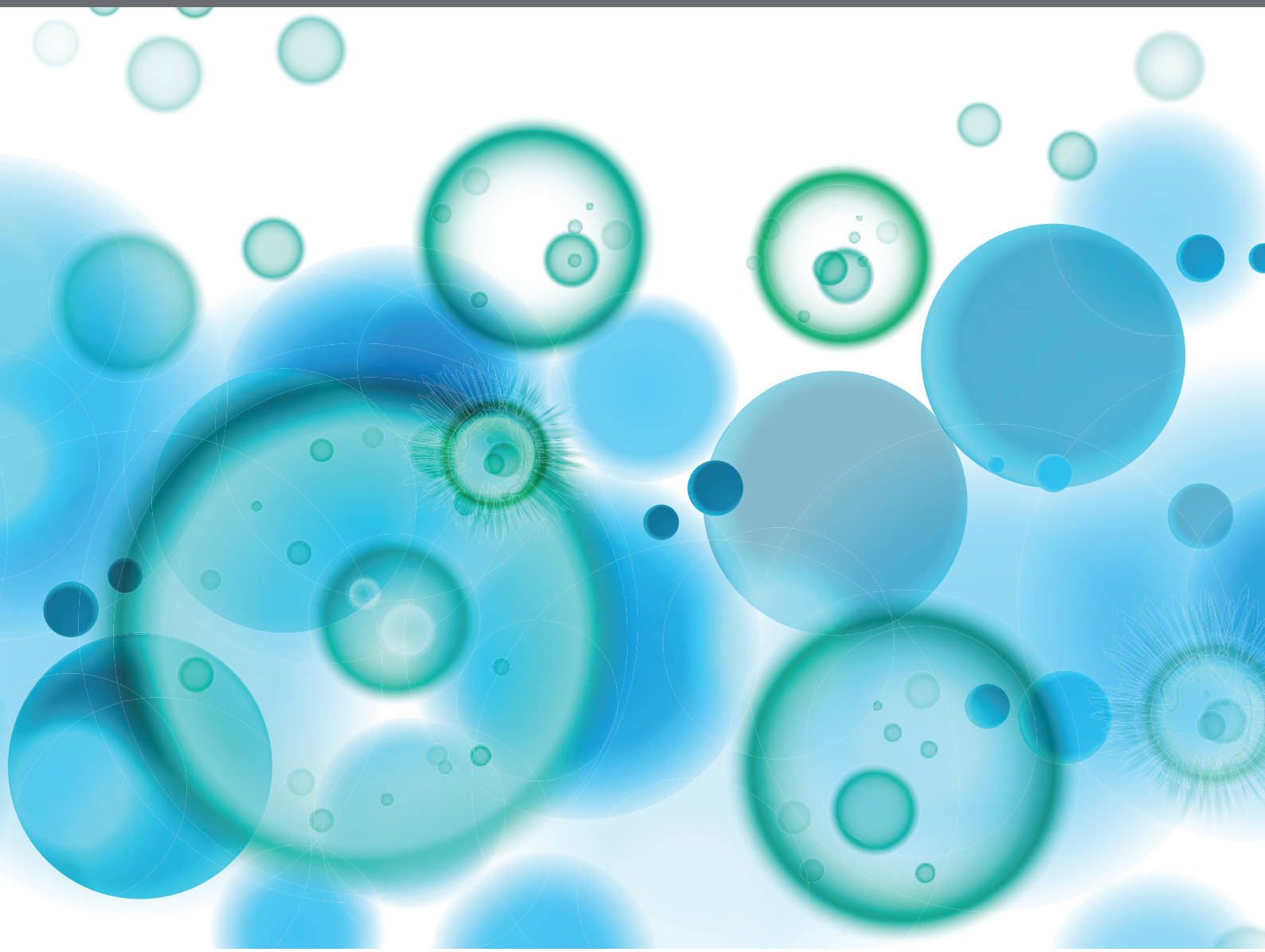
FETAL-MATERNAL IMMUNE INTERACTIONS IN PREGNANCY

EDITED BY: Nandor Gabor Than, Sinuhe Hahn, Simona W. Rossi and Julia Szekeres-Bartho

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FETAL-MATERNAL IMMUNE INTERACTIONS IN PREGNANCY

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EDITORIAL
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Editorial: Fetal-Maternal Immune Interactions in Pregnancy

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Keywords: infection, inflammation, miscarriage, obstetrical syndromes, placenta, pregnancy, maternal-fetal immune tolerance

Editorial on the Research Topic

Fetal-Maternal Immune Interactions in Pregnancy

While contemplating the best way to visually present our Research Topic, the "Tree of Life" concept emerged. Indeed, the developing fetus, attached to the placenta, resembles a tree with its roots. The different cell types, molecules, and their interactions in the placenta that help to nurture, maintain and protect the new life are all important factors in creating a safe environment for the semi-allograft fetus.

Human placentation is unique, as it allows an intimate contact between maternal and fetal cells at the maternal-fetal interface throughout pregnancy, which results in tightly controlled immune interactions between the mother and her child.

Paternal antigens expressed by the fetus are recognized as foreign, which leads to the activation of the maternal immune system. At the same time, several regulatory mechanisms are activated, to create a favorable immunological environment for the developing fetus. The former include altered antigen presentation and T cell differentiation, proliferation and activation of regulatory cells, as well as the action of hormones, cytokines and other soluble factors.

With this Research Topic we have recognized the need to discuss the role of immunological mechanisms, microvesicular and molecular transport of biological information and signaling between the mother and the fetus in promoting immunological tolerance. We have also focused on the effects of maternal infections and local or systemic inflammation that may lead to the failure of these tolerance mechanisms and the development of a spectrum of pregnancy complications, which have an impact on placental and fetal development and health later in life.

Dendritic cells (DC) have a critical role in deciding, whether foreign antigens are to be considered as dangerous or neutral and consequently in the acceptance or rejection of the foreign fetal antigens by the maternal immune system. Ehrentraut et al. report the gestational age-dependent differential regulation of peripheral blood dendritic cell subsets during normal pregnancy. Miscarriage is associated with dysregulations in the myeloid peripheral blood dendritic cell subsets, together with lower regulatory T cell frequencies.

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By down regulating the immune response, antigen specific **regulatory T cells** play an important role in controlling anti-fetal immune reactions. Antigen-specific regulatory T cells are crucial for the establishment of immunological tolerance. While self-reactive Treg cells contribute to the maintenance of self-tolerance, paternal antigen-specific T cells control the immune response to paternal antigen expressing cells. Tsuda et al. review the role of regulatory T cells in establishing an appropriate immunological relationship between the mother and the fetus. Paternal antigen-specific Treg cells accumulate in the murine placenta, and recent studies have identified these cells at the feto-maternal interface of humans. Abnormal expression or function of antigen specific Treg cells has been observed in miscarriage and preeclampsia. Indeed, in an original research article by the same authors, Tsuda et al. shows that the number of clonally expanded decidual effector regulatory T cells increases in late gestation, but not in preeclampsia.

Kieffer et al. discuss the role of memory T cells in the establishment of tolerance toward allogeneic paternal antigens and their important role in inducing fetal tolerance.

Because inflammation is implicated as a causal factor in preeclampsia and because Treg cells are able to control inflammation, the paper of Robertson et al. discusses the potential therapeutic use of Treg cells. Several possibilities—including pharmacological interventions to target Treg cells and *in vitro* Treg cell generation—are considered, as possible new approaches in the therapy of inflammatory conditions.

Tolerance to antigens are also matter of discussion in the review of Hahn et al. where the role of feto-maternal chimerism is examined in relation to the development of preeclampsia, or later in life, of autoimmune diseases.

Other cellular immunological players are largely responsible for the balance between tolerance and inflammation throughout pregnancy. As elaborated by Vacca et al., decidual innate lymphoid cells (ILCs) (that include NK cells, ILC3, and ILC1), may play a key role in the establishment and maintenance of pregnancy, orchestrating both the tolerogenic and the inflammatory phases, by interacting with stromal cells, neutrophils, myelomonocytic cells, and T lymphocytes.

Köstlin-Gille et al. present novel data about the role of HIF-1α production in myeloid-derived suppressor cells. They show that abrogation of HIF-1α expression in this population results in increased abortion rates in mice.

The review from Reyes and Golos discusses the duplicitous nature of Hofbauer cells. These villous macrophages with M2-like profile play a role in placental development; however, they may produce pro-inflammatory cytokines and mediators that damage the villous cell barrier. Hofbauer cells are ineffective in controlling most TORCH infections, while contributing to vertical transmission of pathogens by harboring them as placental reservoirs.

The paper by Pollheimer et al. reviews how **invasive extravillous trophoblasts (EVTs)** develop and migrate into the uterus where these fetal cells remodel maternal spiral arteries, a process critical for adapting blood flow and nutrient transport to the developing fetus. In the decidua, EVTs encounter various maternal cells including decidual macrophages and uNK cells, which regulate EVT functions by growth factors and cytokines. Failures in this mechanism provides the basis for pregnancy complications such as preeclampsia or recurrent abortion.

Among **proteins involved in modulation of immune responses** during pregnancy, several act as checkpoint molecules. CTLA-4 (Cytotoxic T-lymphocyte-associated protein 4), PD-1 (Programmed Cell Death 1), and TIM-3 (T Cell Immunoglobulin Mucin 3), play key roles in immune defense against infections, prevention of autoimmunity, and tumor immune evasion. Here, Miko et al. review the involvement of these molecules in immuno-regulatory processes during normal pregnancy and in pregnancy complications.

The inhibitory ligand of PD-1, PD-L1 (Programmed Cell Death 1 Ligand 1) has a critical role in the induction and maintenance of immune tolerance to self by modulating the activation threshold of T-cells and limiting T-cell effector responses. Okuyama et al. report on higher levels of soluble PD-L1 in the serum of pregnant women compared to non-pregnant women. The results of functional assays support that sPD-L1 may exert immunoregulatory functions during pregnancy.

Galectin-9 (the inhibitory ligand of TIM-3) belongs to the large immunomodulatory family of galectins, reviewed by Blois et al. Galectins constitute a phylogenetically conserved family of soluble β-galactoside binding proteins, which contribute to placentation by regulating trophoblast development, migration and invasion, angiogenesis, and maternal tolerance to the semi-allogeneic fetus. The altered expression of galectins is associated with infertility and pregnancy complications, and their potential therapeutic role in pregnancy complications is also suggested.

Balogh et al. investigated placenta-specific members of this galectin family, which are expressed by a gene cluster on Chromosome 19 that emerged in anthropoid primates. Gal-13 and gal-14 are predominantly expressed by the syncytiotrophoblast at the lining of the maternal-fetal interface, and their expression is down-regulated in miscarriages. Gal-13 and gal-14 bind to T cells, where they inhibit activation, induce apoptosis, and enhance IL-8 production, suggesting that these galectins are key players in regulating the maternal adaptive immune response.

Progesterone is indispensable for both the establishment and maintenance of pregnancy in most mammals. As shown by Shah et al. the immune system is increasingly activated during pregnancy, which is counterbalanced by a tolerant immune environment (IL-10 and regulatory-T cells) that gradually reverses prior to the onset of labor. Progesterone suppresses while progesterone receptor blockers enhance the release of inflammatory cytokines and cytotoxic molecules by antigen-specific CD4 and CD8 T cells. Furthermore, progesterone regulates the sensitivity of differentiated memory T cells to antigen stimulation.

