Cellular Immune Responses in the Pathophysiology of Preeclampsia

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Summary sentence

Innate and adaptive immune cell responses in women with preeclampsia

Short title

Cellular Immune Responses in Preeclampsia

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Key words

Pregnancy, hypertensive disorders of pregnancy (HDP), gestational hypertension, immune cell, T cell, NK cell, regulatory T cell, neutrophil, natural killer cell, macrophage, monocyte

ABBREVIATIONS

APC: antigen presenting cell

cDC: conventional DC

CM: central memory

CTB: cytotrophoblast

DC: dendritic cell

EM: effector memory

EVT: extravillous trophoblast

HELLP: hemolysis, elevated liver enzymes, and low platelet count syndrome

IL: interleukin

iNKT: invariant Natural Killer T cell

iTreg: induced Treg

IUGR: intra-uterine growth restriction

KIR: killer cell immunoglobulin-like receptor

KLR: killer cell lectin-like receptor

LPS: lipopolysaccharide

LTi cell: lymphoid tissue inducer cell

mDC: myeloid DC

MDSC: myeloid-derived suppressor cell

NET: neutrophil extracellular trap

NK cells: Natural killer cell

NKT: Natural killer T cell

nTreg: natural Treg

PBMCs: peripheral blood mononuclear cells

pDC: plasmacytoid DC

ROS: reactive oxygen species

RTE: recent thymic immigrant

RUPP: reduced uteroplacental perfusion

STBM: syncytiotrophoblast microparticles

TCR: T-cell receptor

TLR: Toll-like receptor

Treg: regulatory T cell

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ABSTRACT

Preeclampsia, defined as new-onset hypertension accompanied by proteinuria occurring at 20 weeks of gestation or later, is a leading cause of perinatal morbidity and mortality worldwide. The pathophysiology of this major multi-systemic syndrome includes defective deep placentation, oxidative stress, endothelial dysfunction, the presence of an anti-angiogenic state, and intra-vascular inflammation, among others. In this review, we provide a comprehensive overview of the cellular immune responses involved in the pathogenesis of preeclampsia. Specifically, we summarize the role of innate and adaptive immune cells in the maternal circulation, reproductive tissues, and at the maternal-fetal interface of women affected by this pregnancy complication. The major cellular components involved in the pathogenesis of preeclampsia are regulatory T cells, effector T cells, natural killer cells, monocytes, macrophages, and neutrophils. We also summarize the literature on those immune cells that have been less characterized in this clinical condition, such as γδ T cells, invariant natural killer T cells, dendritic cells, mast cells, and B cells. Moreover, we discuss *in vivo* studies utilizing a variety of animal models of preeclampsia to further support the role of immune cells in this disease. Finally,

we highlight the existing gaps in knowledge of the immunobiology of preeclampsia that require further investigation. The goal of this review is to promote translational research leading to clinically relevant strategies that can improve adverse perinatal outcomes resulting from the obstetrical syndrome of preeclampsia.

INTRODUCTION

Preeclampsia is one of the most common pregnancy complications, occurring in 3-5% of all pregnancies [1-3], and is considered one of the "Great Obstetrical Syndromes" [4, 5]. Preeclampsia is diagnosed as new-onset hypertension (≥140/90 mmHg) accompanied by proteinuria occurring at 20 weeks of gestation or later [6]. Preeclampsia can escalate to eclampsia, characterized by new-onset seizures [7, 8]. Yet, even if eclampsia does not occur, mothers with preeclampsia are at increased risk of stroke [9, 10], acute cardiovascular complications [11, 12], and hemolysis, elevated liver enzymes, and low platelet count (HELLP) syndrome [13], among others. Preeclampsia can also directly affect the fetus by causing fetal distress, fetal growth restriction, and even fetal death [14, 15]. Moreover, preeclampsia is associated with long-term adverse outcomes for the mother, such as increased risk of cardiovascular disease [16, 17], as well as for the offspring [18]. Therefore, preeclampsia is one of the primary causes of maternal and neonatal morbidity and mortality worldwide.

Preeclampsia has been proposed to occur in two distinct stages [19-21]. The first is represented by poor placentation and defective remodeling of the spiral arteries, resulting in insufficient placental circulation [19-22]. The second stage results from such poor blood flow and is characterized by a placental stress response [19-21]. This chain of events culminates in a systemic maternal response that manifests as preeclampsia [19-21]. Thus, the pathophysiology of preeclampsia

is largely driven by the placenta, as evidenced by the fact that delivery of the placenta is the only effective treatment for this condition [2]. In addition, the systemic effects of preeclampsia are thought to result from the release of mediators by the placenta, which includes soluble factors [e.g. soluble FMS-like tyrosine kinase 1 (sFlt-1) and endoglin] [23-25], trophoblast-derived extracellular vesicles and/or microparticles [26, 27], and reactive oxygen species (ROS) [28]. Such mediators exert specific immune functions, contributing to the maternal systemic inflammatory state associated with preeclampsia [29, 30]. Indeed, a large body of research has demonstrated that preeclampsia is characterized by quantitative and qualitative modifications of both systemic and local immune cell responses. Therefore, understanding the complex cellular immune changes and interactions underlying the pathophysiology of preeclampsia may foster research focused on the development of immune therapeutic strategies to tackle this syndrome.

In this review, we provide a comprehensive overview of the cellular immunology of preeclampsia, given the large body of evidence implicating immune cells in the pathogenesis of this great obstetrical syndrome. Herein, we summarize prior reports of innate and adaptive immune cells in the circulation, reproductive tissues, and at the maternal-fetal interface of women with preeclampsia. The maternal-fetal interface includes multiple anatomically-distinct sites of immunological interaction between the mother and fetus: the decidua basalis, where maternal cells interact with the fetal extravillous cytotrophoblast; the intervillous space, where circulating maternal immune cells are in contact with the fetal syncytiotrophoblast; and the interface between the decidua parietalis and amniochorion [31, 32]. We have focused our attention on the immune cell subsets that are thought to be most involved in the pathogenesis of preeclampsia such as regulatory T cells, effector T

cells, and natural killer (NK) cells; yet, we also summarize literature on those subsets that have been less characterized in this clinical condition (Figure 1). Such research includes relevant studies demonstrating the putative functional contributions of different immune cell subsets to the pathogenesis of preeclampsia as well as their interactions with other physiological processes. Moreover, we lay out an overview of *in vivo* studies utilizing a variety of animal models of preeclampsia to offer further mechanistic evidence of immune cellular involvement in this pregnancy complication. Through this review, we provide insight into the immune cellular mechanisms of preeclampsia and potentially highlight gaps in knowledge that can drive future research.

THE ROLE OF ADAPTIVE IMMUNE CELL SUBSETS IN PREECLAMPSIA

Regulatory T cells

Regulatory T cells or 'Tregs' are specialized CD4+ T cells with immunosuppressive activity that are best characterized by their expression of the forkhead/winged-helix transcription factor 3 (Foxp3), which is essential for their development and function [33, 34]. Under physiological conditions, Tregs carry out important functions as part of the mechanisms of peripheral tolerance through the suppression of aberrant T-cell activation [35, 36]. During pregnancy, unique and specific immune interactions take place between the mother and the semi-allogeneic fetus, a concept that is referred to as "maternal-fetal tolerance" [37-39]. At the molecular level, maternal-fetal tolerance arises from maternal immune recognition of fetal antigens, which activates a series of immunological mechanisms to regulate subsequent maternal antigen-specific responses. Tregs are a central component of such mechanisms of maternal-fetal tolerance [40-47], not only throughout pregnancy

but also as part of successful implantation [45, 48, 49] and even prior to fertilization [50, 51].

Overall, current literature suggests that preeclampsia occurs in the context of a systemic and local reduction in Treg numbers and/or proportions. Several studies have consistently demonstrated a reduced presence of Tregs in the placental bed [52] and decidua [53, 54] of women with preeclampsia, although one report did not observe such a reduction [55]. In addition to cellular measurements, tissue-based gene expression analyses have also demonstrated a downregulation of Treg-related transcription factors such as *FOXP3* and *GATA3* in the decidua or placenta of women with preeclampsia compared to those with normotensive term pregnancies [56-58]. Thus, preeclampsia seems to be characterized by an overall reduction of Tregs in the decidua and placenta, and such deficiency is mirrored by a concomitant increase in effector T cells [53, 58], particularly T helper (Th)17 cells [54], as well as the upregulation of Th1-associated molecules such as *TBET* [56, 58]. Notably, early-onset preeclampsia (preeclampsia diagnosed at <34 weeks of gestation) seems to be characterized by a more severe decrease in the proportion of decidual Tregs compared to late-onset preeclampsia [53], suggesting differences between the pathophysiology of these two disease subsets.

Several potential explanations for the local reduction of Tregs in women with preeclampsia have been proposed. First, there may be reduced differentiation of decidual Tregs due to defective signaling and/or antigen presentation by local antigen presenting cells (APCs) [59]. This concept is supported by the demonstration that peripheral DCs from women with preeclampsia exhibit greater capacity for promoting Th1/Th17-like T-cell responses *in vitro* compared to those from healthy pregnant women [60], and that altered circulating DCs were associated with

decreased proportions of peripheral Tregs in women with preeclampsia [61] and with elevated proportions of Th17 cells in women with early-onset preeclampsia [62]. Second, the impaired formation of decidual lymphatic vessels described in preeclampsia may prevent the migration of immune cells into this compartment [63]. Indeed, decidual lymphatic vessel density was shown to correlate with decidual Treg numbers, and thus it is possible that in preeclampsia the routes by which circulating Tregs enter the decidua are impeded [63]. Lastly, recent investigation of the T-cell receptor (TCR) repertoires of Tregs in the decidua revealed that the fraction of clonally-expanded Tregs was significantly decreased in women with preeclampsia [64], possibly indicating a larger issue of impaired mechanisms of maternal-fetal tolerance.

