigen-specific memory T cell generation: possibly contribute to tolerance generation, although their exact contribution to pregnancy is unclear(129, 165)[Reviewed in (163)]</td><td>Unchanged memory-like $CD4^+$ and $CD8^+$ T cell frequencies in the peripheral blood(124)</td></tr>
<tr><td>Unaltered peripheral blood $CD4^+$ T_{CM} population(166-168)
• ↑ after parturition, suggesting persistence of cells(167)
• ↑ activation during and after pregnancy(167)</td><td rowspan="2">Non-pregnant: ↑ total and $CD4^+$ T_{CM} cells in peripheral blood(123, 169)</td></tr>
<tr><td>Unaltered peripheral blood $CD8^+$ T_{CM} population(166-168)
• Unaltered activation during and after pregnancy(167)</td></tr>
<tr><td>Peripheral blood $CD4^+$ T_{EM} population:
• ↑ at the 2nd and 3rd trimesters and after parturition(166, 167)
• <u>But</u> in one study found to be unaltered at the 3rd trimester(168)
• ↑ activation and reduced susceptibility to apoptosis(166, 167)</td><td rowspan="3">Non-pregnant: ↑ total, but not $CD4^+$, T_{EM} cells in peripheral blood(169)</td></tr>
<tr><td>Peripheral blood $CD8^+$ T_{EM} population:
• Unaltered during pregnancy but increased after parturition(166-168)
• Unaltered activation and susceptibility to apoptosis(166, 167)</td></tr>
<tr><td>In pregnant women with a male fetus: T_{EM} cells dominate the peripheral blood fetal HY-specific $CD8^+$ T cell population, exhibiting a functional inflammatory response to HY peptide presentation(129)</td></tr>
<tr><td>In the decidua: $CD8^+$ cells compose the major T cell population(121) with T_{EM} cells constituting the major $CD8^+$ T cell subset(165)</td><td rowspan="2">Endometrial $CD8^+$ memory T cells(170):
• Unaltered prevalence
• Unaltered IFN-γ production upon stimulation</td></tr>
<tr><td>Decidual T_{EM} cells:
• Unusual cytokine profile (IFN-γ^{high}IL-4^{high}) and ↓ perforin and granzyme B expression compared to peripheral blood(44, 165)
• High proliferative activity(131)
• ↑ CTLA-4, Tim-3, LAG-3, and PD-1 checkpoint protein expression(44, 165), indicating a strict control of local activity</td></tr>
</table>

B cells

Despite the small uterine B cell numbers, dysregulation of humoral immunity may be associated with RPL. B cells are thought to contribute to pregnancy success through the downregulation of poly-reactive natural antibody secretion and the production of protective blocking asymmetric antibodies. B cells with regulatory activity and distinct memory subsets have also been described, with potential roles in reproductive success presented. Due to the small number of uterine B cell, the properties of these B cells have not been extensively studied (Table 7).

Table 7. Evidence of the role of B cells in human pregnancy and recurrent pregnancy loss (RPL), including the role of different subsets, antibodies, and B cell memory. Information about total B cells, B cell subtypes and their antibody production, and B cell memory are displayed in the respective table sections. AAb: asymmetric Ab, Ab: antibody, Bregs: regulatory B cells, CD: cluster of differentiation, IL: interleukin, NAb: natural antibody, RA: rheumatoid arthritis, SLE: systemic lupus erythematosus, Th: T helper