Progesterone and a progesterone induced protein, PIBF, are important players in re-adjusting the functioning of the maternal immune system during pregnancy. Szekeres-Bartho

et al. review the role of PIBF (carried by extracellular vesicles) in embryo-maternal immune-interactions. PIBF mediates the immunological actions of progesterone. By upregulating Th2 type cytokine production and by down-regulating NK activity, PIBF contributes to the altered attitude of the maternal immune system. Aberrant production of PIBF isoforms results in the loss of immuno-regulatory functions, and pregnancy failure. They also show pre-implantation embryos produce EVs both *in vitro*, and *in vivo*. PIBF transported by the EVs from the embryo to maternal lymphocytes induces increased IL-10 production by the latter, this way contributing to the Th2 dominant immune responses.

Littauer and Skountzou review several studies that demonstrated the vulnerability of pregnant women to infectious diseases, concluding that modulation of inflammation by pregnancy hormones might be the reason.

Other immunomodulatory molecules include miRNAs that control inflammation and tolerance in pregnancy as reviewed by Kamity et al. The paradigm of a sterile intrauterine microenvironment was challenged by the detection of microflora in gestational tissues and amniotic fluid in the absence of inflammation. Therefore, adaptation to microbial products may be critical for the prevention of excessive maternal inflammatory responses and fetal rejection. The presented model herein suggests that repeated exposures to microbial products induce a tolerant phenotype at the maternal-fetal interface mediated by specific miRNAs mostly contained within placental EVs, and that the impairment of this mechanism will result in pathological inflammatory responses contributing to pregnancy complications.

Original research by Twisselmann et al. presents that IgG Fc glycosylation patterns of infants depend on their gestational ages. Preterm infants acquire reduced amounts of IgG via trans-placental transfer which might explain their high susceptibility for infections. Moreover, there is a qualitative shift in the type of IgG Fc glycosylation toward a pro-inflammatory pattern in preterm infants that might contribute to their increased risk for chronic inflammatory diseases such as bronchopulmonary dysplasia.

Immune tolerance toward paternal and fetal antigens is crucial for reproductive success and the breakdown of this mechanism is implicated in the pathophysiology of **pregnancy complications,** including miscarriage, preterm birth and preeclampsia.

van der Zwan et al. show that pregnancy outcomes can be influenced by the presence of allo-reactive HLA-C CD8+ T cells originating from viral memory response (e.g., Influenza, Epstein-Barr virus, Cytomegalovirus, Varicella).

The review paper by Schepanski et al. focuses on the effect of the maternal immune environment on fetal brain development. Vertical transmission of hormones, maternal immune cells and cytokines might affect brain development as well as cognitive and intellectual performances of the offspring. Recent data underpin that brain development in response to prenatal stress challenges can be altered across several generations, independent of a genetic predisposition, supporting an epigenetic inheritance.

Spontaneous preterm birth is the leading cause of newborn deaths; therefore, there is a huge unmet clinical need for its prevention. In a cross-study meta-analysis, Vora et al. evaluate genome-wide differential gene expression signals in maternal and cord blood taken from women with preterm or term births, and identified genes differentially expressed in preterm birth. These were enriched in immune-related pathways, showing up-regulation of innate immunity and down-regulation of adaptive immunity. Several genes were differentially expressed at mid-gestation, suggesting their potential clinical utility as biomarkers.

Preeclampsia is one of the deadliest obstetrical syndrome. The placenta has a key role in the pathogenesis of preeclampsia, characterized by maternal systemic inflammation, which may be triggered by distinct underlying mechanisms in early pregnancy. Immunological incompatibility between the mother and the fetus is strongly indicated, and genetic factors linking immunological pathways to preeclampsia predisposition have been identified. In a mini-review, Lokki et al. discuss genetic variations in immunological factors in the context of preeclampsia and explore immunogenetic and immunomodulary mechanisms contributing to loss the of tolerance, inflammation, and autoimmunity that may lead to preeclampsia.

In a review, Geldenhuys et al. summarize cellular and molecular background of normal placentation and give details on how placentation is disrupted in the "placental subtype" of preeclampsia as a result of failure of tolerance or infections. These lead to aberrant activation of innate immune cells and imbalanced differentiation of T-helper cell subsets, creating a cytotoxic environment *in utero,* placental developmental problems, and eventually excessive maternal systemic inflammation.

In a complex systems biology study, Than et al. integrated different "omics," clinical, placental, and functional data to gain insights into the early molecular pathways of preeclampsia. Distinct maternal and placental disease pathways were revealed to interact and influence the clinical presentation. As a paradigm shift, the activation of maternal disease pathways, including inflammatory changes, was detected upstream of placental dysfunction, and placental disease pathways to be superimposed on these maternal pathways. This warrants for the central pathologic role of pre-existing maternal diseases or perturbed maternal-fetal-placental immune interactions. The description of these novel pathways in the "molecular phase" of preeclampsia and the discovery of new biomarkers by this study may enable the early identification of patients with distinct molecular preeclampsia phenotypes.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication. All authors have contributed equally to this work.

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Meta-Analysis of Maternal and Fetal Transcriptomic Data Elucidates the Role of Adaptive and Innate Immunity in Preterm Birth

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Preterm birth (PTB) is the leading cause of newborn deaths around the world. Spontaneous preterm birth (sPTB) accounts for two-thirds of all PTBs; however, there remains an unmet need of detecting and preventing sPTB. Although the dysregulation of the immune system has been implicated in various studies, small sizes and irreproducibility of results have limited identification of its role. Here, we present a cross-study meta-analysis to evaluate genome-wide differential gene expression signals in sPTB. A comprehensive search of the NIH genomic database for studies related to sPTB with maternal whole blood samples resulted in data from three separate studies consisting of 339 samples. After aggregating and normalizing these transcriptomic datasets and performing a meta-analysis, we identified 210 genes that were differentially expressed in sPTB relative to term birth. These genes were enriched in immune-related pathways, showing upregulation of innate immunity and downregulation of adaptive immunity in women who delivered preterm. An additional analysis found several of these differentially expressed at mid-gestation, suggesting their potential to be clinically relevant biomarkers. Furthermore, a complementary analysis identified 473 genes differentially expressed in preterm cord blood samples. However, these genes demonstrated downregulation of the innate immune system, a stark contrast to findings using maternal blood samples. These immune-related findings were further confirmed by cell deconvolution as well as upstream transcription and cytokine regulation analyses. Overall, this study identified a strong immune signature related to sPTB as well as several potential biomarkers that could be translated to clinical use.

Keywords: preterm birth, meta-analysis, transcriptomics, immunology, pregnancy

INTRODUCTION

Preterm birth (PTB), which is defined as giving birth before completion of 37 weeks of gestation, is the leading cause of newborn deaths worldwide. In 2010, 14.9 million babies were born preterm, accounting for 11.1% of all births across 184 countries, with the highest PTB rates occurring in Africa and North America (1). This high incidence of PTB is concerning since 29% of all neonatal deaths worldwide, approximately 1 million deaths total, are accounted to complications in PTB (2). Furthermore, children born prematurely are at increased risk for a milieu of short- and long-term complications including motor, cognitive, and behavioral impairments (3, 4).

Approximately 30% of PTBs are medically indicated due to maternal or fetal conditions; the other two-thirds are categorized as spontaneous preterm births (sPTB) that include spontaneous preterm labor and preterm premature rupture of the membranes (5). PTB is a syndrome with multiple etiologies. Numerous signs point to genetic factors as playing a role in birth timing including the observations that PTBs are likely to recur in mothers, women who are born preterm are more likely to deliver prematurely, and sisters of women who have delivered prematurely are at an increased risk of delivering preterm. Furthermore, twin studies suggest that genetics account for approximately one-third of the variation in PTB (6, 7). Other factors shown to influence risk for PTB include those associated with adverse lifestyle and behavior, such as stress, smoking, drug use, and nutrition (8). Although a variety of social (9, 10), environmental, and maternal factors have been implicated in PTB, causes of sPTB have remained largely mysterious and therefore, in most instances, not amenable to effective interventions. Thus far, there exists no universal detection method to predict sPTB or intervention approach to prolong labor and extend the pregnancy to term. The complexity and multiple etiologies of sPTB, along with the inconsistency in clinical phenotyping and non-uniform classification system, have limited the identification of genetic factors and clinically relevant biomarkers (11).

Over the years, many different mechanisms have been identified to be associated with sPTB, including breakdown of maternal–fetal tolerance, decidual senescence, uterine overdistension, and procoagulant activity (12, 13). One particularly interesting mechanism that has been implicated is the dysregulation of the interplay between the maternal innate and adaptive immune systems. The innate immune system, also known as the non-specific immune system, comprises cells and mechanisms including but not limited to macrophages, toll-like receptors, neutrophils, and cytokines which aid in host defense from infection (14, 15). This sub-system is responsible for the generalized, non-specific immune response, inflammation, and activation of the adaptive immune system through antigen presentation (14, 15). Contrastingly, the adaptive immune system comprises lymphocytes, specifically T cells and B cells, which are specialized white blood cells that provide long-term immunity (14, 15). In pregnancy, regulatory T-cells proliferate after implantation and function to prevent rejection of the fetus by creating an anti-inflammatory environment (16, 17). However, for labor to initiate and progress, the maternal immune system switches to a pro-inflammatory state by activating the pro-inflammatory nuclear factor-kB signaling pathway, which leads to an increase in the production of cytokines, chemokines, and interleukins and allows for infiltration of the fetal/maternal interface by activating leukocytes (16–19). The location and function of each immune cell is critical to sustain pregnancy to term; it has been proposed that a premature shift from the anti-inflammatory to the pro-inflammatory state, and therefore a disruption in the balance of innate and adaptive immunity, could result in preterm labor and delivery (19).

There is a need to understand the mechanisms by which preterm labor is affected which could then lead to identification, intervention, and prevention. Identifying immune-related genetic signatures as well as clinically relevant diagnostic biomarkers specific to sPTB would enhance our ability to discern women who are at an elevated risk for delivering prematurely. However, findings have been limited due to small sample size and issues with irreproducibility (20). Meta-analysis, which combines information from multiple existing studies, is a powerful tool that improves reliability, generalizability, and ability to detect differential gene expression by larger statistical power (20). With the development of databases such as the National Institute of Health Gene Expression Omnibus (NIH GEO) and Array Express, gene expression meta-analysis has been applied to investigate different disease subtypes and discover novel biomarkers (21–24). In the area of obstetrics, a recent study performed a meta-analysis which integrated diverse types of genomic data, overlaying evolutionary data, and placental expression data in an effort to elucidate genes that may be involved in parturition and disrupt pregnancy (25).

As discussed in a recent systematic review (26), although there have been 134 genome-wide transcriptomic studies related to pregnancy and PTB, most of these studies have focused on PTB related to preeclampsia (one of the medical indications of PTB). sPTB was investigated in only 7% of all studies and 18% of preterm studies, even though sPTB is responsible for over two-thirds of all PTBs. Furthermore, 61% of the studies focused on placental tissue, which has limited utility in the diagnostic setting and upon comparison of results from the different studies, there was very limited overlap among differentially expressed genes; only 2 genes of 6,444 differentially expressed genes identified were present in 10 or more gene expression studies (26). Therefore, there exists a need to aggregate data and perform meta-analyses to elucidate gene signatures that are robust and can be reproduced in studies of maternal blood, which allows for discovery of biomarkers that can be implemented as part of the standard prenatal care. The NIH GEO database has three sPTB related, publicly available datasets which have all been analyzed separately before. The first study, which included women who were diagnosed with threatened preterm labor (median gestational age: 32 weeks), found 469 differentially expressed genes and significantly increased leukocyte and neutrophil counts in women who had sPTB within 48 h after initiation of labor (27). The second study, also by Heng et al., collected samples at two different time points and found no differentially expressed genes in the second trimester and 26 differentially expressed genes in the third trimester when comparing sPTB and term birth (28). The last study analyzed eight tissue

types, comparing women who delivered preterm and term with or without labor; they found that pregnancy was maintained by downregulation of chemokines at the maternal–fetal interface but the work has not been published.