Consistent with the observed local deficiency of Tregs, decreased numbers of circulating Tregs [52, 61, 65-79] and CD8+CD25+Foxp3+ cells [80] have been reported in women with preeclampsia, although several studies did not observe differences [55, 81, 82], and such cells may also display lower suppressive capacity [55, 66, 70, 73, 78]. The changes in circulating Tregs are reflected by elevated proportions of conventional T cells, particularly Th17 cells [73, 75, 77, 79], as well as by the reduced *FOXP3* expression and IL-35 levels accompanied by elevated *RORC* expression and IL-17A levels in women with preeclampsia [83]. In particular, women with early-onset preeclampsia show increased disparity between CD4+ T cells expressing *RORC* and those expressing *FOXP3* compared to those with late-onset disease or healthy pregnancies [84]. The enhanced Th17-like responses observed in women with preeclampsia may not be solely driven by Th17 cells in all cases, but could also involve type 3 innate lymphoid cells (ILC3s) that have similar cytokine profiles and functions [85]. Regardless of origin, the administration of soluble IL-17

receptor C (IL-17RC) in a rat reduced uteroplacental perfusion (RUPP) model of preeclampsia was shown to at least partially mitigate the effects of an aberrant Th17 response [86]. Thus, the combination of reduced Tregs and inefficient suppression may result in a systemic environment that encourages aberrant Th17-dominant inflammation in women with preeclampsia.

One question that arises from the study of Tregs concerns the contributions of thymic/natural Tregs and peripheral/induced Tregs to pregnancy success, a topic that was recently reviewed [87]. Deficiency of conserved noncoding sequence 1 (CNS1), a *Foxp3* element with a prominent role in peripheral Treg generation, increased immune cell infiltration of the placenta and caused fetal loss [46], suggesting an important role for peripheral Tregs in pregnancy maintenance. More recent studies have indicated that the generation of thymic Tregs continues during pregnancy [88, 89], although this concept is still controversial [90]. Notably, in women with preeclampsia, the proportions of circulating recent thymic immigrant (RTE) and mature naïve Tregs were decreased while that of memory Tregs was elevated, suggesting that the differentiation process of circulating Tregs may be impaired [90, 91]. Further supporting these observations, the proportions of $CD25^{hi}$ Tregs were increased in women with preeclampsia while $Foxp3^{hi}$ Tregs decreased, and it was suggested that the $Foxp3^{hi}$ subset represents natural or thymic Tregs whereas the $CD25^{hi}$ subset may be induced from peripheral naïve T cells [66]. Moreover, another report showed that the proportions of naïve Tregs were decreased while those of CD45RA-$HLA\text{-}DR^{hi}$ and CD45RA-$HLA\text{-}DR^{lo}$ Tregs increased in preeclampsia [70], providing evidence that naïve Tregs may undergo an accelerated maturation or differentiation towards a highly suppressive phenotype [92, 93] in women with this condition. Finally, reports have indicated a general

reduction of Tregs [72] or the loss of specific subsets [94] in women with preeclampsia. Taken together, these studies point to the altered differentiation of circulating Tregs as a hallmark of the pathophysiology of preeclampsia. This concept has been recently strengthened by high-dimensional longitudinal studies investigating the dynamics of peripheral Tregs in women with preeclampsia [95]. Notably, signatures corresponding to Tregs and effector T cells stood out as being differentially modulated in women with preeclampsia [95]. Specifically, STAT5 signaling in Th1 cells was consistently decreased in women who ultimately developed preeclampsia [95], which is notable since the IL-2/STAT5 pathway has been implicated in T helper [96] and Treg [97] differentiation, and may also inhibit Th17 differentiation [98]. In addition, p38 signaling (required for the suppressive function of Tregs [99]) was increased in Tregs from women with normal pregnancies, but not those with preeclampsia [95]. These observations provide a more translational perspective on Treg dysfunction and Treg/Th17 imbalance in women with preeclampsia, and demonstrate potential for the use of specific signatures in the maternal circulation to evaluate preeclampsia.

The immune checkpoint PD-1/PD-L1 pathway has also been implicated in the aberrant Treg responses observed in women with preeclampsia [54]. Peripheral Tregs from women with preeclampsia demonstrated higher expression of PD-1 together with reduced expression on Th17 cells compared to those from normal pregnancies [54]. I*n vitro* experiments revealed that inhibition of the PD-1/PD-L1 pathway promoted the expression of *RORC* and *IL17A* by Tregs, and supplementation with PD-L1 Fc skewed naïve CD4+ T-cell differentiation towards a Treg phenotype [54]. Similar findings were obtained using a rat model of L-N^G-Nitroarginine methyl ester (L-NAME)-induced preeclampsia, in which the

administration of PD-L1-Fc restored the imbalanced Treg/Th17 ratio and, more importantly, improved fetal outcomes [75]. Thus, dysfunctional or inhibited PD-1/PD-L1 pathway signaling may contribute to the Treg/Th17 imbalance observed in women with preeclampsia. In addition to the PD-1/PD-L1 pathway, the altered expression of apoptotic molecules may predispose Tregs to premature deletion, thereby contributing in part to their diminished numbers in women with preeclampsia [71]. The proportion of Tregs expressing the anti-apoptotic molecule Bcl-2 was greatly reduced in women with preeclampsia compared to normal pregnancies, while the intensity of pro-apoptotic Bax expression was highly increased on those Tregs displaying this molecule [71]. These findings suggest that Tregs may be more susceptible to apoptotic cell death in the context of preeclampsia, which is in tandem with the reduced numbers of Tregs found in this clinical condition.

Several *in vivo* studies have explored methods of boosting maternal Treg populations and/or function as part of ongoing efforts to find safe and effective treatments for preeclampsia [100, 101]. Treatment with an anti-CD28 “superagonist” has been tested in rats using both angiotensin-renin and RUPP models [100, 101]. In the former, anti-CD28 treatment resulted in an expansion of Tregs in the placenta, spleen, and periphery of affected rats and improved fetal outcomes [100], whereas in the latter model, increased proportions of circulating Tregs were observed together with elevated systemic concentrations of IL-10 and transforming growth factor β (TGFβ) without significantly improving neonatal weight [101]. Using the same RUPP rat model, the infusion of IL-10 [102] or the adoptive transfer Tregs derived from healthy pregnant rats [103] both improved maternal symptoms; yet, no benefits to impaired fetal growth were observed. Thus, while treatments that directly promote Treg expansion or function may have short-term benefits for women with

preeclampsia, additional investigation to determine potential fetal/neonatal benefits is required.

Taken together, the evidence presented above demonstrates that a deficiency or imbalance of Tregs may contribute to the pathophysiology of preeclampsia. Tregs have been typically considered as most important in early pregnancy; indeed, the depletion of Tregs in early pregnancy causes preeclampsia and fetal loss. Yet, the studies summarized herein indicate that the adverse effects of impaired or defective Tregs can be detected throughout pregnancy. In line with this concept, we recently demonstrated that the loss of Tregs in late gestation causes preterm birth together with fetal growth restriction and bradycardia without altering umbilical or uterine arterial blood flow [104]. Moreover, the loss of Tregs causes dysregulation of multiple cellular and developmental processes in the placenta [104], providing further evidence that Tregs play an important role in fetal development in late pregnancy. Yet, whether Tregs represent a viable target for treatment in the second or third trimester, after the diagnosis of preeclampsia, remains unclear. It is likely that such a treatment may be most effective earlier in gestation; yet, given the demonstrated importance of Tregs for continued fetal growth, targeting Tregs in late pregnancy may also provide some benefits. Regardless, further research is required to determine whether therapies to boost Tregs or enhance their functions could be attainable in women with preeclampsia.

Effector T cells

Preeclampsia is characterized by a systemic and local imbalance between Tregs and effector T cells, resulting in a pro-inflammatory state that favors Th1/Th17 cell activity. Yet, the details of how effector T cells are dysregulated are less clear.

Here, we focus on reports of the general CD4+ and CD8+ effector T-cell populations in women with preeclampsia as well as changes in Th1/Th2 balance, given that multiple Treg studies described above simultaneously reported on Th17 responses.

Studies have indicated that the systemic effector T-cell pool is enhanced in women with preeclampsia, either in terms of increased numbers/proportions or a higher degree of activation [105-109]. The proportions of circulating CD4+ T-cells were increased in women with preeclampsia compared to those with healthy pregnancies [105], which is consistent with the observation that the proportion of Th1 cells (and subsequently the Th1/Th2 ratio) is also higher in this disease [106]. This increase in CD4+ T cells may be driven by the expansion of memory T cells, as the proportion of this subset was higher (and that of naïve T cells reduced) in women with preeclampsia compared to normal pregnant women [107]. Besides CD4+ T cells, studies have also indicated dysregulation of CD8+ T cells in women with preeclampsia compared to normal pregnancies [108]. Moreover, CD8+ T cells demonstrated enhanced cytotoxic activity in women with preeclampsia compared to those from non-preeclamptic women [109], potentially due to the loss of Treg-mediated suppression (as described above). Consistent with such enhanced cytotoxic activity, the fraction of circulating microparticles derived from cytotoxic T cells was significantly increased in women with preeclampsia compared to non-pregnant controls [110].