B cells	
Normal pregnancy:	**In women with RPL:**
During pregnancy cell-mediated autoimmunity, like RA, is ameliorated, while antibody-mediated autoimmunity, such as SLE, is often aggravated(46, 171, 172), suggesting a role of humoral immunity in pregnancy	Pre-conception B cell frequencies in the peripheral blood were unaltered(123)
Endometrium and decidual: low B cell levels(173, 174)	↑ Endometrial $CD20^+$ B cell levels(80)
• The role of B cells in early pregnancy is unclear • In the 3rd trimester and during parturition peripheral B cell lymphopenia is reported(175), indicating an absence of B cell involvement in the later pregnancy stages	↑ Endometrial B cell prevalence in women with RPL and a following 1st-trimester miscarriage, compared to those with an intact conceptus at least until the 36th week of their following pregnancy(80)
B cell subsets and antibody (Ab) production	
3 major B cell subsets(176-178): • B-1 cells detected in peripheral and umbilical cord blood(179): produce low-affinity polyreactive, often autoreactive, NAbs in the absence of exogenous immunization(177, 179, 180). Auto-Ab production is thought to be mediated by $CD5^+$ B-1 cells(181, 182), although $CD5^-$ NAb-producing B cells were also detected(183) • B-2 "conventional" cells: upon antigen recognition can differentiate into high-affinity Ab-secreting plasma or memory cells(184) • Bregs: mainly characterized by their IL-10 secretion(176, 185). IL-10-producing Bregs inhibit Th1 activation and Th17 differentiation and promote $CD4^+$ T cell conversion into Tregs, promoting tolerance(178, 186)	
Normal pregnancy:	**In women with RPL:**
B cell subsets have been poorly defined in the context of pregnancy	B cell subset number alterations not associated with miscarriage
↓ Circulating $CD5^+$ B cell prevalence is during pregnancy, indicating a possible protective mechanism through ↓ NAb production(187)	Failure to limit NAb production has been linked to pregnancy complications but not RPL(176, 177)
Protective effect of maternally-derived anti-paternal Abs during pregnancy suggested(177): • Pregnancy-protective Ab production by human term placenta-derived B cells upon stimulation reported(173). • Anti-paternal Ab AAbs, binding antigen but lacking the capacity for immune effector activation(176), could acquire a protective blocking role(188).	• Anti-paternal or anti-firstborn HLA Ab presence was linked to ↓ likelihood of subsequent live birth(189). • ↓ AAb production detected in the placenta and maternal serum in RPL, compared to normal pregnancy(177, 190-192)
Bregs may contribute to the maintenance of tolerance during pregnancy through their IL-10 secretion(176).	Human Bregs not studied in the context of RPL
B cell memory	
Upon antigenic stimulation, a small B cell percentage forms a persisting memory population that upon secondary stimulation with the same antigen secretes large amounts of high-affinity, class-switched, monospecific antibodies(176). B cell memory is attributed to the B-2 subset(176, 184).	
Normal pregnancy:	**In women with RPL:**
• 3rd trimester and at the day of delivery: ↓ peripheral blood memory B cell counts, compared to non-pregnant controls(175). • 6 weeks post-partum: ↑ memory B cell percentages in the peripheral blood, compared to the 3rd trimester(175).	B cell memory not studied in the context of RPL