Using these three datasets, we performed a cross-study meta-analysis which identified a set of significant differentially expressed genes in maternal blood, many of which were immune related and a few of which could translate to clinically relevant biomarkers. An additional analysis of measurements collected during mid-gestation in one study revealed a smaller set of significant genes that were differentially expressed over time. Finally, a complementary analysis of fetal cord blood (CB) revealed that there were a number of differentially expressed genes on the fetal side, many of which overlapped with the significant genes in maternal blood and showed opposing changes in regulation.

RESULTS

Datasets

We identified three datasets, from the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database (23, 24), which were comprised of whole blood gene expression profiles from women who delivered preterm and term, respectively. These three studies (GSE46510, GSE59491, and GSE73685) included 339 maternal whole blood samples, 134 from women who delivered preterm, and 205 from those delivered at term. The gestational age of the preterm deliveries ranged from 24.4 to 36.9 weeks with a median of 34 weeks. One study (GSE59491) collected blood samples at two different time points, second trimester (17–23 weeks) and third trimester (27–33 weeks), respectively. In addition to whole blood samples, another study used in the meta-analysis (GSE73685) collected RNA samples from seven other different types of tissues including amnion, CB, chorion, decidua, fundus, lower segment, and placenta (**Table 1**).

Overview

Our primary goal was to perform a meta-analysis to identify potential maternal plasma biomarkers by evaluating differentially expressed genes associated with sPTB and investigating whether certain cell types are enriched in sPTB, using time-matched maternal data from the three independent studies. Taking advantage of the repeated samples collected in mid-gestation from study GSE59491 and samples collected from seven additional tissues in study GSE73685, we performed secondary analyses to identify potential common gene expression signatures across different gestational stages and different tissues and to investigate the potential maternal–fetal interplay at the transcriptomic level. We investigated and compared the transcriptomic signature that was identified as part of the maternal meta-analysis to what was observed earlier in the pregnancy. The second additional analysis investigated differential gene expression in various tissue types to identify tissue specific transcriptomic signatures (**Figure 1**). Each of the signatures was further interrogated through pathway and transcriptional regulation analysis.

Cross-Study Gene Expression Meta-Analysis in Maternal Blood

Samples from the three studies were pooled together based on gestational age at time of sample collection. The women were split into two groups based only on whether they delivered before or after 37 weeks of gestation, with no regard to time of delivery relative to the initiation of labor.

When pooling samples from the different studies, study-specific differences in gene expression were seen (**Figure 2A**) and corrected for using ComBat (29) to eliminate such biases (**Figure 2B**). When we imposed a false discovery rate (FDR) of 0.1, the normalized, merged gene dataset of 17,337 was reduced to 4,648 significant genes. Setting a significance threshold at a fold change (FC) of 1.3-fold increase or decrease in gene expression (30) for PTB samples, relative to term birth, condensed our gene list from 4,648 genes to 210 differentially expressed genes (FC range: 0.46–1.94) (**Figure 2C**), with 65 genes upregulated and 145 genes downregulated (Table S1 in Supplementary Material). We saw clustering of preterm samples and term samples based on the 210 significant genes; however, we did not see any clustering by study (**Figure 2D**). Only third

TABLE 1 | Datasets used in discovery analyses.

Dataset	Year	Author	Platform	Sample types	Preterm births	Term births	Gestational age at sampling*
GSE46510	2014	Heng	GPL16311	Maternal whole blood	75	79	32 (24–36)
GSE59491	2016	Heng	GPL18964	Maternal whole blood	51 (T2)	114 (T2)	19 (17–20)
					47 (T3)	114 (T3)	29 (27–33)
GSE73685	2016	Baldwin	GPL6244	Amnion (A)	12	12	NR
				Cord blood	11	12	
				Chorion (C)	12	12	
				Decidua (D)	11	12	
				Fundus (F)	10	10	
				Lower segment	12	12	
				Placenta (P)	12	9	
				Maternal whole blood (WB)	12	12	

Median (range) reported.
NR, not reported; T2, second trimester; T3, third trimester.

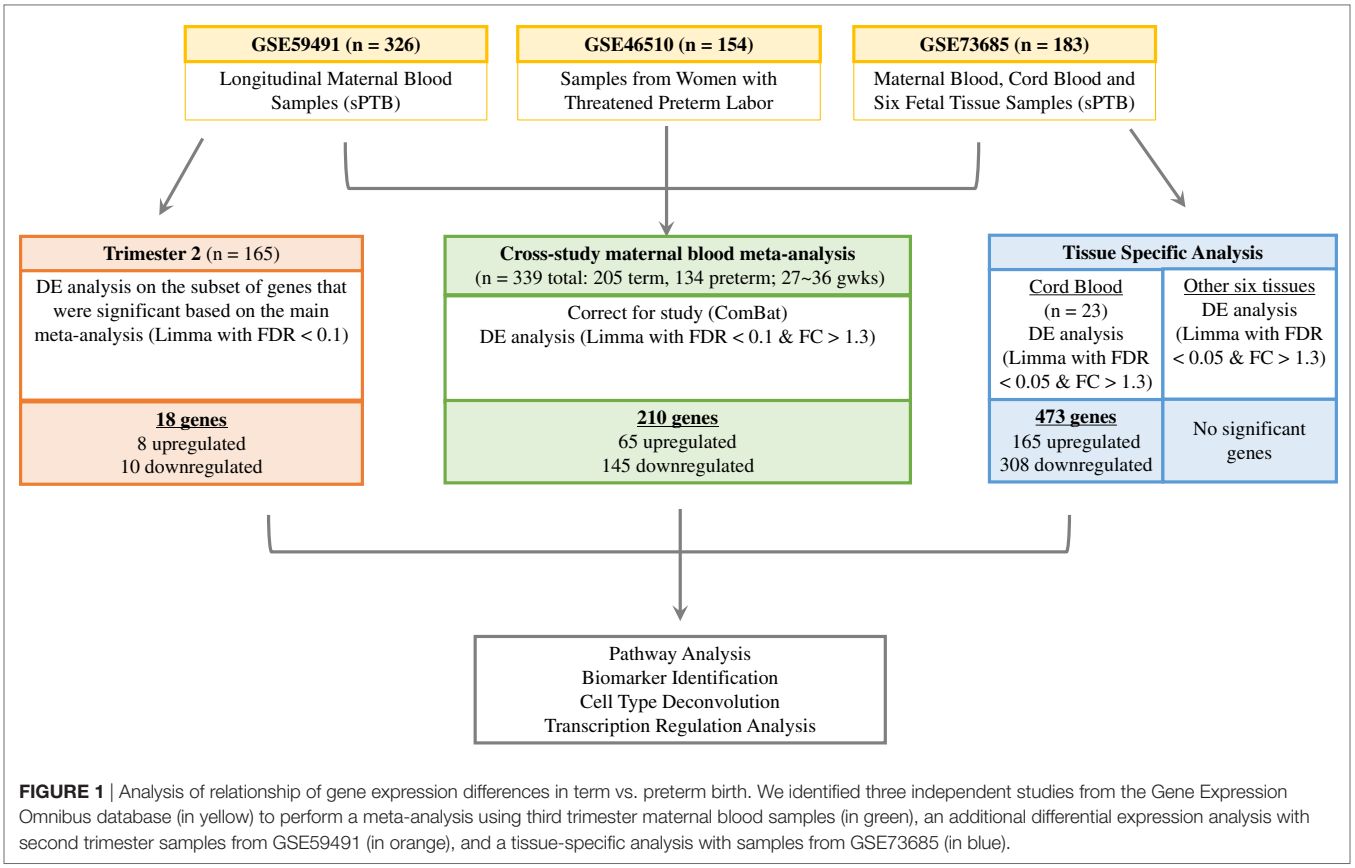


FIGURE 1 | Analysis of relationship of gene expression differences in term vs. preterm birth. We identified three independent studies from the Gene Expression Omnibus database (in yellow) to perform a meta-analysis using third trimester maternal blood samples (in green), an additional differential expression analysis with second trimester samples from GSE59491 (in orange), and a tissue-specific analysis with samples from GSE73685 (in blue).

trimester samples from GSE59491 were used in the meta-analysis to better time-match all samples (**Figure 2E**).

Splitting our list of 210 genes into two sub-groups based on whether they were upregulated or downregulated in PTB, we found that the downregulated genes demonstrated strong network connectivity using the STRING database (31–33) (**Figure 3A**) and were functionally enriched in 36 different pathways using the ToppFun database (34) (**Table 2**), more than half of which were immune related. Specifically, the downregulated genes were highly involved in the adaptive immune response, showing significant clustering and connectivity in Gene Ontology Consortium (GO) biological processes (Table S2 in Supplementary Material) including antigen receptor-mediated signaling pathway, leukocyte activation, lymphocyte activation, and T-cell activation (**Figures 4A–D**). Furthermore, there were six genes (*CD8B, CLC, DPP4, NELL2, SERPINI1*, and *NUCB2*) of 145 downregulated genes that were found to be secreted as proteins in humans from the UnitProt database (35) (**Table 3**). The 65 upregulated genes showed less network connectivity relative to the downregulated genes (**Figure 3B**). Although the majority (5 of 6) of the functionally enriched pathways were immune related (**Table 2**), the upregulated genes were specifically involved in the innate immune response, a stark contrast to the downregulated genes. In addition, there were 9 genes from the 65 upregulated genes that were found be secreted as proteins in humans [IL-1 receptor type I (*IL-1R1*), *IL-1R2, IL-1RAP, HPSE, NLRP3,* tissue factor pathway inhibitor (*TFPI*), *LRG1, CST7, LAMB2*] in the UniProt database (35) (**Table 3**).

These immune pathways and secreted proteins associated with sPTB could have been missed in the single-study analysis due to limited sample size and thus not reaching statistical significance: only 26 significant genes were identified (FDR < 0.10) in the GSE59491 study and no significant genes were identified (FDR < 0.10) in the GSE73685 study (analysis of maternal blood sample only). This highlights the importance and power of aggregating the data and performing a meta-analysis.