Together with the reported increases in peripheral T-cell populations and cytotoxic function, lymphocytes from women with preeclampsia were shown to display higher intracellular free Ca^{2+} [111], and CD3+ T cells showed greater expression of pyruvate kinase [112], suggesting enhanced activation of circulating T cells. The expression of the Th1- or Th17-associated transcription factors Tbet and

RORγt was increased in peripheral blood mononuclear cells (PBMCs) from preeclamptic women compared to those from normotensive pregnancies [113], and the *in vitro* knockdown of these factors caused a shift towards greater Foxp3 expression [113], further supporting a preeclampsia-associated imbalance in the differentiation of effector and regulatory T cells. Accordingly, phenotypic changes in effector T cells from women with preeclampsia included the increased expression of activation-associated markers such as HLA-DR on both CD4+ and CD8+ T-cell subsets [114, 115] together with greater *in vitro* secretion of relevant cytokines such as IL-2, tumor necrosis factor alpha (TNF), and IFNγ [115]. Interestingly, the surface expression of CD40L by peripheral CD4+ T cells and serum concentrations of soluble CD40L were both elevated in women with preeclampsia [116], potentially promoting immune cell activation and increasing antigen-presenting cell activity, and the administration of anti-CD40L antibody alleviated maternal preeclampsia symptoms in a rat model of adoptive transfer of CD4+ T cells [117]. Together with human data, these *in vivo* studies provide further evidence that peripheral T cells may participate in the pathophysiology of preeclampsia; indeed, the adoptive transfer of Th1-differentiated splenocytes into pregnant mice has been used as an *in vivo* model of this syndrome due to the resulting preeclamptic features (e.g. elevated blood pressure and proteinuria) [118]. This model has since been replicated, as lymphocytes activated via culture with anti-CD3 antibody and IL-2/IL-12 were shown to induce hypertensive symptoms in mice [119], and both IL-12-stimulated and IL-4-stimulated splenocytes were shown to cause fetal resorption and hypertension/proteinuria in mice [120]. These findings further indicate that skewed differentiation of peripheral T-cells towards pro-inflammatory subsets takes place in

women with preeclampsia, and that the aberrant activation of these cells can contribute to the disease pathophysiology.

Although the majority of investigations have pointed to increased T-cell activity in women with preeclampsia, several reports have indicated the opposite phenomenon. An early study indicated low peripheral T cell/B cell counts in women with preeclampsia together with reduced *in vitro* T-cell responses to stimulation compared to normal pregnancies [121], and lymphocytic cytotoxic activity was also shown to decrease in preeclamptic women [122]. More recently, it was reported that, while total CD4+ and CD8+ T-cell proportions did not differ between normal and preeclamptic pregnancies, proportions of CD4+ memory, CD4+ effector memory (EM), and CD4+ central memory (CM) subsets were all decreased in women with preeclampsia compared to those with healthy pregnancies [123]. Such reports of decreased T-cell populations may reflect a diminished presence of specific subsets, rather than a general reduction. Indeed, the proportion of peripheral CD4+HLA-G+ T cells was specifically decreased in pregnancies complicated by preeclampsia [124], as were the maternal plasma concentrations of soluble HLA-G [125]. This observation is notable since HLA-G, which is primarily expressed by fetal tissues [126] and promotes immune tolerance [127, 128], can also be expressed by a subset of immunosuppressive T cells [129, 130]. Thus, in the context of preeclampsia a reduction of specific subsets of peripheral T cells may occur that is distinct from the enhanced presence of inflammatory T cells reported by other studies.

It should be pointed out that several studies have not reported significant alterations in circulating T-cell proportions, phenotype, or function in women with preeclampsia [131-133]. These studies highlight the fact that, unlike Tregs, changes in the composition, proportion, or activity of peripheral T cells may not be a

ubiquitous phenomenon in women with preeclampsia, nor may the degree of such changes be consistent among patients. Similarly, a lack of detectable differences in the expression of lymphocyte markers between normal and preeclamptic pregnant rats [134] could be due to the specific markers investigated, or potentially the model utilized [lipopolysaccharide (LPS) infusion]. Thus, homogenization of patient groups or animal models, including in-depth characterization and categorization of patient/animal parameters, is essential for the determination of the specific immune changes taking place in preeclampsia.

Investigations of effector T cells in the decidua and placenta largely provided inconsistent results, likely due to variation in patient groups, experimental methods, and the specific phenotypes or functions investigated. Flow cytometric [135] and immunohistochemical [136] studies of the decidual tissues demonstrated reduced proportions of T cells in women with preeclampsia compared to those who underwent preterm birth or delivery at term, respectively. However, a later immunohistochemistry study showed increased infiltration of CD8+ T cells in decidual tissues from preeclamptic women compared to those without this disease [137]. Similarly, the proportion of CD8+CD28+ T cells was elevated in the decidual tissues from preeclamptic women, despite an observed overall reduction in total T cell proportions [138]. Yet, another study reported increased numbers of both CD8+ T cells and total T cells in placental bed biopsies from women with preeclampsia compared to those with normotensive term pregnancies [139], and the volume fraction of CD8+ T cells was increased in placentas from pregnancies complicated by preeclampsia or fetal growth restriction [140]. Similar to changes observed in the periphery, preeclampsia-specific alterations in local effector T cells may be driven by individual subsets: flow cytometry studies of early-onset and late-onset preeclampsia

showed a reduction in the proportions of CD4+ CM T cells and CD8+CD45RO+ T cells in both study groups, but increased proportions of activated CD4+ and CD8+ memory cells in women with early-onset preeclampsia compared to those with normal term pregnancies [141]. Thus, not only do preeclampsia-associated alterations in the local T-cell repertoire seem to be based on individual subsets, but it seems that early-onset preeclampsia is characterized by more significant (and potentially more severe) immunological changes.

The increased presence of effector T cells in the decidua of women with preeclampsia may also occur as a secondary response to other local events. A subset of clonally-expanded CD8+ EM T cells expressing reduced PD-1 was demonstrated to be present in the decidual tissues of women with preeclampsia compared to those from women with healthy term pregnancies [142], which is consistent with a previous report demonstrating that fetal antigen-specific CD8+ T cells persist throughout multiple pregnancies and typically display an exhausted-like PD-1+ phenotype [143]. Thus, in some cases of preeclampsia the local expansion of effector T cells may occur as a result of impaired suppressive activity. Alternatively, the development of placental histological lesions such as acute atherosis, which is associated with preeclampsia, may also promote T-cell infiltration. Indeed, increased numbers of such cells were observed in the decidual tissues of women with preeclampsia and acute atherosis compared to those from preeclamptic women without this lesion [144], highlighting the importance of considering confounding variables such as placental lesions when evaluating the immune changes that take place in women with preeclampsia.

Collectively, the above reports indicate that T-cell activation in both the maternal circulation and in the decidua or placenta is a characteristic of

preeclampsia, likely driven by the prevalence of systemic inflammation together with the decreased presence and function of Tregs and immunosuppressive HLA-G+ T cells (Figure 2). Importantly, women with early-onset preeclampsia seem to present more severe T-cell responses, and the presence of associated conditions such as acute atherosis may also exacerbate T cell-driven pathology. Such variability highlights the complex and heterogeneous nature of the immunological responses that occur in preeclampsia, which should be taken into consideration when evaluating this disease and potential treatments.

Gamma Delta (γδ) T cells

A small proportion of the T-cell population [145] is composed of T cells that express a TCR composed of a γ-chain and δ-chain rather than the conventional α/β TCRs found on most T cells [146-148]. The investigation of such γδ T cells in the context of preeclampsia has been limited, but deserves discussion. One study found that the proportions of circulating Vγ9Vδ2+ γδ T cells, which form the majority of γδ T cells in humans [149], did not differ between women with preeclampsia and those with normal pregnancies [150]. However, such cells showed increased production of perforin and IFNγ in women with preeclampsia, suggesting polarization towards a pro-inflammatory state, and were less susceptible to apoptosis compared to those from women with normal pregnancies [150]. In contrast, the expression of the γδ TCR was increased in the placentas of women with preeclampsia compared to those from non-preeclamptic women, and *in vivo* studies using Toll-like receptor 3 (TLR3) or TLR7 activation-induced mouse models of preeclampsia suggested that splenic γδ T cells are enriched in this clinical condition [151]. The involvement of γδ T cells in preeclampsia was further supported by the *in vivo* deletion of such cells, which

reduced the maternal symptoms resulting from TLR activation [151]. Interestingly, a longitudinal systems biology approach demonstrated that p38 signaling decreased in γδ T cells from the first to second trimester in women who eventually developed preeclampsia [95]; however, mechanistic investigations are required to further establish the relevance of this finding.

Taken together, the above studies provide a preview of γδ T-cell responses in women with preeclampsia, suggesting that this minor yet unique T-cell subset undergoes altered activation and signaling in the context of this pregnancy disease. Future studies may provide further insights into the relative contribution of these cells to local and systemic inflammation in women with preeclampsia.

B cells

B cells, particularly B1 cells, are considered capable of participating in autoimmune disease through the production of autoantibodies [152]; thus, given the relevance of autoantibody production in the pathophysiology of preeclampsia [153], multiple studies have investigated B cells in the context of this obstetrical syndrome. One of the earliest of these studies found that the total proportions of peripheral B cells did not differ between women with preeclampsia and those with healthy pregnancies [154]. However, the authors surmised that, if immunological changes occurred in preeclampsia, such changes were likely subtler than the parameters determined in the current study. Accordingly, later immunophenotyping studies showed that the proportions of peripheral memory B cells and plasma cell precursors were both elevated in women with preeclampsia compared to healthy pregnant women [155]. *In vitro* mitogenic stimulation assays also demonstrated that the proportions of generated plasma cells and the numbers of Ig-producing cells were

greater in samples from such preeclamptic women [155]. Subsequently, the frequencies of peripheral B1a cells [156] as well as memory B cells and non-class-switched memory B cells [157] were also shown to increase in women with preeclampsia compared to those with normal pregnancies, and the general proliferative capacity of the latter cells was enhanced by *in vitro* LPS stimulation [157]. Thus, preeclampsia is associated with enhanced circulating B cells with potentially greater capacity for antibody production.