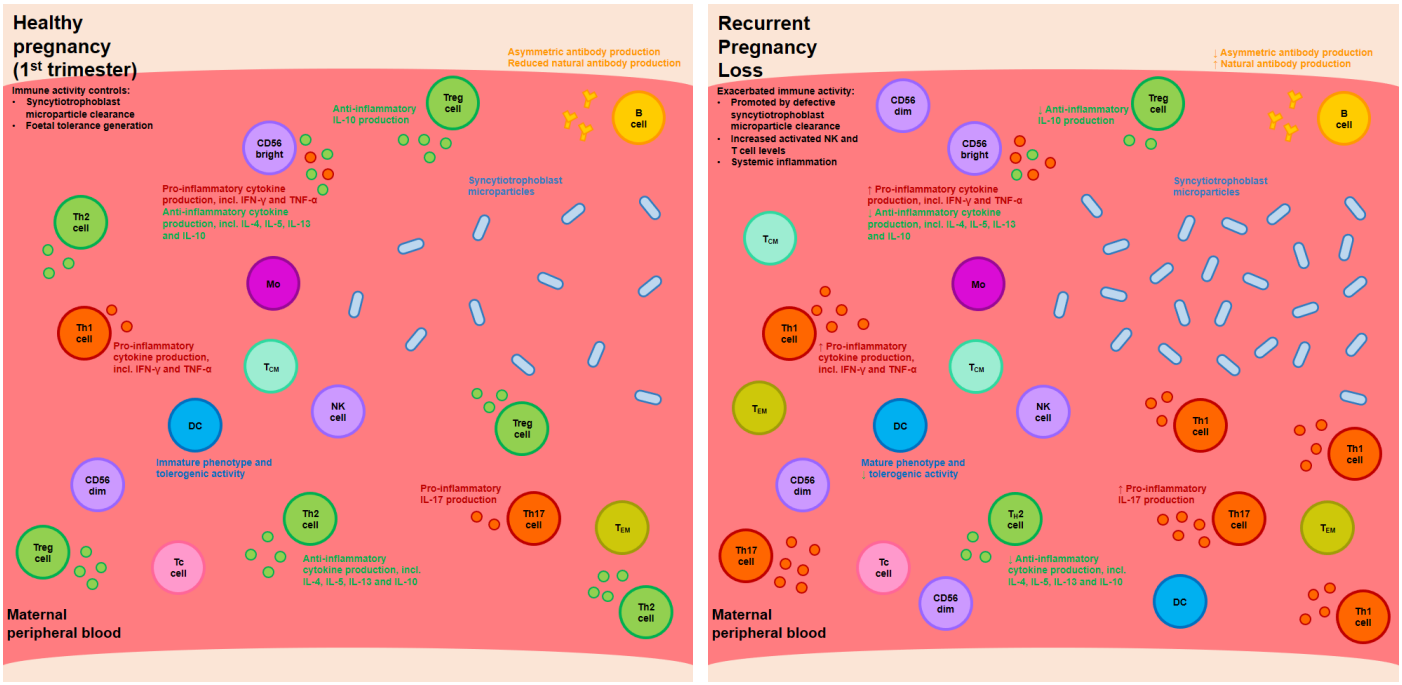

Figure 1. Overview of peripheral immunity during the first trimester in women with healthy pregnancy and women with recurrent pregnancy loss. CD: cluster of differentiation, DC: dendritic cell, IFN: interferon, IL: interleukin, Mo: monocyte, NK cell: natural killer cell, Tc cell: cytotoxic T cell, T_{CM}: central memory T cell, T_{EM} cell: effector

(coloured in print)

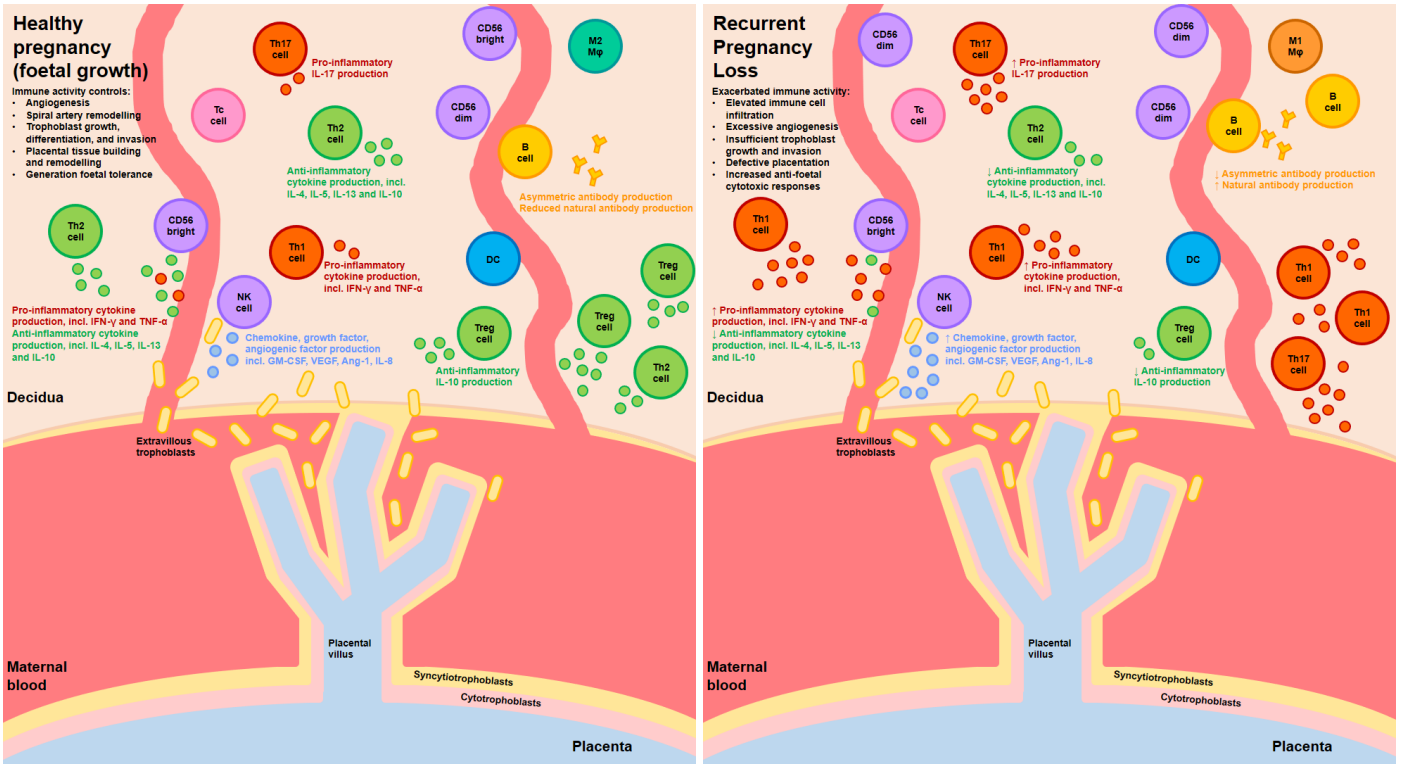

Figure 2. Overview of decidual immunity during healthy pregnancy, at the stage of foetal growth, and in women with recurrent pregnancy loss. Ang-1: angiopoietin 1, CD: cluster of differentiation, DC: dendritic cell, GM-CSF: granulocyte-macrophage colony-stimulating factor, IFN: interferon, IL: interleukin Mφ: macrophage, NK cell: natural killer cell, Tc cell: cytotoxic T cell, Th cell: T helper cell, TNF: tumor necrosis factor, *Treg cell: regulatory T cell, VEGF: vascular endothelial growth factor*