Cell-Type Deconvolution Analysis in Maternal Blood

Due to the heterogeneity of plasma samples, it is important to identify and quantify the various cell types that comprise peripheral blood. If not taken into account, the variability in cell composition in each sample can confound the results and limit interpretability (36). To examine the reproducibility of our experiments and test the hypothesis of aligning our pathways with cell type abundance when comparing preterm and term birth, we performed a cell-type deconvolution analysis. Specifically, since immune cells constitute a large portion of cell types in plasma, and a large part of differentially expressed pathways were immune related, we utilized xCell, a computational method that

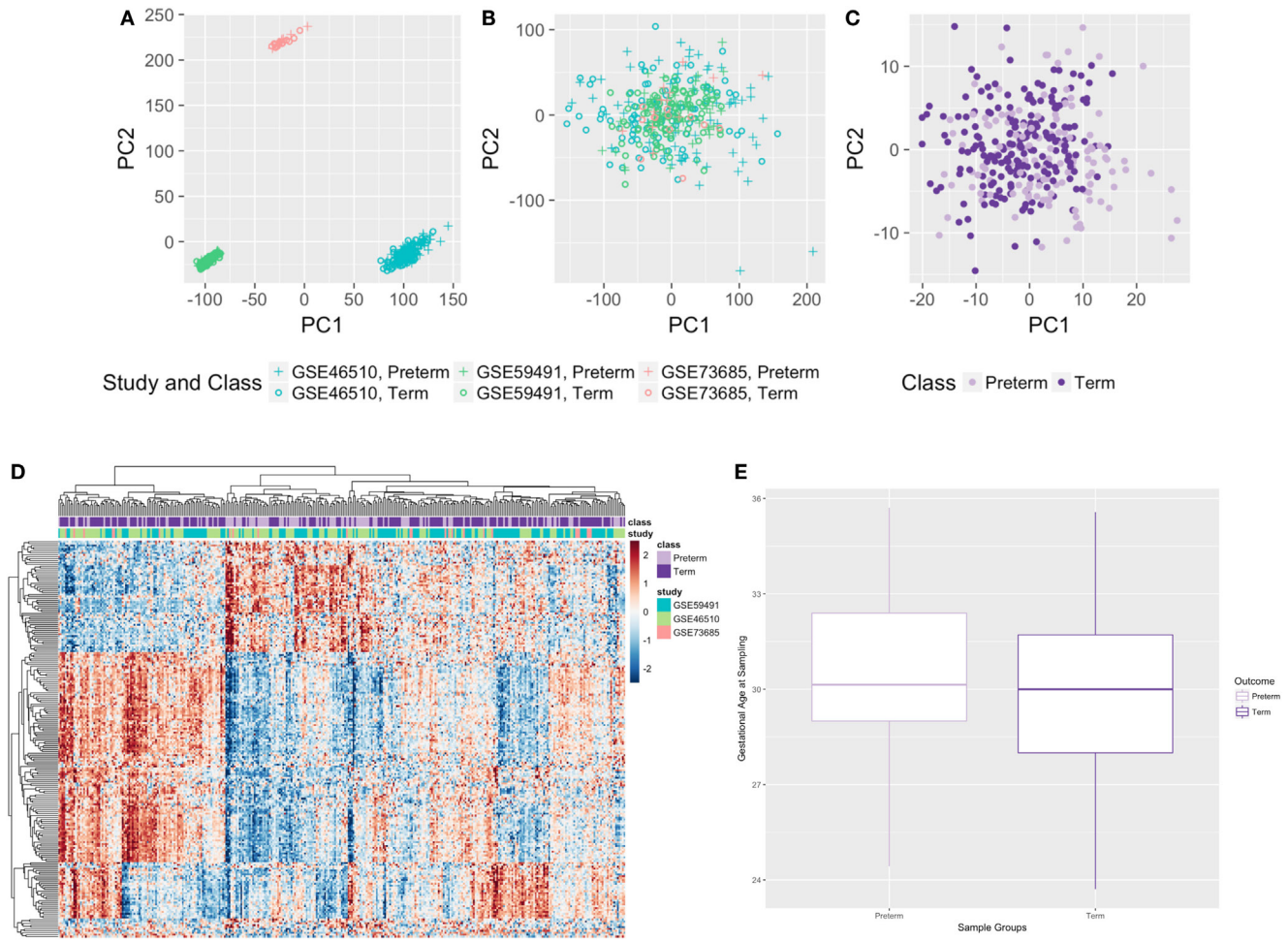


FIGURE 2 | Results from the cross-study meta-analysis and distribution of gestational age at sampling. **(A,B)** Principal component analysis plots with all genes before **(A)** and after **(B)** ComBat. **(C,D)** Principal component analysis plot **(C)** and heatmap **(D)** of all samples based on 210 significant differentially expressed genes. **(E)** Gestational age at sampling was not significantly different between preterm and term maternal whole blood samples (n = 315, p-value = 0.125).

is able to infer 64 various immune and stroma cell types using gene signatures (37).

Our xCell analysis of all 339 samples revealed that there were 27 cell types that were enriched and significantly differentially expressed between preterm and term birth. Macrophages (M2 type) and microvascular endothelial cells demonstrated the largest and most significant (FDR = 0.003) difference in enrichment between preterm and term maternal blood samples (**Figure 5A**) when comparing average xCell scores. We also saw some clustering of preterm and term birth samples by immune cell type, with term birth samples showing some clustering and upregulation of adaptive immune cells, such as Th2 cells, CD8+ T-cells, CD4+ T-cells, and B-cells, and PTB samples showing some clustering and upregulation of innate immune cells, such as NKT, macrophages M2, basophils, and neutrophils (**Figure 5B**). Adjusting for significant cell types as a covariate in our differential expression analysis for T3 samples resulted in 334 genes that were differentially expressed in PTB compared with term birth. Upon pathway analysis, we found that the innate immune pathway was upregulated in the preterm samples, which is consistent with our initial results.

Additional Analysis of Maternal Signatures in the Second Trimester

To investigate and compare expression profiles at two different time points in pregnancy, we utilized the samples collected at second trimester in the GSE59491 study and performed an additional analysis investigating whether any of our significant genes from the third trimester analysis were differentially expressed at an earlier time point to facilitate potential biomarker identification. Implementing an FDR < 0.1 on the filtered list of 210 genes, there were 18 genes (8 upregulated and 10 downregulated) that were significantly differentially expressed (**Figure 6A**; Table S3 in Supplementary Material).

These 18 genes, which were differentially expressed in PTB relative to term birth at the second trimester (17–20 weeks) and the third trimester (24–36 weeks), showed similar FC direction

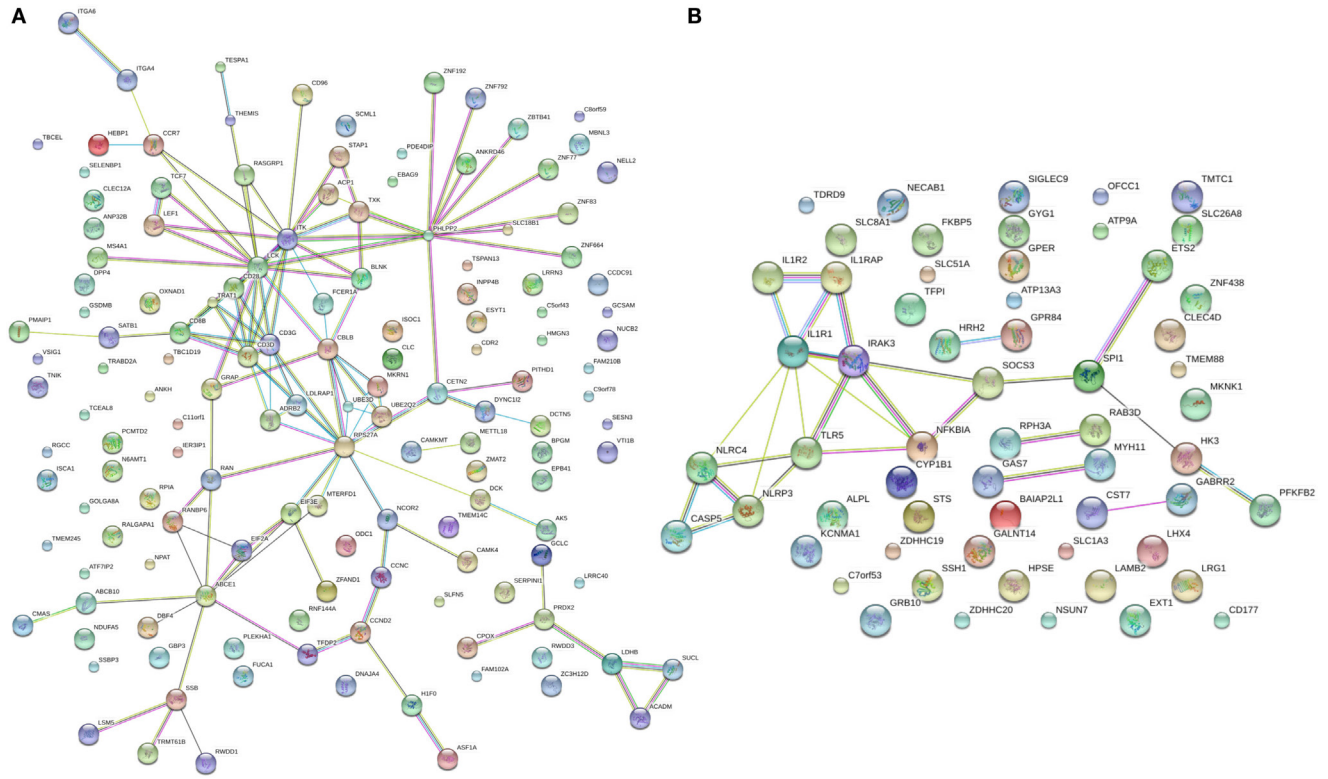


FIGURE 3 | STRING connectivity networks based on 210 differentially expressed genes. **(A,B)** Connectivity networks for significantly downregulated **(A)** and upregulated **(B)** genes from meta-analysis.

and values when comparing second trimester samples from GSE59491 and the samples from the cross-study meta-analysis (Table S4 in Supplementary Material). Furthermore, when plotting the raw expression data for the second and third trimester samples from GSE59491 for these 18 genes, the same trends were upheld, demonstrating similar FC direction and values between the two groups (Figure S1 in Supplementary Material). 2 of these 18 genes (*IL-1R1* and *TFPI*) showed potential as diagnostic biomarkers; they were found to be secreted and detectable in human plasma in the UniProt database (35) (**Table 3**) and upheld the same fold-change directionality in both second and third trimester samples (**Figures 6B,C**).

Upstream Transcription and Cytokine Regulation Analysis in Maternal Signatures

To better understand the differential expression patterns, we explored the upstream regulation of differentially expressed upregulated genes for the second and third trimester separately. We first created a transcription factor regulation network for the second and third trimester (**Figures 7A,B**). In **Figure 7A**, we found four regulators for only two of the second trimester differently expressed genes. Out of the four regulators, one transcription factor, *BCL6* (38), has been shown before to regulate components of the immune system and another, *MXD1*, is involved in cell proliferation (39). In **Figure 7B**, we found nine regulators for 46 of the third trimester differently expressed genes. Out of those nine, a few are known to be involved in development of the immune system, such as *SPI1* (40), *BCL6*, and *UXT* (41) while others are involved in embryonic cell development such as *CBX5* (42), *RUNX2* (43), and *TCF3* (44). The overlap between the groups is two transcription factors, *BCL6* and *MXD1*.

We then explored cytokine regulation in differentially expressed upregulated genes in second and third trimesters. In both trimesters, as shown clearly in **Figures 7C,D**, *IL-7* is the only cytokine we found to be involved in the regulation. Given the known role for *IL-7* signaling in lymphocyte differentiation, this finding is also consistent with the immune signature we observed.

Based on those four regulatory networks and two modes of regulation, we see enrichment of transcription factors involved with the immune system and with cell proliferation.

Differential Gene Expression Analysis in Samples From Other Tissues

Since GSE73685 contained a set of diverse tissues, we also evaluated transcriptional signal in various maternal and fetal

TABLE 2 | Functionally enriched pathways from cross-study meta-analysis.