A hallmark characteristic of preeclampsia is the production of agonistic autoantibodies directed against the angiotensin II type 1 receptor [153]. The elevated proportions of systemic B1a cells found in preeclamptic women were shown to participate in the production of such autoantibodies [156]. Notably, B1a cells were also found exclusively in placental samples from preeclamptic patients, but not in tissues from normal pregnancies [156]. In addition to autoantibody production, such B1a cells may be intrinsically dysfunctional in preeclampsia, as this B-cell subset in an abortion-prone mouse model failed to inhibit Th17 cell differentiation and induced Th1 polarization [158]. Moreover, in normal pregnant mice the proportion of peritoneal CD86+ B1a cells was reduced compared to non-pregnant mice, but this population was maintained in the abortion-prone model [158]. The above studies suggest that specific B-cell subsets are expanded in women with preeclampsia and can produce autoantibodies, potentially including those against the angiotensin II type 1 receptor, thereby promoting hypertension and inflammation. Further *in vivo* studies have strengthened this concept by showing that treatment with the anti-CD20 antibody Rituximab in a RUPP rat model reduced circulating angiotensin II type 1 receptor autoantibodies, subsequently ameliorating some maternal symptoms without improving fetal outcomes [159]. Consistently, other reports in animals

confirmed that autoantibodies were produced by B cells as part of the hypertensive disorders caused by an environmental pollutant, cadmium [160, 161]. However, not all studies investigating the contribution of B cells to preeclampsia have been consistent, as peripheral, splenic, and placental B1 and B2 cells were largely unaltered in a RUPP rat model, and anti-CD20 B-cell depletion did not improve maternal symptoms [162]. The above studies, together with other reports using animal models of hypertensive disorders [163, 164], indicate that the depletion of B cells can improve some maternal parameters but may not be sufficient to rescue pregnancy outcomes.

B cell-associated dysfunction may also have a genetic basis in some women with preeclampsia, as the presence of a polymorphism in the Fc fragment of IgG receptor IIb (FcγRIIB) was significantly associated with preeclampsia occurrence [165]. This finding is of particular interest, given that FcγRIIB is the only known inhibitory Fc receptor; moreover, it is the only FcγR found on B cells in mice [166]. FcγRIIB has thus been considered as an “antibody checkpoint”, mirroring in some ways the functions of multiple immune checkpoint markers found on T cells [167]. Therefore, it is plausible that the presence of polymorphisms that result in the reduced or impaired function of FcγRIIB may allow for uncontrolled B-cell responses such as the enhanced production of autoantibodies.

The above studies demonstrate that B cells contribute to the pathophysiology of preeclampsia through the production of autoantibodies that can further exacerbate ongoing immune responses. Thus, the systemic immune response that characterizes preeclampsia is a multivariate condition involving the dysregulation of several distinct immune pathways. This is further highlighted by the fact that the use of treatments that target B cells was only partially successful in animal models. Therefore, future

efforts to prevent or treat preeclampsia will likely need to consider each individual immune pathway implicated in this clinical disease.

THE ROLE OF INNATE IMMUNE CELL SUBSETS IN PREECLAMPSIA

Natural Killer cells

During early pregnancy, Natural Killer (NK) cells represent a large population of leukocytes in the decidual tissues [168] and perform a critical role in the remodeling of the spiral arteries during this period [169-171]. Thus, it was presumed that NK cells may be implicated in diseases related to poor placentation such as preeclampsia. However, there is some controversy surrounding the importance of NK cells in the pathogenesis of preeclampsia due to multiple studies that appear to provide conflicting data. One factor that may contribute to such seeming discrepancy could be that, since NK cells provide their most important contributions during early pregnancy, deficiencies in these cells leading to preeclampsia may not be reflected in the NK cell populations observed at delivery (when most studies of human preeclampsia are performed).

Several studies have reported an increased NK cell numbers or frequencies in the gestational and reproductive tissues in women with preeclampsia [172-174], while others report a diminished presence [175, 176] compared to normal pregnancies. Alterations in specific NK cell phenotypes may contribute to such discrepancies; for example, the proportions of decidual CD56+NKp46+ cells were increased in women with preeclampsia, but subsets identified using other NK cell markers such as NKp44, NKp30, NKp80, or NKG2D were not [173]. Differences between the disease mechanisms of early-onset and late-onset preeclampsia could also contribute to disparities between studies, given that the increase in both

decidual and peripheral NK cell proportions was more significant in women with early-onset preeclampsia than in those with late-onset preeclampsia when compared to term pregnancies [174]. A potentially limiting factor when evaluating tissue-wide immune cell populations may be the use of microscopy-based techniques, as both immunohistochemistry [176] and immunofluorescence microscopy [175] of decidual tissues revealed lower numbers of NK cells in preeclamptic patients compared to healthy pregnancies.

Preeclampsia is also accompanied by alterations in local NK cell function. Decidual NK cells displayed a distinct gene expression profile compared to peripheral NK cells, and it was found that a subset of these transcripts were downregulated in chorionic villi tissue from women who subsequently developed preeclampsia compared to those with normotensive pregnancy [177]. Interestingly, the proportions of decidual NK cells expressing IFNγ, perforin, or granzyme B were shown to be elevated in early- and late-onset preeclampsia compared to those with healthy term pregnancies, with early-onset having the greatest increase [174]. In contrast, specific markers of decidual NK cell activation (IFNγ, IL-8, and CD107a) were shown to be decreased in women with preeclampsia compared to those with healthy term pregnancies [173]. Thus, the activation and functional status of local NK cells in women with preeclampsia remains to be established. Nonetheless, it has been suggested that the reduced expression of Fas on decidual NK cells leads to reduced NK cell apoptosis in women with preeclampsia [178], thereby prolonging any potential state of dysfunction.

Aberrant NK cell responses during pregnancy may have a genetic basis in some cases. Mothers with the KIR genotype *KIR AA* showed an increased propensity to undergo pregnancy disorders such as preeclampsia when the fetus

presented more group 2 *HLA-C* genes than the mother [179]. In contrast, the presence of the telomeric (*Tel-B*) region in mothers with the *KIR B* haplotype was associated with a significant protective effect against pregnancy complications, particularly when paired with an *HLA-C2* fetus [179]. Notably, the *Tel-B* region includes *KIR2DS1*, which binds HLA-C2+ trophoblasts and is expressed by uterine NK cells [179]. However, the complexity of the interactions between maternal KIRs and fetal HLA-C was heightened by observations showing the combination of maternal *KIR B* and fetal *HLA-C2* to be a risk factor for acute atherosis in women with preeclampsia [180]. A separate report showed that both uterine and peripheral NK cells expressed *KIR2DL1A* and *KIR2DL1B*, with a predominance of *KIR2DL1A* variants being associated with enhanced NK cell function as well as an increased risk for preeclampsia [181]. In line with these findings, the expression of the immune tolerance-promoting HLA-G [127, 128] and its receptors such as LILRB1 [182] was decreased in placentas from women with preeclampsia [183]. A link between fetal HLA genotype and maternal preeclampsia risk was further supported by the reported association between a poly-T stretch within the downstream region of the HLA-G*01:01:01:01 allele and the occurrence of preeclampsia [184]. Based on these observations, the determination of local NK cell status in early pregnancy (e.g. the evaluation of maternal KIRs and paternal/fetal HLA genotype) could potentially have predictive value for preeclampsia.

Human and animal studies have identified specific factors that may participate in altered NK cell functions in women with preeclampsia. Placental expression of the adhesion molecule *NECTIN4* was shown to be elevated in women with preeclampsia compared to those with normotensive pregnancies, and the overexpression of this molecule in a trophoblast cell line resulted in enhanced susceptibility to NK cell-

mediated cytotoxicity [185]. In mice, uterine NK cells were shown to express placental growth factor (PlGF), with immature NK cells having greatest expression [186], and the deletion of PlGF resulted in the altered composition of local NK cells [186]. Given that PlGF has also been shown to promote apoptosis and exhaustion of effector T cells [187], altered levels of this factor may contribute to dysregulated T-cell responses in women with preeclampsia. Lastly, mice with homozygous or heterozygous deficiency of heme oxygenase 1 (HMOX-1) had fewer uterine NK cells and presented IUGR and gestational hypertension, which were ameliorated by the application of carbon monoxide (the primary metabolite of HMOX-1) [188].

Several studies have provided a mechanistic link between NK cells and pathological changes during pregnancy by depleting NK cells using the anti-asialo GM1 antibody [189-191] in different models of preeclampsia. In a rat renin-angiotensin model, deletion of NK cells resulted in the degeneration of vessels in the mesometrial triangle (the distinct lymphoid aggregate formed between the uterus and decidua in rodents and other species [192] that is comparable to the human placental bed) together with a reduced presence of trophoblasts in the vessel lumen [193]. The vasculopathy resulting from NK cell depletion manifested as lower fetal weight in normal wild type rats and a reduced brain/liver weight ratio in preeclamptic rats [193]. However, in a RUPP rat model, the depletion of NK cells improved the mean arterial pressure of dams and fetal weight [194], and a similar study also demonstrated improved maternal parameters upon depletion of NK cells [195]. These reports highlight key differences in the animal models utilized: in the renin-angiotensin model, maternal hypertension, tissue damage, and fetal growth impairment begin early in pregnancy [196-199], and NK cell depletion was performed on 5 and 10 days *post coitum* (dpc) [193]. In the latter two studies, RUPP was

surgically performed at 14 dpc with NK cell depletion being carried out over the following days [194, 195]. Given that NK cells have been suggested to be more involved in early-onset preeclampsia [174], it is likely that interference with NK cell function in early pregnancy may have more severe outcomes than in a model of late-onset preeclampsia.