(coloured in print)

Immunological testing in idiopathic RPL - progress and challenges

Immune cell testing is without question an expanding field with the exciting potential to offer important diagnostic and prognostic information for couples with RM. For reasons clearly outlined within this review, at present no accepted immune-based tests exist outside of the research setting(3). Enumeration of circulating cytotoxic and helper T, B, and NK cells and assays evaluating the Th cell-associated cytokine production and the cytotoxic activity of NK cells may provide important prognostic markers, however taking these tests forward from 'bench to bedside' requires further validation. The comparative value of these tests also remains to be elucidated(55, 64). Albeit beyond the scope of this review, we suggest that only then should targeted immunomodulatory treatment trials, such as human immunoglobulins (IVIgG), paternal/donor leukocyte immunization, and/ or TNF-α inhibitors, be performed(193, 194).

NK cell testing has gained particular interest. Although commercially available, international consensus regarding "normal" NK cell ranges is required. High-quality prospective case-control studies with sufficient power to account for confounding factors such as age(195) are required in both healthy controls and women with RPL. The key role of NK cells in implantation and vessel remodeling has been highlighted in the present review. However, the suggested changes in their numbers and activity in RPL remain uncertain. Hence, the role of these cells in RPL should be fully elucidated prior to targeting immune activity for therapeutic purposes. Similarly, the value of cytokine testing therefore remains contentious due to data heterogeneity(156, 196) and lack of a universal definition of "increased" Type-1/Type-2 ratios.

An important factor contributing to experimental finding variation is the methodological differences between studies. Apart from the lack of a universal RPL definition, leading to diverse inclusion criteria, the timing of immunological testing remains inconsistent. Immune cell variation during the menstrual cycle and upon pregnancy establishment has been observed, possibly due to the influence of hormone level fluctuation, including progesterone, estrogen, and human chorionic gonadotropin(197, 198). Studies accounting for menstrual cycle phase may also be informative in order to accurately identify pre-conception immune cell markers in women with RPL.

Variation can also be seen between studies examining the total RPL population and those focusing on women with the idiopathic form of this condition. The pathogenesis of non-idiopathic RPL may include co-morbidities like antiphospholipid syndrome and infection, which are underlined by specific patterns of immune dysregulation. Thus, inclusion of women with non-idiopathic RPL may predispose towards the detection of immune abnormalities. To the contrary, it remains uncertain whether idiopathic and non-idiopathic cases are underlined by the same immunological pathogenesis (7). In addition, RPL may be underlined by a multifactorial pathogenic mechanism, with different factors including ethnicity, BMI, and male parameters likely contributing to the reproductive outcome. Moving forward it is important to investigate the total RPL population, with well-powerd idiopathic and non-idiopathic subpopulations analysed separately. This would also help determine the optimal timing of immunological testing in clinical practice, i.e. in relation to other diagnostic tests performed.

Sampling methods are also of certain importance and interest. Although uterine endometrial/decidual sampling poses the opportunity to characterise the local immune environment, the introduction of an invasive

endometrial/decidual procedure is not without risk. Peripheral blood tests offer a less invasive, favourable option, however their suitability remains to be fully validated in terms of RPL pathogenesis and prognostic accuracy. Moving forward, paired blood-uterus samples would help bridge this knowledge gap.

Given the potential psychological impact of miscarriage, the impact of stress upon circulating immune cell types, including NK and T cells, is of probable significance(4, 5, 199, 200). The effectiveness of supportive care for women with idiopathic RPL is already shown(201). Studies measuring the value of supportive care upon immune cell function in women with RPL remain to be established.

Final Remarks

Immune dysfunction is associated with idiopathic RPL. Prospective, well-powered studies to elucidate the exact mechanisms underlying this association and evaluate the diagnostic and prognostic capacity of immunological testing are urgently required. We anticipate this will inform potential future targeted immunotherapies. Albiet beyond the scope of this review, given the potential side-effects and risks of some of these therapies, well conducted pre- and post-conception research studies should precede their routine clinical implementation in the management of idiopathic RPL(202). Current trials, examining the use of antibitotics, steroids, and biologics are expected to offer a new insight on the management of RPL(203-205).

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