ID	Name	Source	*p*-Value	FDR B&H	Genes from input	Genes in annotation
Upregulated						
M12095	Signal transduction through *IL1R**	MSigDB C2 BIOCARTA (v5.1)	6.17E−06	3.04E−03	4	33
1269320	Interleukin-1 signaling*	BioSystems: REACTOME	2.38E−05	5.86E−03	4	46
1457780	Neutrophil degranulation*	BioSystems: REACTOME	6.52E−05	1.07E−02	9	492
1269203	Innate Immune System*	BioSystems: REACTOME	1.81E−04	2.23E−02	14	1312
137944	*IL1*-mediated signaling events*	BioSystems: Pathway Interaction Database	2.84E−04	2.54E−02	3	35
82974	Starch and sucrose metabolism	BioSystems: KEGG	3.10E−04	2.54E−02	3	36
Downregulated						
M1467	The Co-Stimulatory Signal During T-cell Activation*	MSigDB C2 BIOCARTA (v5.1)	3.46E−07	3.21E−04	5	21
83080	T cell receptor signaling pathway*	BioSystems: KEGG	8.04E−07	3.66E−04	8	103
138055	TCR signaling in naive CD8+ T cells*	BioSystems: Pathway Interaction Database	1.24E−06	3.66E−04	6	48
1269171	Adaptive Immune System*	BioSystems: REACTOME	1.58E−06	3.66E−04	20	826
137998	TCR signaling in naive CD4+ T cells*	BioSystems: Pathway Interaction Database	4.71E−06	8.73E−04	6	60
1269175	Generation of second messenger molecules	BioSystems: REACTOME	5.89E−06	9.10E−04	5	36
1269174	Translocation of ZAP-70 to Immunological synapse*	BioSystems: REACTOME	1.77E−05	2.10E−03	4	22
M9526	T Cell Signal Transduction*	MSigDB C2 BIOCARTA (v5.1)	1.81E−05	2.10E−03	5	45
1269173	Phosphorylation of CD3 and TCR zeta chains*	BioSystems: REACTOME	3.00E−05	2.98E−03	4	25
1269172	TCR signaling*	BioSystems: REACTOME	3.32E−05	2.98E−03	7	124
1269182	PD-1 signaling*	BioSystems: REACTOME	3.53E−05	2.98E−03	4	26
M16519	HIV Induced T Cell Apoptosis*	MSigDB C2 BIOCARTA (v5.1)	5.98E−05	4.62E−03	3	11
83078	Hematopoietic cell lineage*	BioSystems: KEGG	7.48E−05	5.34E−03	6	97
M10765	Lck and Fyn tyrosine kinases in initiation of TCR Activation*	MSigDB C2 BIOCARTA (v5.1)	1.03E−04	6.46E−03	3	13
1269176	Downstream TCR signaling*	BioSystems: REACTOME	1.05E−04	6.46E−03	6	103
M13247	T Cytotoxic Cell Surface Molecules*	MSigDB C2 BIOCARTA (v5.1)	1.30E−04	7.07E−03	3	14
M6427	T Helper Cell Surface Molecules*	MSigDB C2 BIOCARTA (v5.1)	1.30E−04	7.07E−03	3	14
83125	Primary immunodeficiency*	BioSystems: KEGG	1.47E−04	7.55E−03	4	37
1269177	Costimulation by the CD28 family*	BioSystems: REACTOME	1.90E−04	9.25E−03	5	73
169352	Regulation of Wnt-mediated beta catenin signaling and target gene transcription	BioSystems: Pathway Interaction Database	2.75E−04	1.27E−02	5	79
1269183	Signaling by the B Cell Receptor (BCR)*	BioSystems: REACTOME	3.25E−04	1.42E−02	8	236
M16966	Stathmin and breast cancer resistance to antimicrotubule agents	MSigDB C2 BIOCARTA (v5.1)	3.36E−04	1.42E−02	3	19
M18215	Role of Tob in T-cell activation*	MSigDB C2 BIOCARTA (v5.1)	4.57E−04	1.83E−02	3	21
1269201	Immunoregulatory interactions between a Lymphoid and a non-Lymphoid cell*	BioSystems: REACTOME	4.74E−04	1.83E−02	6	136
1270272	Activation of NOXA and translocation to mitochondria	BioSystems: REACTOME	5.21E−04	1.88E−02	2	5
1269102	Nef-mediates down modulation of cell surface receptors by recruiting them to clathrin adapters	BioSystems: REACTOME	5.26E−04	1.88E−02	3	22
M6327	Activation of Csk by cAMP-dependent Protein Kinase Inhibits Signaling through the T Cell Receptor*	MSigDB C2 BIOCARTA (v5.1)	6.84E−04	2.35E−02	3	24
1427859	Cargo recognition for clathrin-mediated endocytosis	BioSystems: REACTOME	7.78E−04	2.58E−02	5	99
137922	*IL12*-mediated signaling events*	BioSystems: Pathway Interaction Database	1.01E−03	3.24E−02	4	61
1269603	Binding of TCF/LEF:CTNNB1 to target gene promoters	BioSystems: REACTOME	1.08E−03	3.24E−02	2	7
137936	*IL12* signaling mediated by STAT4*	BioSystems: Pathway Interaction Database	1.08E−03	3.24E−02	3	28
1269100	The role of Nef in HIV-1 replication and disease pathogenesis*	BioSystems: REACTOME	1.20E−03	3.49E−02	3	29
83004	Propanoate metabolism	BioSystems: KEGG	1.61E−03	4.52E−02	3	32
1269298	Fc epsilon receptor (FCERI) signaling*	BioSystems: REACTOME	1.84E−03	4.94E−02	9	381
117293	Arrhythmogenic right ventricular cardiomyopathy (ARVC)	BioSystems: KEGG	1.88E−03	4.94E−02	4	72
1269528	SMAD2/SMAD3:SMAD4 heterotrimer regulates transcription	BioSystems: REACTOME	1.92E−03	4.94E−02	3	34

*Pathways annotated with a * are immune related.*

FDR B&H, false discovery rate using Benjamini–Hochberg method; Genes from input, number of significant genes included in given pathways; Genes in annotation, number of genes involved in functional pathway; MSigDB C2 BIOCARTA, Molecular Signatures Database curated gene set derived from BIOCARTA database; KEGG, Kyoto Encyclopedia of Genes and Genomes.

tissues separately. With an FDR < 0.05, only one of the tissue types, CB, showed significant differentially expressed genes. Imposing a fold-change cutoff of 1.3 on the 507 genes that were identified from the differential expression analysis resulted in 473 significant genes (Table S5 in Supplementary Material), 165 upregulated and 308 downregulated genes in PTB relative to term birth, which clustered to create a distinct separation between PTB and term birth (**Figure 8A**). Based on the ToppFun database, 308

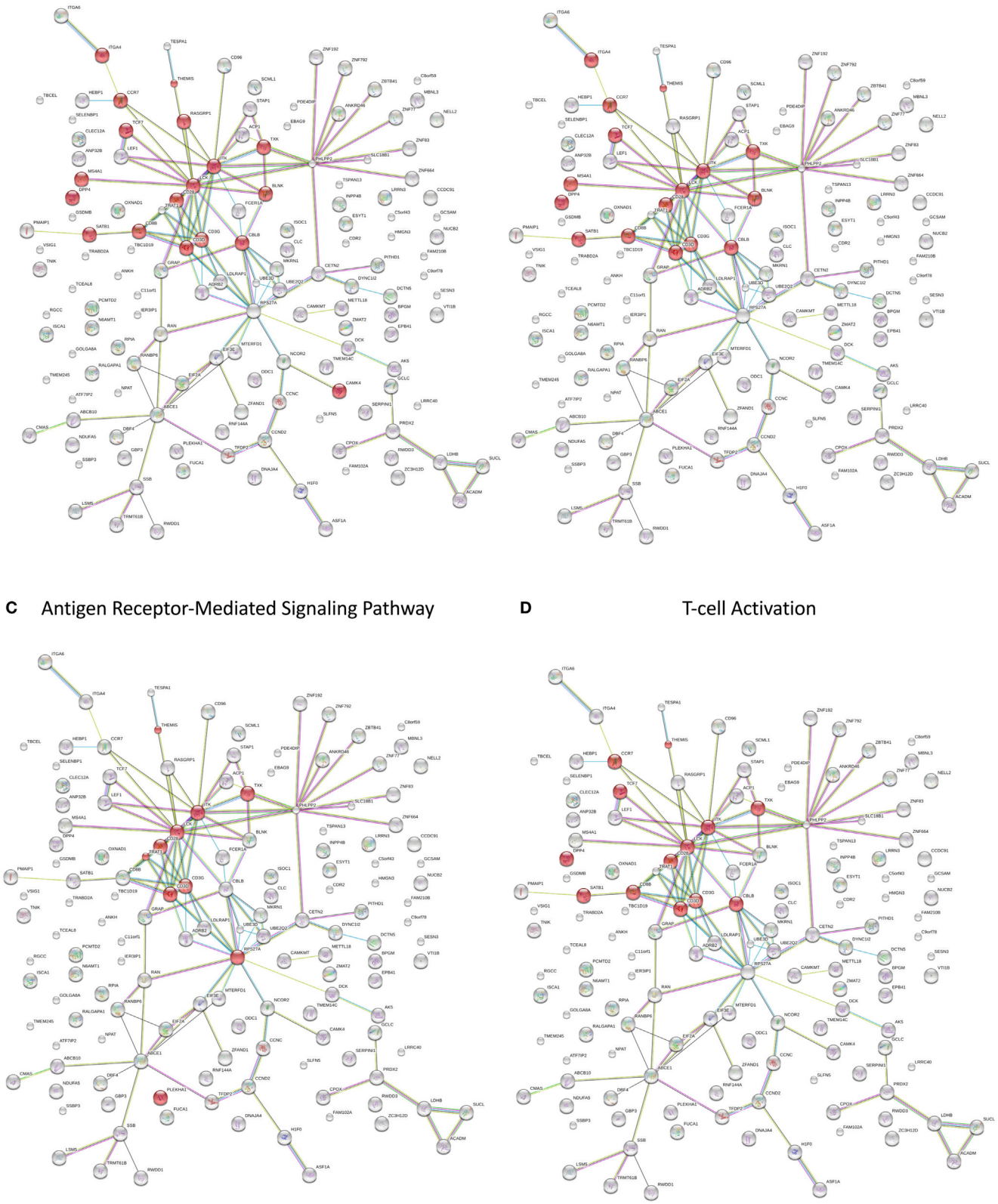


FIGURE 4 | **(A–D)** Network visualization of functionally enriched GO biological processes in significantly downregulated genes from the meta-analysis.

TABLE 3 | Secreted proteins from meta-analysis and T2 *ad hoc* analysis.

Genes	FC_GSE46510	FC_GSE59491	FC_GSE73685	Directionality	*p*-Value	Adj *p* value
HPSE	1.12502566	1.029755132	1.302481986	Upregulated	0.01472767	0.072407187
NLRP3	1.120388112	1.045936747	1.463420748	Upregulated	0.008637567	0.055169509
LRG1	1.304600844	1.012782905	1.266657092	Upregulated	0.001694397	0.02445227
CLC	0.77816604	0.925657768	0.460056969	Downregulated	0.013608709	0.06964973
DPP4	0.906503094	0.939578805	0.7254311	Downregulated	0.007598762	0.051200829
*IL1R1**	1.088237667	1.085324456	1.324171306	Upregulated	0.006616252	0.047794152
IL1RAP	1.163741033	1.016218763	1.310389926	Upregulated	0.017000877	0.07821447
LAMB2	1.05178463	1.022848768	1.305601216	Upregulated	0.004096624	0.037683905
NELL2	0.785324082	0.91373226	0.642718858	Downregulated	0.000198362	0.010174565
NUCB2	0.749613912	0.965573738	0.800992598	Downregulated	0.000657135	0.01646351
SERPINI1	0.920203336	0.872420873	0.755891687	Downregulated	0.000180817	0.009596761
*TFPI**	1.044097212	1.117168095	0.750437685	Upregulated	0.009325712	0.057394342
IL1R2	1.231921476	1.051452709	1.625438109	Upregulated	0.004772589	0.040619724
CST7	1.188969828	1.033458464	1.334064851	Upregulated	0.003500353	0.034868265
CD8B	0.856761369	0.909815767	0.742642806	Downregulated	0.006032779	0.045913205

*Genes with * annotation are also found to be significant in the T2 analysis.*
FC_GSE46510, fold-change calculated using GSE46510 samples; FC_GSE59491, fold-change calculated using GSE59491 samples; FC_GSE73685, fold-change calculated using GSE73685 samples; Adj p val, adjusted p-value.