Circulating NK cells are also affected in women with preeclampsia, although the extent and direction of such changes are still under debate. Specifically, the cytotoxicity of NK cells has been separately reported to increase [200-202], decrease [203], or remain consistent [204] in women with preeclampsia compared to those with healthy pregnancies. Women who later developed preeclampsia had increased numbers of circulating NK cells in the first trimester compared to women who had uncomplicated pregnancies [205], while another report found that elevated proportions of NK cells were associated with postpartum preeclampsia, but not with preeclampsia during pregnancy [206]. Yet, it has also been shown that the proportions of specific NK cell subsets are increased in the third trimester in women with preeclampsia compared to normotensive pregnant women [207]. Thus, there seems to be some overall consensus that peripheral NK cells are enhanced in women who had or are at risk for preeclampsia. In line with this concept, several studies have indicated that the functionality of such cells is similarly enhanced. The expression of IFNγ by peripheral NK cells tended to increase in women with preeclampsia [208, 209], as did the expression of NKG2A and NKG2C [210, 211], compared to those from women with healthy pregnancies. The ratio of “type 1” to “type 2” NK cells also increased in the circulation of women with preeclampsia [133], whereas the proportions of such cells expressing protective factors such as vascular endothelial growth factor (VEGF) [212] and galectin-1 [213] were significantly

decreased. Mucin-16 (CA-125), a glycoprotein that can be expressed by decidual cells [214, 215], was more frequently bound to circulating NK cells in women with preeclampsia compared to those from term uncomplicated pregnancies [216]. Finally, the intracellular expression of multiple cytokines was enhanced in peripheral NK cells from women with severe early-onset preeclampsia compared to those from healthy pregnancies [211]. Together, these studies support a dysregulated and potentially activated status for peripheral NK cells in preeclamptic women.

In search of potential treatments for preeclampsia, several studies investigating the beneficial effects of anti-inflammatory compounds have also reported alterations in local or systemic NK cell populations as secondary outcomes. The administration of the anti-inflammatory cytokine IL-4 in a RUPP rat model reduced the proportions of total and cytolytic NK cells in the placenta [217], as did treatment with hydroxyprogesterone caproate (17-OHPC) [218]. Targeting reactive oxygen species by treatment with the superoxide dismutase mimetic tempol also lowered the proportions of circulating total and cytotoxic NK cells [219]. Thus, one of the mechanisms by which anti-inflammatory treatments improved preeclampsia symptoms may be through the modulation of NK cells.

Collectively, the studies described above provide evidence of two distinct but connected phenomena: first, dysfunctional local NK cell responses leading to poor placentation and/or lack of spiral artery remodeling may lay the groundwork for the eventual manifestation of preeclampsia later in pregnancy. Poor spiral artery remodeling alone may not lead to preeclampsia, and may require the superimposition of other insults or mechanisms for disease escalation [220]. This model is based on observations in *Rag2*$^{-/-}\gamma_c^{-/-}$ mice, in which the presence of thickened spiral artery walls and reduced lumen diameter was associated with the

loss of NK cells [221, 222] and did not lead to maternal hypertension [223]. Thus, while NK cells are important for proper remodeling of the spiral arteries in early pregnancy, disruption of this mechanism may not be the sole driver of preeclampsia. Yet, human studies demonstrated that a high uterine artery resistance index in the first trimester as determined by Doppler ultrasound was associated with reduced proportions of NK cells expressing specific KIRs and LILRB1, suggesting altered NK cell-trophoblast interactions in such cases [224]. Second, the studies of NK cells reviewed above suggest that the immunological manifestations of preeclampsia include elevated proportions and activity of peripheral NK cells as part of the overall inflammatory profile that characterizes this clinical condition. Therefore, changes in local or systemic NK cell populations observed after the diagnosis of preeclampsia (in late pregnancy) are likely the result of upregulated inflammatory signaling, rather than the cause, and should therefore be considered as distinct phenomena.

Invariant Natural Killer T cells

Natural Killer T (NKT) cells are a unique population of lymphocytes that express a TCR together with NK cell markers [225]. Among this population, invariant NKT (iNKT) cells represent the largest and best studied subset [226]. Although several studies have proposed a role for iNKT cells in the pathophysiology of pregnancy complications such as preterm birth [227-230], the contribution of these cells to preeclampsia is less well defined. The ratio of circulating type 1 to type 2 iNKT cells was shown to increase in women with preeclampsia compared to those with a normal pregnancy, suggesting a balance shift towards a pro-inflammatory phenotype [133]. Moreover, the expression of the early activation marker CD69 and the effector molecules perforin and IFNγ was also shown to be increased in

circulating iNKT cells in women with preeclampsia compared to those with healthy pregnancies [231], further indicating cell activation. The expression of CD95 was reduced on such circulating iNKT cells [231], potentially indicating reduced rates of iNKT-cell apoptosis that could result in a more prolonged state of activation. However, a separate study found no differences in the counts, proportions, or CD69 expression of iNKT cells between women with preeclampsia and those with normal pregnancies [232]. Based on these limited data, forming a firm conclusion as to the participation of iNKT cells in the pathophysiology of preeclampsia remains difficult. It is possible that iNKT-cell activity is altered (potentially enhanced) in preeclampsia without fluctuations in the overall population; yet, additional studies are required to establish this concept.

Dendritic cells

Dendritic cells (DCs) are professional antigen-presenting cells that play a critical role in both central and peripheral tolerance [233] and thus represent a central component of the innate immune system. A primary function of DCs during pregnancy is to uptake and present the paternal/fetal antigen to Tregs in the secondary lymphoid tissues, promoting maternal tolerance of the fetus [234]. Thus, multiple studies have proposed that alterations in the populations and functionality of local and circulating DCs may contribute to a breakdown of maternal-fetal tolerance, thereby promoting inflammation as part of the pathogenesis of preeclampsia. Indeed, the proportions of plasmacytoid DCs (pDCs) were decreased [235, 236] and those of myeloid DCs (mDCs) were increased [236] in women with preeclampsia compared to those with healthy pregnancies. As a result, the peripheral ratio of mDCs to pDCs was also shown to be elevated [61, 235]. Consistent with reports of other immune

cell subsets, early- and late-onset preeclampsia may each be associated with different DC responses, as one study found that pDCs were reduced only in women with early-onset preeclampsia compared to those with normal pregnancies [62].

Peripheral DCs also exhibit a more activated phenotype in preeclampsia. Higher basal expression of TLR3 and TLR4 was observed on mDCs, and increased TLR9 on pDCs, in women with preeclampsia compared to those from healthy pregnancies. This was accompanied by elevated basal expression of cytokines such as IL-6, TNF, and IFNα by mDCs as well as elevated IFNα and TNF by pDCs [237]. Such high basal cytokine expression could indicate prior activation of circulating DCs, particularly since these cells isolated from women with preeclampsia showed weaker responses to *in vitro* stimulation with TLR ligands [237]. Notably, both mDCs and pDCs also showed greater expression of the immune checkpoint molecule CD200 in women with preeclampsia compared to those with normal pregnancies [238], potentially as a compensatory mechanism to restore immune regulation. However, the same authors showed that the expression of another immune checkpoint marker, PD-L1, was decreased on both subsets [239]. Thus, the expression of immune checkpoint markers by DCs seems to be dysregulated in a specific manner in women with preeclampsia. Finally, one report demonstrated upregulation of CD80, CD86, and CD83 on peripheral DCs in women with preeclampsia compared to those with uncomplicated pregnancies [60], indicating greater potential for antigen presentation. This observation was supported by *in vitro* evidence that VEGF, which is downregulated in women with preeclampsia, reduces the expression of CD80, CD86, and CD83 on DCs [236]. Therefore, these findings indicate an activated and pro-inflammatory state for circulating DCs in women with

preeclampsia, and suggest that reduced VEGF signaling may contribute to this process.

Consistent with human studies, several investigations in animals have also reported altered systemic DC populations using preeclampsia models. Pregnant mice treated with Poly:IC (a TLR3 ligand) develop hypertension [240], which is in line with the increased placental expression of TLR3 in women with preeclampsia [241], and these mice displayed higher numbers of splenic DCs and other systemic immune changes that were largely abrogated by treatment with IL-4 and IL-10 [240]. In a model of arginine vasopressin-induced preeclampsia, increased expression of MHC-II, CD80, and CD86 was observed on splenic DCs together with reduced PD-L1 and PIR-B [242], which is similar to the findings reported in pregnant women [60].