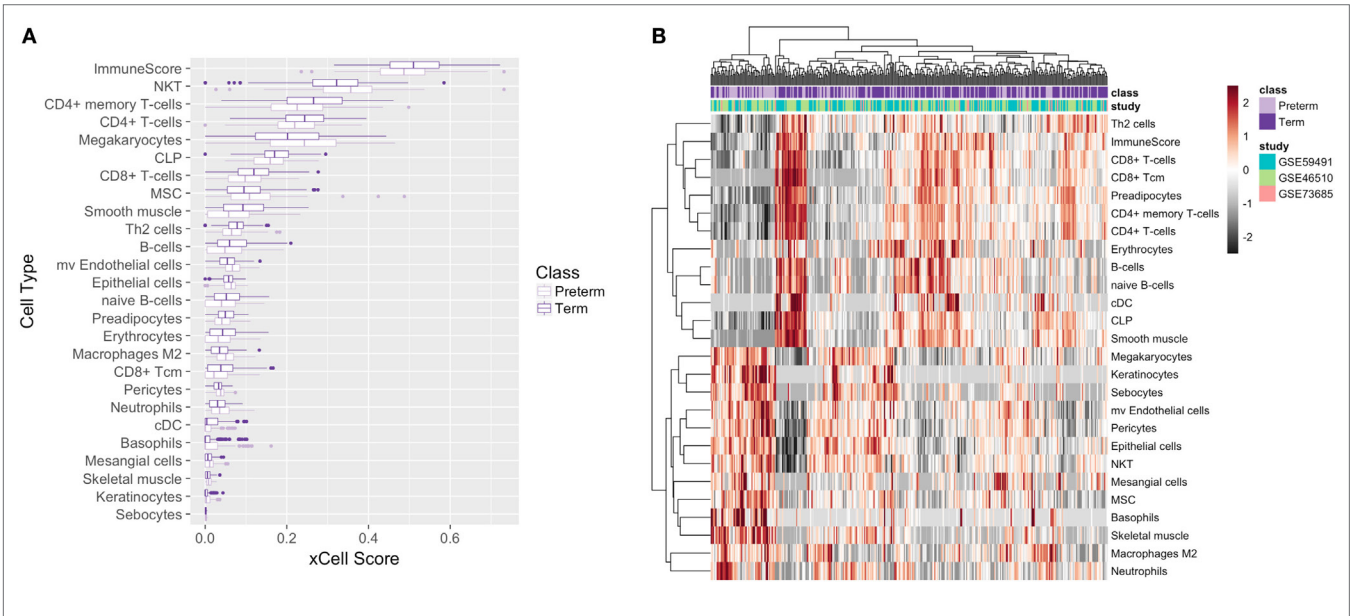


FIGURE 5 | Cell deconvolution of 339 meta-analysis samples. Boxplot **(A)** and heatmap **(B)** of average xCell scores for enriched cell types.

downregulated genes were highly enriched in multiple functional pathways, many of which were immune related (**Table 4**) (34). Specifically, PTB samples showed downregulation of many innate immune-related pathways relative to term birth samples. Conversely, the 165 upregulated genes showed low-functional pathway enrichment (**Table 4**) (34).

Comparing these 473 significant genes from the CB analysis to the 210 significant genes output from the maternal blood meta-analysis, we found that there were 13 genes including toll-like receptor 5 (*TLR5*) and other immune transcripts which overlapped and were significant in both analyses. Plotting the raw data for these 13 genes from GSE73685 revealed opposite directionality comparing preterm and term birth for CB and maternal blood, respectively (**Figure 8B**). While some genes were upregulated in preterm maternal whole blood samples (in both the meta-analysis and GSE73685 only samples), those same genes were downregulated in preterm CB samples; the same was true for many genes which were downregulated in preterm maternal whole blood samples but upregulated in preterm CB samples.

All the results and the data are available as an RShiny Application for the benefit of the research community: http://comphealth.ucsf.edu/preterm_transcriptomics/.

DISCUSSION

Given the role of the immune system in pregnancy, there exists a need to elucidate immune signatures specific to PTB at both the

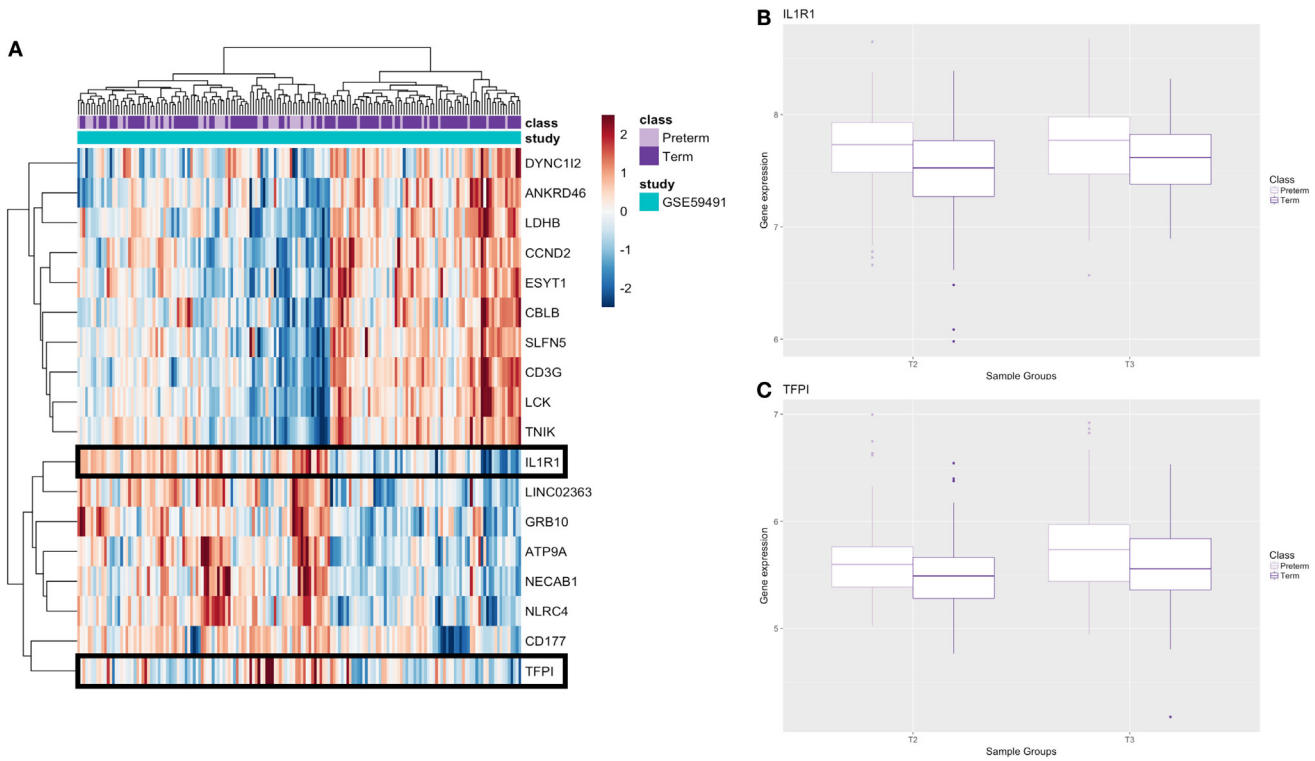


FIGURE 6 | Results from additional second trimester analysis. **(A)** Heatmap of significant genes from second trimester analysis; genes which are secreted as proteins are boxed. **(B,C)** Boxplots of genes that encode secreted proteins at second (T2) and third (T3) trimester; raw gene expression values from GSE59491 are plotted.

maternal and fetal level. This study was thus designed to answer these questions by aggregating data from multiple independent experiments in an attempt to discover significant, differential genetic signatures in women who deliver preterm. Our cross-study meta-analyses revealed 210 differentially expressed genes, 15 of which were found to be secreted in the plasma. Interestingly, 18 of these 210 genes also demonstrated differential expression in the second trimester, suggesting a possibility for early identification of patients who might deliver preterm. *IL-1R1* and *TFPI*, both of which encode immune-related proteins, were found to be differentially expressed and secreted longitudinally. CB analysis also revealed significant differential gene expression and had clustering in immune related pathways. In contrast to preterm maternal whole blood, which showed upregulation of innate immunity and downregulation of adaptive immunity, CB showed downregulation in innate immunity. This juxtaposition, as well as the heavy involvement of immune-related pathways and biomarkers, bring to light novel findings which coincide with previous literature.

Leveraging Transcriptomics to Identify New Biomarkers for sPTB

There is a crucial need to find biomarkers for PTB. There are classic negative predictors such as the absence of fetal fibronectin in the cervicovaginal fluid, but they are less useful as a routine screening tool to identify women with high risk of PTB (45–47). Identifying biomarkers predictive of PTB in maternal blood seems like an easier target as blood is easily accessible and can be collected in most women as part of the standard prenatal care (27). In our study, we found nine upregulated genes that encode secreted proteins in human (48). These markers may be further investigated regarding their values as biomarkers for identifying high-risk women for PTB, especially *IL-1R1* and *TFPI* that were significantly over expressed among PTB cases as early as during second trimester.

IL-1 receptor type I belongs to the *IL-1* family of receptors which contains 10 distinct but related gene products all of which are heavily involved in the innate immune response. This receptor has a variety of ligands which are involved in the initiation (*IL-1α* and *IL-1β*) and inhibition (*IL-1Ra*) of the immune and inflammatory responses (49). *IL-1α* belongs to a group of dual-function cytokines, constitutively present inside cells under normal homeostatic condition and playing a role as a transcription regulator to trigger inflammation and immunity extracellularly (50). This ligand has been shown to induce an inflammatory response in absence of infection as well as is responsible for the stimulation and release of *IL-1β* from monocytes (51). Conversely, *IL-1β* is not expressed in homeostatic conditions and is active only upon cleavage of its precursor caspase-1 (50). Although *IL-1Iα* is the

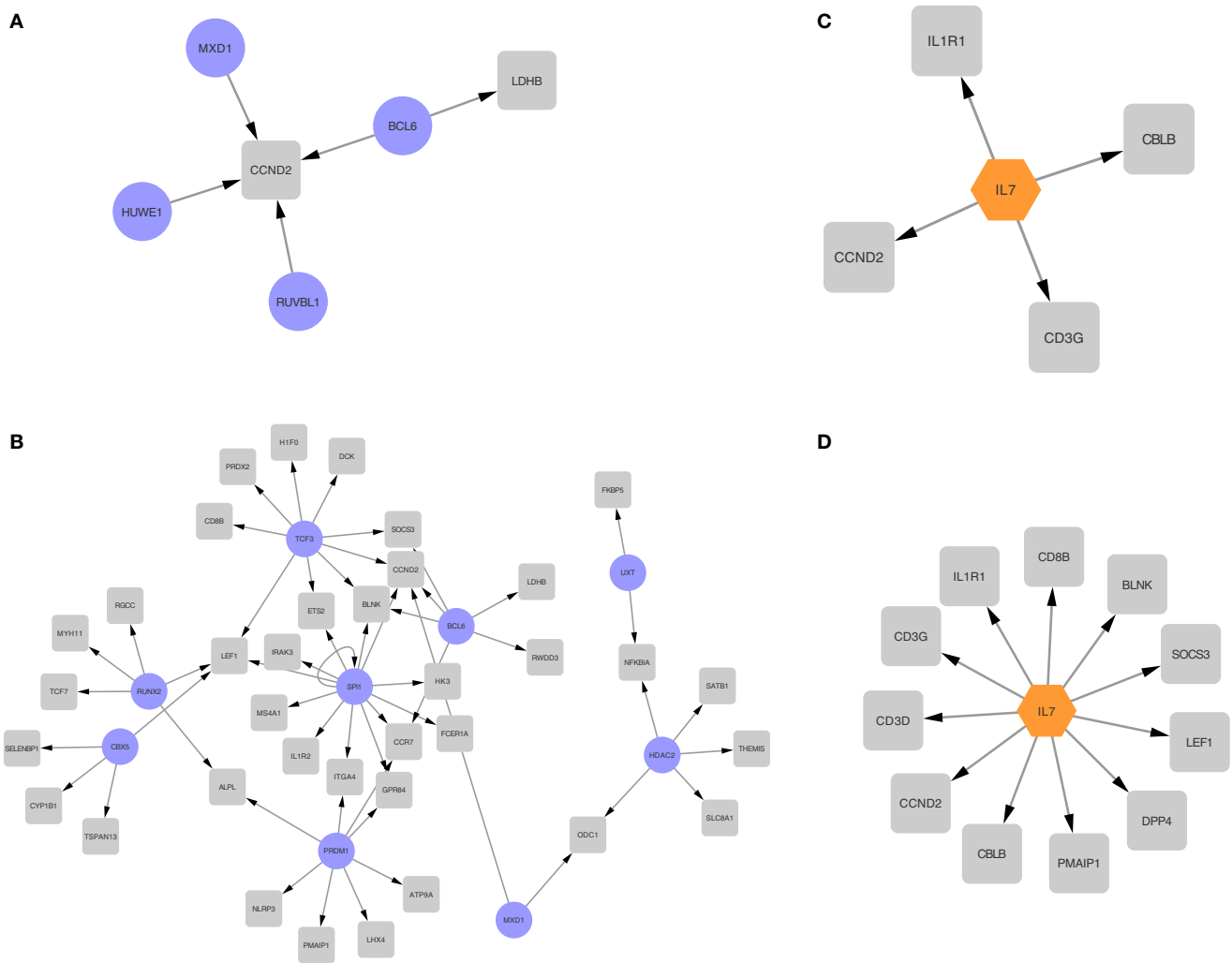


FIGURE 7 | Regulatory networks for second and third trimester differentially expressed genes. Transcription regulation networks for differentially expressed genes in the second trimester **(A)** and third trimester **(B)**, where the transcription factors are represented with a purple round node and the differentially expressed targets are represented with a gray square node. Cytokine networks for second trimester **(C)** and third trimester **(D)**, where the transcription factors are represented with an orange hexagon node and the differentially expressed targets are represented with a gray square node.

initiator of sterile inflammation *IL-1β* has been shown to play a role as an amplifier of inflammation (50, 51). The binding of either of these molecules to *IL-1R1* leads to the activation of many transcription factors including nuclear factor-kappa B (*NF-kB*) and ultimately leads to an inflammatory response (49).

IL-1 receptor type I has been studied as one of the potential biomarkers to predict heart failure in hypertensive patients (52) and was proposed as a candidate molecular target for rheumatoid arthritis treatment (53). In the pregnancy space, *IL-1R1* has been investigated in endometrial tissues and chorioamnionitis (54, 55) and has been found to be increased in PTBs stimulated by RU486 in rats (56). One study found an aberrant placental expression of interleukin 1 receptor-like 1 (*IL-1RL1*) in PTB cases (compared with spontaneous term births) whose mRNA transcript were of higher detection in maternal plasma samples than their gestational age-matched controls that had term birth, suggesting *IL-1RL1* to be a candidate PTB-associated marker (57). Other cytokines have also been identified as PTB biomarkers, including *IL6*, *IL-1β*, and *IL2* (26). In case of infection, blocking a single factor on the pathway may not be sufficient to prevent preterm delivery (58). Our finding suggests that *IL-1R1* could be one of the detectable markers of the dysregulated inflammatory network associated with PTB that bears further investigation.

The overall signature we observed is consistent with previously published literature supporting a role for the inflammasome and activation of the innate immune system in the onset of spontaneous preterm labor. For example, activation of the *NLRP3* inflammasome, which ultimately results in increased levels of mature *IL-1β*, has also been implicated in patients (59). There are increased levels of *IL-1β* in the amniotic fluid of patients with preterm labor (60) as well as in the chorioamniotic membranes (59). A GWAS study also reported that polymorphisms in the *IL1R*

antagonist locus were associated with PTB (61, 62). In mouse models, introduction of *IL1* can induce PTB by activating the innate immune system, and blockade of *IL1R* can abrogate this phenotype (63). Given the extensive downstream effects of this signaling pathway in influencing neonatal morbidity in preterm infants (64), our findings have clinical relevance for discovering targetable molecular pathways.

Tissue factor is a key element for normal gestation (65). Maternal plasma concentrations of total *TFPI*, the main physiological inhibitor of the tissue factor-dependent pathway of blood coagulation, is shown to increase during the first half of pregnancy, remain relatively constant in the remaining half, and decrease during labor (66–68). Different profiles of maternal plasma tissue factor and *TFPI* concentrations have been observed among several obstetrical syndromes including preeclampsia (69), preterm prelabor rupture of membranes (70), and small for gestational age (69).

Maternal and Fetal Signals Elucidate the Role of Adaptive and Innate Immunity in PTB

Immunity and inflammation have been shown to play an important role in parturition timing (71–75). Specifically, infection and breakdown of maternal–fetal tolerance (rejection) are the two most important in this respect. These have different association