Preeclampsia has also been associated with local changes in DC populations. Placental bed biopsies revealed increased numbers of CD209+ or CD83+ leukocytes (termed immature DCs and activated DCs, respectively) in women with preeclampsia compared to tissues from normal pregnancies [243]. In the decidua, tissue-specific upregulation of the chemokines CCL2, CCL4, CCL7, and CCL20 was observed in women with preeclampsia, providing a potential mechanism whereby immune cells, including DCs, may migrate to this compartment [243]. Indeed, *in vitro* studies of first trimester decidual cells suggested that the upregulation of CCL2 and CCL5 is particularly impactful in promoting the chemotaxis of DCs and macrophages [244]. Decidual cells from women with preeclampsia also expressed high levels of GM-CSF compared to those from normal pregnancies, and stimulated first trimester decidual cells released GM-CSF that promoted the *in vitro* differentiation of peripheral monocytes to dendritic cells and macrophages [245]. Moreover, *in vivo* experiments revealed that the observed increase in decidual GM-CSF expression was

accompanied by the infiltration of DCs and macrophages into this compartment [245]. Dendritic cells may also respond to signals released by apoptotic extravillous trophoblasts (EVTs), as immunohistochemistry analyses showed the co-localization of these cells in the decidua/myometrium of women with IUGR [246]. The increased number of mature decidual DCs identified in women with preeclampsia was attributed in part to phosphorylated STAT3 and the DC-specific long noncoding RNA lnc-DC [247], both of which showed increased expression in the decidual tissue [248]. In addition, *in vitro* studies suggested that lnc-DC signaling by mature decidual DCs may participate in the regulation of trophoblast invasion [249].

The combination of a greater number of infiltrating DCs together with their enhanced activation and/or accelerated maturation may contribute to the pathogenesis of preeclampsia. Yet, how the dysregulated activity of DCs in women with preeclampsia affects the processing and presentation of paternal/fetal antigens, and the extent of the relationship between DCs and impaired Tregs in such women, requires further research.

Neutrophils

The majority of investigations into the role of neutrophils in preeclampsia have focused on these cells in the maternal circulation, given the systemic intravascular inflammation associated with this pregnancy complication [29, 30, 107, 250]. Early reports noted elevated numbers of polymorphonuclear cells expressing complement and immunoglobulins in the circulation of preeclamptic women compared to those with normal pregnancies [251] as well as elevated plasma concentrations of neutrophil elastase [252-255], suggesting neutrophil activation. Subsequently, several other studies provided demonstrations of neutrophilia in women with

preeclampsia [256, 257] and noted that the systemic numbers of other immune cell subsets were not as drastically altered [257], given that the neutrophil to lymphocyte ratio was also significantly increased [258]. Moreover, preeclampsia-associated neutrophilia was likely further exacerbated by the reported impaired or delayed neutrophil apoptosis occurring in such patients [259]. Elevated neutrophil proportions, adhesion, and infiltration were reported in subcutaneous fat microvessels from women with preeclampsia compared to those from normal pregnant women [260, 261], further indicating the systemic nature of preeclampsia-associated neutrophilia. Moreover, correlations between increased isovolumetric venous pressure and maternal plasma levels of neutrophil elastase, VCAM-1, and E-selectin were also demonstrated [255].

Peripheral neutrophils are phenotypically altered in women with preeclampsia as well [250, 262-265], showing greater nuclear translocation of NF-κB [264, 266] and increased expression of surface markers such as CD11b [250, 263] together with reduced expression of CD62L [250, 263-265] compared to those from normal pregnancies, thus indicating neutrophil activation. Circulating neutrophils were also shown to have reduced expression of TLR2 and TLR4 in women with preeclampsia compared to normal pregnant women [267]. However, a later study reported increased mRNA and surface protein expression of TLR2 and TLR4 in neutrophils from women with early onset preeclampsia and HELLP syndrome, whereas as those from women with late onset preeclampsia were similar to controls [268]. Thus, it is possible that the two studies differed due to heterogeneous study populations as well as disease severity.

Neutrophil functions are also altered in women with preeclampsia, likely driven by the presence of circulating placenta-derived factors [269-271]. Indeed,

conditioned media obtained from placental explants from women with preeclampsia increased neutrophil-endothelial cell adhesion compared to that from healthy placentas, which seemed to be driven by the endothelial-derived platelet-activating factor (PAF) [269]. Circulating endothelial microparticles were linked to the greater systemic levels of dsDNA, myeloperoxidase, and histones (components of neutrophil extracellular traps or NETs) found in women with preeclampsia [271]. Consistently, it was recently reported that the elevated levels of the proteases neutrophil elastase and matrix metalloproteinase 1 (MMP-1) found in the plasma of women with preeclampsia can also contribute to enhanced neutrophil activation [272]. Superoxide production by circulating neutrophils is also increased in women with preeclampsia compared to those with healthy pregnancies [270, 273], and such a phenomenon was reproduced *in vitro* by the co-culture of neutrophils and syncytiotrophoblast microparticles (STBM) isolated from preeclamptic women [270]. One report noted that superoxide-anion production by circulating granulocytes typically decreases in normal pregnancy, but not preeclampsia, compared to the non-pregnant state, and may thus contribute to the subsequent systemic inflammation and/or endothelial damage [274]. Finally, neutrophil migration is affected by the excess levels of sFlt-1 in women with preeclampsia, since high concentrations of this molecule may prevent VEGF-mediated signaling in these cells [275]. Neutrophils themselves likely contribute to the systemic inflammatory response of preeclampsia in a positive feedback manner, as two studies have indicated an increase in neutrophil-derived microparticles in women with this obstetrical syndrome [276, 277]. Importantly, the contribution of placenta-derived and vascular circulating microparticles to the pathophysiology of preeclampsia is likely

further exaggerated by the reduced phagocytic clearance of such microparticles by circulating neutrophils [278, 279].

The above studies strongly suggest that the factors initiating systemic neutrophil responses likely originate from the placenta. In line with this proposed model, an *ex vivo* study reported that activated neutrophils are found in the uterine vein, but not antecubital placental vein, of women who underwent cesarean section due to preeclampsia [280]. Moreover, the co-culture of neutrophils and endothelial cells in conditioned medium from the placentas of preeclamptic women resulted in enhanced neutrophil-endothelial cell adhesion compared to conditioned media from healthy pregnancies [281]. The stratification of preeclampsia cases into clusters based on their placental transcriptional signatures demonstrated an increased presence of neutrophils in the placentas of women with “immunological” (transcriptional/epigenetic signature corresponding to increased immune responses) preeclampsia compared to those from women with a milder form of preeclampsia (characterized by a healthy placenta and term delivery) [282]. In addition, one study demonstrated the formation of NETs in the placental intervillous space from women with preeclampsia, and placenta-derived IL-8 and STBM caused NET formation *in vitro* [283]. Thus, while the consideration of local mechanisms occurring in preeclampsia is important, understanding the systemic immune responses driven by placenta-derived factors or patient co-morbidities also warrants attention. Together, the above reports demonstrate the enhanced activation of circulating neutrophils in women with preeclampsia and emphasize the role of placenta-derived circulating factors in the activation of such cells.

Mechanistic studies have explored whether neutrophils could be targeted to treat preeclampsia. The depletion of neutrophils using an anti-polymorphonuclear

leukocyte (anti-PMN) antibody in a RUPP rat model of preeclampsia lowered mean arterial pressure in RUPP rats without having noticeable effects in sham rats; however, systemic C3a levels and RUPP-induced fetal loss were unmitigated [284]. This finding suggests that neutrophils are not the major cause of fetal damage resulting from preeclampsia, and may be primarily involved in propagating maternal systemic responses.

Myeloid-derived suppressor cells (MDSCs) represent a heterogeneous population of immature neutrophils [granulocytic (g)-MDSCs] and monocytes [monocytic (m)-MDSCs] that display immunosuppressive properties [285, 286]. Several prior studies have implicated MDSCs in the maintenance of maternal-fetal tolerance [287-289], and one report demonstrated a significant reduction of g-MDSCs in the circulation of preeclamptic women compared to those with healthy pregnancies, which was accompanied by a systemic decrease in arginase-1 (Arg1) levels [290]. Thus, the loss of g-MDSC-mediated immunomodulation may allow for the further dysregulation of peripheral neutrophil responses in preeclampsia; however, this concept requires further investigation.

The above studies demonstrate the massive activation of neutrophils that occurs as part of the pathophysiology of preeclampsia and is likely exacerbated by reduced or impaired MDSC activity. Given their systemic dominance, neutrophils may act as important propagators of inflammation by rapidly responding to placenta-derived factors present in the circulation in women with this clinical condition.

Monocytes

After neutrophils, monocytes are the most frequent circulating immune cells, and thus likely participate in the vascular immune responses that are a hallmark of

preeclampsia [250]. Indeed, the proportions of total CD14+ monocytes were elevated in women with preeclampsia or post-partum preeclampsia compared to normotensive controls [206]. Such an increase was likely driven by changes in the composition of the monocyte population, as the proportions of non-classical $CD14^{lo}CD16^{hi}$ monocytes were elevated in women with preeclampsia [291], and the proportions of this monocyte subset increased towards the end of pregnancy in a rat ATP infusion model of preeclampsia [292, 293]. In contrast, significantly higher proportions of intermediate ($CD14^{hi}CD16^{int}$) monocytes were reported in women with preeclampsia without changes in the classical or non-classical subsets [292]. Therefore, the proportions of systemic monocytes seem to be enhanced in women with preeclampsia, although the specific affected subsets remain to be confirmed. As an alternative approach, the categorization of monocytes as M1-like (CD14+CD11c+CD163-) or M2-like (CD14+CD11c-CD163+) cells demonstrated that a significant increase in the M1-like subset occurs in women with preeclampsia compared to normal pregnancies, which is accompanied by reduced levels of M2-like monocytes [294]. Such a bias towards M1-like responses is supported by the decreased expression of CD163 on peripheral monocytes from women with preeclampsia [295]. Studies have also pointed out enhanced expression of TLR4 by monocytes in women with preeclampsia [291, 296]; moreover, total monocytes from preeclamptic women demonstrated significantly increased cytokine secretion in response to *in vitro* stimulation with TLR ligands [291, 296], further supporting the pro-inflammatory phenotype of such cells. Thus, the immune response in women with preeclampsia is characterized by alterations in the phenotypic composition of circulating monocytes.

Due to the importance of immune-endothelial interactions in the pathophysiology of preeclampsia, studies have also examined the monocyte-specific expression of potentially involved surface molecules. One report found no significant differences in the expression of integrins such as CD11a, CD11b, and CD11c between monocytes from women with preeclamptic or normal pregnancies; however, *in vitro* treatment of endothelial cells with serum from preeclamptic women increased their expression of ICAM-1, an adhesion receptor that regulates the recruitment of circulating leukocytes [297], suggesting that soluble factors in the maternal serum rather than monocyte-endothelial cell interaction may drive endothelial activation [298]. In contrast, other studies demonstrated increased CD11b expression [250, 299, 300] together with decreased expression of CD62L [250, 299] on monocytes from women with preeclampsia. Interestingly, such a discrepancy may be explained by the fact that the expression of integrins CD11a, CD11c, and CD49d as well as the complement-related markers CD46 and CD59 on monocytes was significantly higher in samples obtained from uterine veins compared to antecubital veins in women with preeclampsia [280]. Thus, monocytes migrating in close proximity to the placenta may undergo greater exposure to factors released by this organ that may contribute to cellular activation.

Elevated baseline levels of ROS production have also been observed in monocytes derived from women with preeclampsia compared to those from healthy pregnancies [250, 299]. Pregnancy is characterized by a physiological elevation in arginine uptake by peripheral leukocytes, mediated primarily through system y+ (encoded by the genes *CAT1*, *CAT2*, and *CAT3*) [301]. Notably, in peripheral leukocytes from preeclamptic women, system y+-mediated arginine uptake was significantly lower than in normal pregnancy despite increased transcription of *CAT2*,

thus suggesting impairment of this pathway [301]. It was proposed that such arginine deficiency would favor the production of harmful O_2^- and $ONOO^-$ radicals [301], thereby contributing to systemic oxidative stress in women with preeclampsia. Yet, this phenomenon could be partially remedied by the activation of compensatory glutathione peroxidases (GPx), as elevated mRNA and protein levels of GPx-1 and GPx-4 were detected in peripheral mononuclear cells from preeclamptic women compared to those from normotensive pregnancies [302].

A large component of monocyte function is the production of cytokines. Multiple studies have shown that, in women with preeclampsia, monocyte cytokine production is heavily skewed towards the release of pro-inflammatory mediators such as TNF and IL-12 [27, 296, 303, 304] as well as IL-8, IL-6, and IL-1β [305, 306], typically accompanied by reduced IL-10 levels [296]. This general upregulation of inflammatory responses is likely driven by upstream signaling, which may include the silencing or inhibition of immunomodulatory factors such as α7 nicotinic acetylcholine receptors (α7nAChR) that suppress pro-inflammatory cytokine pathways [307]. Accordingly, monocytes isolated from women with preeclampsia displayed higher expression of inflammasome-related molecules (e.g. NLRP3, NLRP1, and caspase-1) and NF-κB together with reduced expression of the NF-κB-inhibitor IκBα compared to those from normotensive women [308]. *In vitro* assays demonstrated that multiple alarmins (i.e. endogenous molecules that initiate non-infectious or sterile inflammation [309, 310]) such as monosodium urate [308], heat shock protein 70 [306], and hyaluronan [306] can propagate pro-inflammatory monocyte responses, thereby implicating the inflammasome pathway in the pathophysiology of preeclampsia, a topic that was recently reviewed [311]. It is tempting to suggest that alarmins could be released from apoptotic trophoblasts or damaged endothelium in

women with preeclampsia, leading to monocyte activation; yet, this concept requires further investigation.

In light of current knowledge of the pathogenesis of preeclampsia, the relationship between monocytes and other involved tissues (i.e. the placenta/trophoblast and vascular endothelium) is not entirely clear. Monocytes from preeclamptic women were shown to inhibit trophoblast proliferation in an *in vitro* system, and such monocytes also induced higher rates of trophoblast apoptosis than those from healthy pregnancies [303]. However, monocytes from both preeclamptic and normal pregnancies showed similar binding of syncytiotrophoblast-derived microparticles [27]; thus, it is possible that such microparticles are not the primary drivers of monocyte activation in this context. This concept is further suggested by the fact that incubation of peripheral monocytes with preeclamptic or normal pregnancy-derived STBMs caused only modest changes in surface marker expression or cytokine release [312].

Another factor that adds to the complexity of monocyte interactions in women with preeclampsia is the involvement of the coagulation system. Both preeclamptic and healthy pregnant women had higher proportions of monocytes with bound platelets compared to non-pregnant women after *in vitro* adenosine diphosphate (ADP) stimulation, and the numbers of bound platelets per monocyte were also increased [313]. Moreover, platelet-monocyte aggregates from women with preeclampsia released higher levels of sFlt-1 than those from healthy pregnant or non-pregnant women [314]. Fibrinogen, an acute phase protein that is released during inflammation and displays multiple immune interactions [315], was elevated in the circulation of preeclamptic women and increased *in vitro* cytokine production by preeclampsia-derived monocytes compared to those from normotensive pregnancies

[316]. Interestingly, fibrinogen treatment also reduced the levels of sFlt-1 present in a co-culture system of preeclampsia-derived monocytes and endothelial cells, suggesting that fibrinogen exerts distinct and unrelated effects on the cytokine production and angiogenesis pathways as part of the pathophysiology of preeclampsia [305].

The above studies highlight the complex network of interactions that takes place between monocytes, the placenta, and the maternal vasculature, and how dysregulation or impairment of one component can affect monocytes and potentially other circulating immune cells, leading to systemic responses. An important topic of future research in the pathogenesis of preeclampsia will be the unraveling of such interactions to determine cause versus effect, specifically in regards to monocyte activation and polarization.

Macrophages

Distinct macrophage populations are well described in tissues such as the decidua [317-319] and placenta [320]. Such macrophages have also been implicated in several aspects of the pathophysiology of preeclampsia, including the progression of acute atherosis [321, 322], a lesion of the spiral arteries that often accompanies this pregnancy disease [5, 323-326]. One of the first examples of such involvement was the morphological identification of lipid-scavenging macrophages in the uteroplacental arteries [323]. Similarly, immunohistochemical analysis showed that macrophages surround and infiltrate the decidual spiral arteries in preeclamptic women [327, 328], potentially mediated by ICAM-1+ and HLA-DR+ endovascular cytotrophoblasts [328]. Finally, macrophage infiltration of the spiral arteries in the decidua [328] and myometrium [329] was greater in women with preeclampsia than

in those with healthy pregnancies. Whether such macrophage infiltration is a local or tissue-wide phenomenon in preeclampsia remains unclear, as some reports found similar numbers of CD14+ and/or CD68+ macrophages in the placental beds of preeclamptic women and those with healthy pregnancies [330], while others reported an increase in these cells [176, 331]. Regardless, the increased localization of macrophages to the spiral arteries appears to be a common feature in women with preeclampsia.

Macrophages seem to be skewed towards a pro-inflammatory state in women with preeclampsia, although the question of cause versus effect remains. Placentas from women who had preeclampsia contained higher proportions of M1-like CD11b+ inducible nitric oxide synthase (iNOS)+ macrophages and simultaneously lower proportions of M2-like CD11b+Arg1+ macrophages compared to those from women with healthy pregnancies [294]. The expression of iNOS was noted in Hofbauer cells (placental macrophages), but no differences were shown among women with normal pregnancy, preeclampsia, or IUGR [332]. However, the decidual tissues from a preeclampsia-prone BPH/5 mouse model displayed elevated mRNA expression of iNOS [333]. Thus, changes in the expression of iNOS during the pathogenesis of preeclampsia may depend on macrophage polarization status as well as tissue-specific differences. In line with this concept, flow cytometric analyses of decidual tissues with lesions of acute atherosis demonstrated increased proportions of M1-like macrophages expressing CD80, iNOS, or IL-12 compared to unaffected tissues [322]. Similar tendencies were observed in an animal model of LPS-induced hypertension in which decidual M2-like macrophages were reduced, as was the expression of the immune checkpoint marker TIM-3 by such cells [334]. Notably, the administration of the TIM-3 ligand galectin-9 increased M2-like macrophage

polarization and improved maternal/fetal parameters [334], suggesting that the TIM-3/galectin-9 pathway could be implicated in altered macrophage functions in cases of preeclampsia. In tandem with these observations, the proportion of CD206+ M2-like macrophages was decreased in the mesometrial triangle in a rat model of ATP-induced preeclampsia [292]. Thus, the pathogenesis of preeclampsia may be associated with an imbalance between M1-like and M2-like macrophages. Such imbalance could potentially be driven by placental defects, as it was recently reported that a first-trimester trophoblast cell line secretes soluble PD-L1 that promotes an anti-inflammatory M2-like macrophage phenotype *in vitro* [335]. Moreover, soluble PD-L1 levels increased throughout the first trimester in normal pregnant women [335]; yet, whether alterations in the relationship between trophoblasts and macrophages occur in women with preeclampsia requires further investigation.

Outside of the strict M1-M2 paradigm, two less well described macrophage subsets have also been detected in decidual tissues from women with acute atherosis [322]: Mhem [336] and MOX [337] macrophages, both of which can express the anti-inflammatory molecule HMOX-1 [338]. HMOX-1 has been shown to play important roles in placental development [339] and maternal-fetal tolerance [340]; thus, the presence of macrophages expressing this molecule may represent a physiological response to the oxidative stress and inflammation occurring in women with preeclampsia. The molecule CD74 (MHC-II invariant chain Ii [341]) may also be an important regulator of these cells, as CD74+ macrophages were shown to be reduced in the placentas of preeclamptic women compared to those with normotensive pregnancies, and mice deficient for this molecule display disturbed placental development and spiral artery remodeling accompanied by fetal growth

restriction [342]. Such findings may be due in part to the shift towards a pro-inflammatory phenotype that occurs in CD74-deficient macrophages [342]. Macrophage phenotypes were investigated in spontaneously preeclamptic BPH/5 mice based on the decidual CD11c-expressing subsets originally described by Houser et al. [343], and the proportion of IL-10-producing $CD11c^{hi}$ macrophages was decreased in the murine decidua while that of $CD11c^{lo}$ macrophages were elevated [333]. Together, these reports further indicate the plasticity of macrophages in the decidua and placenta, and support the polarization of such cells towards more pro-inflammatory phenotypes outside of the M1-M2 dichotomy in women with preeclampsia.

Immunohistochemistry studies showed poor trophoblast invasion of the spiral arteries as well as the co-localization of apoptotic trophoblasts and macrophages in women with preeclampsia [344], suggesting that macrophages may promote trophoblast apoptosis in such cases. Several *in vitro* studies strengthened this concept by demonstrating that the co-culture of macrophages with a trophoblast cell line led to increased trophoblast apoptosis, which was reversed by the addition of antibodies against TNF receptor 1 (TNF-R1) and tryptophan [344]. Such functions may be restricted to activated or M1-primed macrophages, as LPS treatment of such cells greatly enhanced their TNF-mediated prevention of trophoblast invasion *in vitro* [345] and was largely reversed by exposure of LPS-activated macrophages to IL-10 [346]. Dysregulated corticotropin-releasing hormone (CRH) may contribute to this process, as this molecule was increased in EVTs derived from the placentas of preeclamptic women compared to those from normal pregnancies and upregulated the expression of Fas ligand (FasL) by macrophages *in vitro*, resulting in apoptosis of a trophoblast cell line [347]. The mRNA and protein expression of

cyclooxygenase-1 (COX-1), an important component of the prostaglandin synthesis pathway, was also elevated in the placental beds of preeclamptic women and was localized to placental cells, including macrophages [348]. Such enhanced placental expression of COX-1 may indicate a compensatory response to local and/or systemic inflammation in women with preeclampsia.

In addition to the apoptosis and prostaglandin pathways, previous studies have also implicated the complement system in the pathophysiology of preeclampsia [349-351]. Plasma levels of C5a were elevated in women with preeclampsia compared to non-preeclamptic pregnancies [254, 349, 352], and it was subsequently shown that this factor was localized to placental CD11b+ macrophages and caused dysregulation of trophoblast invasiveness [352]. Thus, it appears that multiple macrophage signaling pathways are affected in women with preeclampsia, subsequently resulting in altered functionality of these cells.

Taken together, the above studies establish the accumulation of macrophages in response to impaired spiral artery remodeling and their enhanced polarization towards a pro-inflammatory phenotype as contributing factors in the pathogenesis of preeclampsia. Given that aberrant M1-like macrophage polarization has been associated with other pregnancy complications such as preterm labor and birth [317], the specific targeting of these cells or restoration of the overall pro-/anti-inflammatory balance may represent potential strategies to improve pregnancy outcomes in women with preeclampsia.

Mast cells

Mast cells participate in the systemic responses observed in women with preeclampsia, although not likely to be primary players [353-355]. An early study

identified increased proportions of mast cells and higher histamine concentrations in placental tissues from preeclamptic women compared to those from normal pregnancies [353]. Such observations were strengthened by the increased mast cell density and reduced mean mast cell area in the placental tissues of preeclamptic women, potentially indicating mast cell degranulation or activation [354]. In contrast, the numbers of cells expressing human mast cell chymase were decreased in the placenta but elevated in the myometrial tissues of women with severe preeclampsia compared to healthy pregnant women [355]. Similarly, the number of cells positive for endothelin-1, a downstream product of human mast cell chymase cleavage of big endothelins [356], was also decreased in the placenta and increased in the myometrium from women with severe preeclampsia [355]. Together, these limited reports indicate the potential involvement of mast cells in the pathophysiology of preeclampsia; however, mechanistic studies are required to demonstrate the participation of these cells.

CLOSING REMARKS

In the current review, we summarize the critical involvement of the cellular immune system in the pathogenesis of preeclampsia. The reviewed literature reveals that preeclampsia involves a complex relationship between the maternal immune system and the placenta as well as other pathophysiological processes. Notably, women with preeclampsia are characterized by an exacerbated intravascular inflammatory response, which is likely triggered by factors released by the dysfunctional placenta. Such dysregulation also includes aberrant cellular immune responses in the reproductive tissues and maternal-fetal interface, which further contributes to the pathophysiology of preeclampsia. Yet, such immune responses may differ depending on disease severity and the early- or late-onset disease type,

which has implications for the diagnosis, management, and treatment of women with preeclampsia.

Despite the large body of evidence demonstrating the close involvement of the cellular immune system in pathophysiology of preeclampsia, efforts to translate such findings and thereby improve the clinical care of women with this obstetrical syndrome are still lacking. Thus, future investigations should seek to generate translationally useful results and models that can advance the prevention, diagnosis, and/or treatment of preeclampsia. Specifically, the incorporation of recently emerging technologies such as single-cell RNA-sequencing together with large-scale longitudinal study designs [319, 357, 358] may be able to provide potential immunological biomarkers or novel therapeutic strategies to treat the devastating effects of this obstetrical syndrome.

AUTHORSHIP

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CONFLICT OF INTEREST DISCLOSURE

The authors declare no conflict of interest.

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FIGURE LEGENDS

Figure 1. The role of innate and adaptive immune cells in the pathophysiology of preeclampsia. In the maternal circulation (upper image), a large body of evidence has implicated adaptive immune cells such as regulatory T cells, effector T cells, γδ T cells, and B cells as well as innate immune cells, namely natural killer cells, invariant natural killer T cells, neutrophils, monocytes, and dendritic cells, in the cellular mechanisms that take place in women with preeclampsia. In the decidua (lower image), studies have described alterations in natural killer cells, neutrophils, dendritic cells, mast cells, and tissue-resident macrophages as well as regulatory T cells, effector T cells, γδ T cells, and B cells that occur in women with preeclampsia. Future studies may focus on crosstalk between local and systemic immune cells to provide a more interconnected picture of cellular immune responses that occur as part of the pathophysiology of preeclampsia.

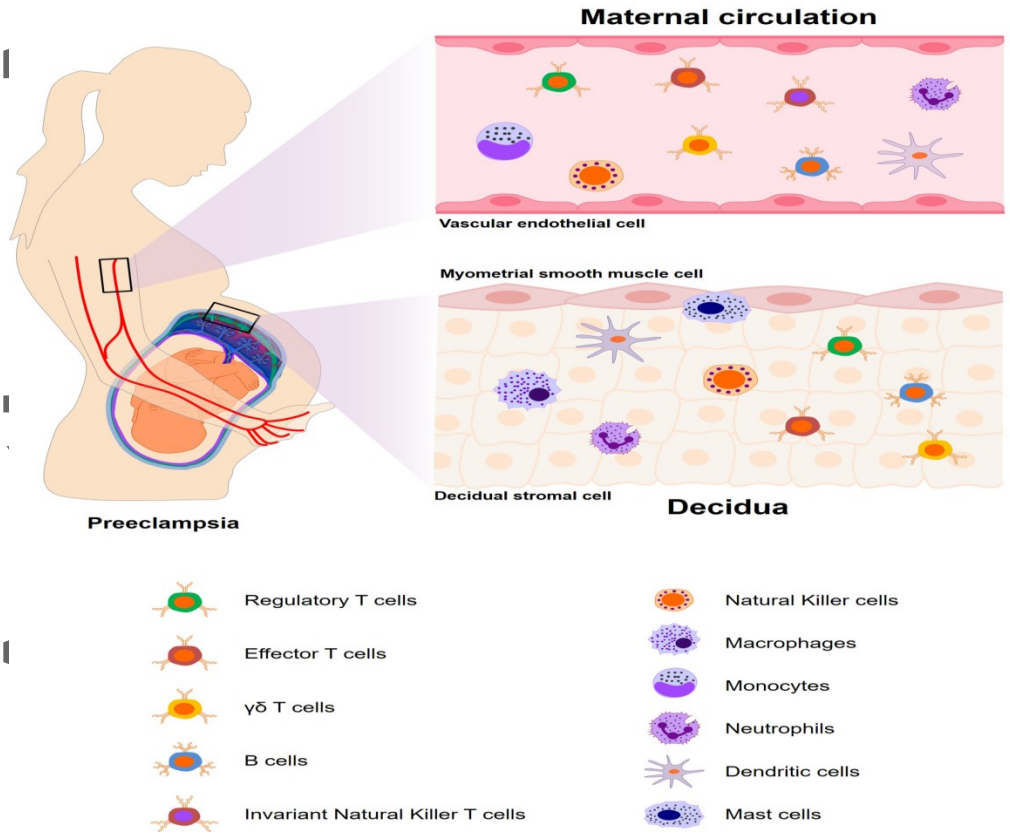

Figure 2. An imbalance between regulatory and effector T cells in the pathophysiology of preeclampsia. During normal pregnancy, regulatory T cells in the maternal circulation (upper panel) and the decidua (lower panel) mediate effector T cell activity to prevent aberrant immune responses. Such balance is disrupted in women with preeclampsia, where systemic and local proportions of regulatory T cells are decreased and effector T cells exhibit greater activation and function.