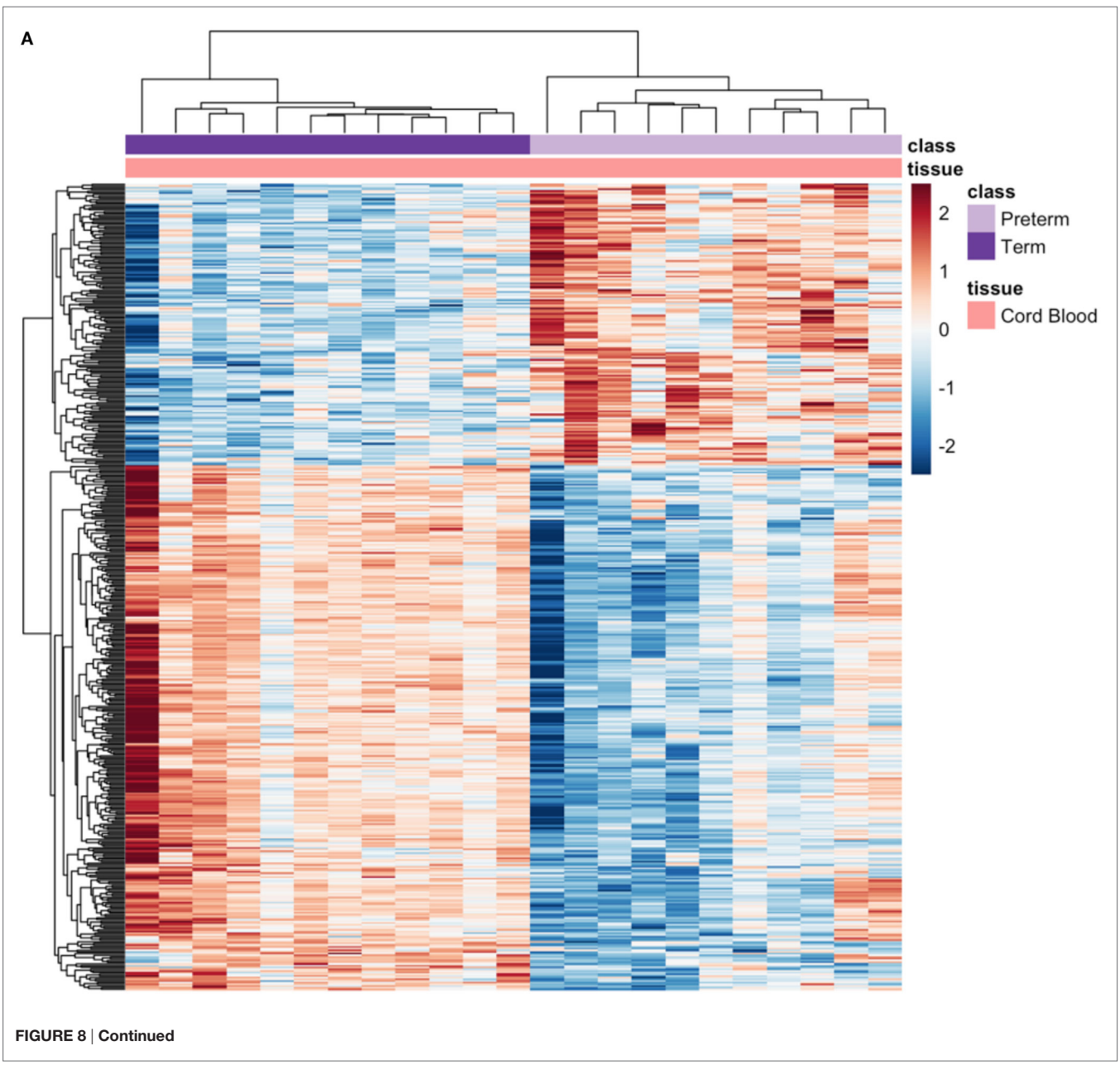


FIGURE 8 | **Continued**

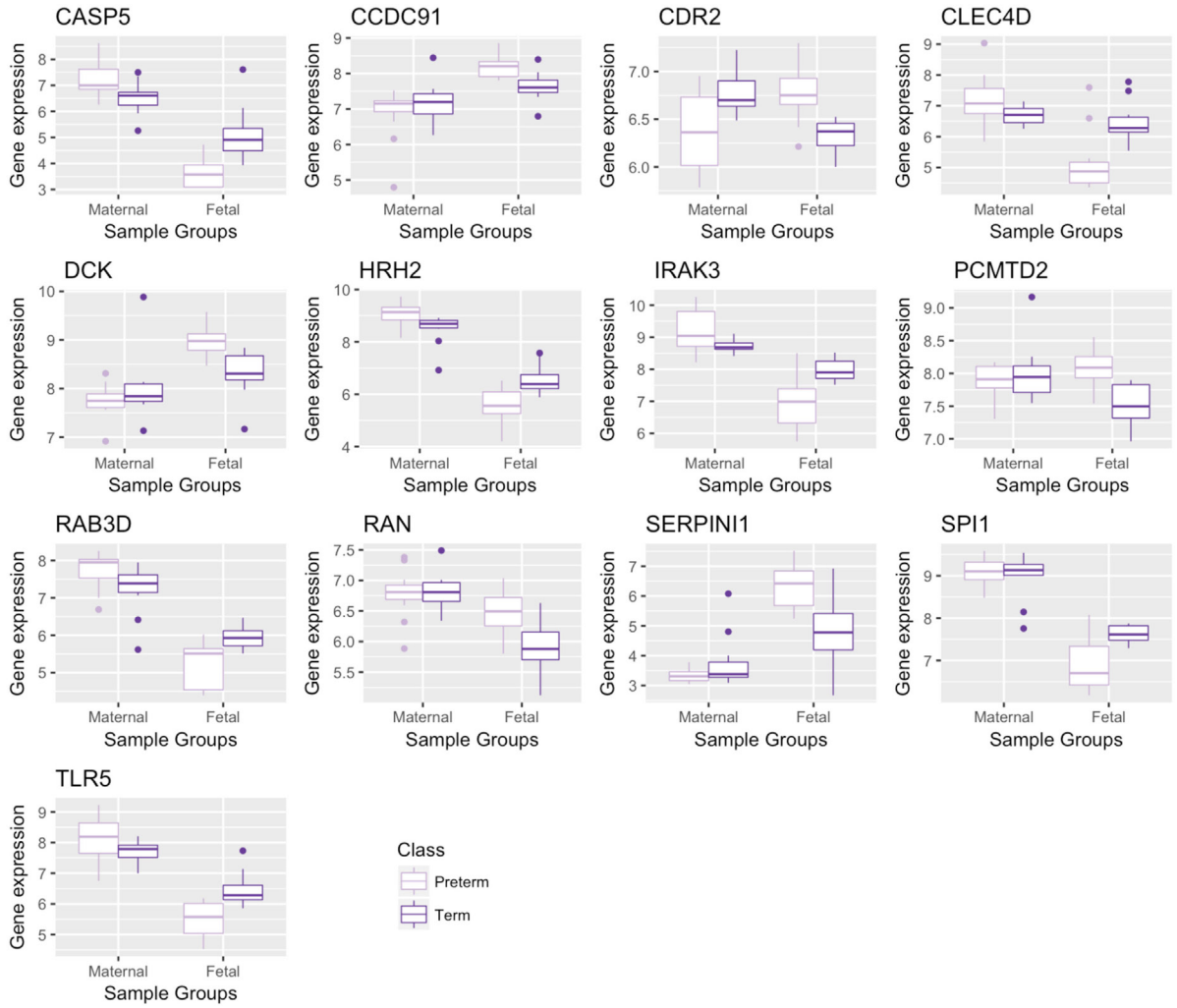


FIGURE 8 | Significant genes from cord blood (CB) tissue analysis and maternal–cord gene signature comparison. **(A)** Heatmap of significant differentially expressed genes from CB analysis. **(B)** Boxplot of overlapping significant genes from meta-analysis and CB analysis; raw gene expression values from GSE73685 plotted.

with gestational age, with infection (76) affecting mainly early PTB while rejection (77) affecting mainly late PTB cases. Healthy pregnancy involves multiple tolerance mechanisms that prevent the maternal and fetal immune systems from recognizing and rejecting each other (78, 79), whereas preterm labor may result from a breakdown in maternal–fetal tolerance (12). Kourtis et al. conclude that aspects of innate immunity are maintained or enhanced during pregnancy, particularly during the second and third trimesters and there are decreases in adaptive immunity seen in later stages of pregnancy (80). Before labor, the maternal immune system modulates inflammatory signaling pathways to avoid rejection of the fetus. Conversely, in pregnancies with PTB, the fetal immune system might undergo activation, resulting in recognition and rejection of maternal antigens. Implications of pregnancy as a modulated immunological condition are vast including prevention of fetal rejection, susceptibility to some infections and maybe even PTB (80).

The upregulated and downregulated gene signatures identified in the maternal meta-analysis demonstrate a clear enrichment in immune-related pathways. When looking at the regulation of the differentially expressed genes, we found that transcription factors regulating the differentially expressed genes were also immune-related transcription factors. Looking at cytokine data, we found *IL-1*-related pathways are upregulated during the third trimester for women who deliver preterm. This supports the upregulation of inflammatory pathways involving cytokines and their receptors among PTB cases reported by Heng et al.'s (28) study, whose data were included in the current meta-analysis. Genes encoding *IL-1α* and *IL-1β*, two founding members of the *IL-1* family that have played a central role in several autoinflammatory diseases (81–83), and other cytokines such as *IL-6* are also upregulated in our study, despite not being statistically significant after multiple testing correction. Past research suggests that pro-inflammatory cytokines *IL-1β* and *TNF-α* play a primary role in inducing

TABLE 4 | Functionally enriched pathways from cord blood tissue analysis.

ID	Name	Source	*p*-Value	FDR B&H	Genes from input	Genes in annotation
Upregulated						
169351	Validated targets of *C-MYC* transcriptional activation	BioSystems: Pathway Interaction Database	7.733E−08	0.00008437	9	81
Downregulated						
1269203	Innate Immune System*	BioSystems: REACTOME	7.768E−26	9.578E−23	81	1312
1457780	Neutrophil degranulation*	BioSystems: REACTOME	8.786E−25	5.417E−22	50	492
213780	Tuberculosis*	BioSystems: KEGG	5.678E−07	0.0002334	15	179
83051	Cytokine-cytokine receptor interaction*	BioSystems: KEGG	0.000001234	0.0003804	18	270
469200	Legionellosis*	BioSystems: KEGG	0.000004663	0.0009906	8	55
144181	Leishmaniasis*	BioSystems: KEGG	0.00000482	0.0009906	9	73
1427857	Regulation of *TLR* by endogenous ligand*	BioSystems: REACTOME	0.000005868	0.001034	5	16
M9546	Chaperones modulate interferon Signaling Pathway*	MSigDB C2 BIOCARTA (v5.1)	0.00001497	0.002122	5	19
1269204	Toll-Like Receptors Cascades*	BioSystems: REACTOME	0.00001549	0.002122	12	153
1269310	Cytokine Signaling in Immune system*	BioSystems: REACTOME	0.00002554	0.002599	30	763
1269158	*IRAK4* deficiency (*TLR2/4*)*	BioSystems: REACTOME	0.0000274	0.002599	4	11
PW:0000234	Innate immune response*	Pathway Ontology	0.0000274	0.002599	4	11
1269160	*MyD88* deficiency (*TLR2/4*)*	BioSystems: REACTOME	0.0000274	0.002599	4	11
634527	*NF-kappa B* signaling pathway*	BioSystems: KEGG	0.00004183	0.003636	9	95
122191	NOD-like receptor signaling pathway*	BioSystems: KEGG	0.00004423	0.003636	12	170
1269156	Diseases of Immune System*	BioSystems: REACTOME	0.00005093	0.003694	5	24
1269157	Diseases associated with the *TLR* signaling cascade*	BioSystems: REACTOME	0.00005093	0.003694	5	24
1269318	Signaling by Interleukins*	BioSystems: REACTOME	0.00005712	0.003913	23	531
M13968	HIV-I Nef: negative effector of Fas and TNF*	MSigDB C2 BIOCARTA (v5.1)	0.00006456	0.00419	7	58
138052	Ephrin B reverse signaling	BioSystems: Pathway Interaction Database	0.00009269	0.005531	5	27
193147	Osteoclast differentiation	BioSystems: KEGG	0.0000942	0.005531	10	130
1383066	*TP53* Regulates Transcription of Cell Death Genes	BioSystems: REACTOME	0.0001239	0.006944	6	45
P00031	Inflammation mediated by chemokine and cytokine signaling pathway*	PantherDB	0.0001357	0.007273	12	191
1269545	Class A/1 (Rhodopsin-like receptors)	BioSystems: REACTOME	0.0001744	0.00896	16	322
1269236	Activated *TLR4* signaling*	BioSystems: REACTOME	0.0001852	0.009001	9	115
114228	Fc gamma R-mediated phagocytosis*	BioSystems: KEGG	0.0001898	0.009001	8	91
217173	Influenza A*	BioSystems: KEGG	0.0002322	0.01017	11	173
1269239	Toll-Like Receptor *TLR1*:*TLR2* Cascade*	BioSystems: REACTOME	0.0002557	0.01017	8	95
1269238	Toll-Like Receptor 2 (*TLR2*) Cascade*	BioSystems: REACTOME	0.0002557	0.01017	8	95
1269237	*MyD88*:Mal cascade initiated on plasma membrane*	BioSystems: REACTOME	0.0002557	0.01017	8	95
1269240	Toll-Like Receptor *TLR6*:*TLR2* Cascade*	BioSystems: REACTOME	0.0002557	0.01017	8	95
137995	HIV-1 Nef: Negative effector of Fas and TNF-alpha*	BioSystems: Pathway Interaction Database	0.0003326	0.01282	5	35
99051	Chemokine signaling pathway*	BioSystems: KEGG	0.0003596	0.01294	11	182
137910	*CXCR4*-mediated signaling events*	BioSystems: Pathway Interaction Database	0.0003598	0.01294	7	76
1269234	Toll-Like Receptor 4 (*TLR4*) Cascade*	BioSystems: REACTOME	0.0003674	0.01294	9	126
147809	Chagas disease (American trypanosomiasis)*	BioSystems: KEGG	0.0004156	0.01403	8	102
172846	Staphylococcus aureus infection*	BioSystems: KEGG	0.0004209	0.01403	6	56
1457777	Antimicrobial peptides*	BioSystems: REACTOME	0.0005053	0.0164	8	105
1269280	*FCGR* activation*	BioSystems: REACTOME	0.0005221	0.01651	4	22
213306	Measles*	BioSystems: KEGG	0.0005771	0.01779	9	134
M15285	*NF-kB* Signaling Pathway*	MSigDB C2 BIOCARTA (v5.1)	0.0006234	0.01875	4	23
138022	Class I *PI3K* signaling events	BioSystems: Pathway Interaction Database	0.0007051	0.02047	5	41
83060	Apoptosis	BioSystems: KEGG	0.0007139	0.02047	9	138
375172	*Salmonella* infection*	BioSystems: KEGG	0.0007631	0.02138	7	86
169642	Toxoplasmosis*	BioSystems: KEGG	0.0008232	0.02256	8	113
1269161	*MyD88* deficiency (*TLR5*)*	BioSystems: REACTOME	0.0009052	0.02375	2	3
1269566	Hydroxycarboxylic acid-binding receptors	BioSystems: REACTOME	0.0009052	0.02375	2	3
1269303	C-type lectin receptors (CLRs)*	BioSystems: REACTOME	0.00112	0.02877	9	147
137964	Regulation of p38-alpha and p38-beta*	BioSystems: Pathway Interaction Database	0.00117	0.02937	4	27
1269576	G alpha (i) signaling events	BioSystems: REACTOME	0.001191	0.02937	12	243
1270241	Signal regulatory protein (*SIRP*) family interactions*	BioSystems: REACTOME	0.00133	0.03216	3	13
1269308	Dectin-2 family*	BioSystems: REACTOME	0.00154	0.03607	4	29

(*Continued*)

TABLE 4 | Continued

ID	Name	Source	*p*-Value	FDR B&H	Genes from input	Genes in annotation
153910	Phagosome*	BioSystems: KEGG	0.001551	0.03607	9	154
1470924	Interleukin-10 signaling*	BioSystems: REACTOME	0.001602	0.03617	5	49
1269546	Peptide ligand-binding receptors	BioSystems: REACTOME	0.001752	0.03617	10	188
1269332	*TNF*s bind their physiological receptors*	BioSystems: REACTOME	0.001753	0.03617	4	30
137974	Caspase cascade in apoptosis*	BioSystems: Pathway Interaction Database	0.001755	0.03617	5	50
138017	Signaling events mediated by *PTP1B*	BioSystems: Pathway Interaction Database	0.001755	0.03617	5	50
PW:0000681	FasL mediated signaling pathway*	Pathway Ontology	0.001789	0.03617	2	4
1269159	*IRAK4* deficiency (*TLR5*)*	BioSystems: REACTOME	0.001789	0.03617	2	4
PW:0000464	leukotriene metabolic*	Pathway Ontology	0.001789	0.03617	2	4
83099	Amyotrophic lateral sclerosis (ALS)	BioSystems: KEGG	0.001919	0.03816	5	51
P00020	*FAS* signaling pathway	PantherDB	0.001986	0.03874	4	31
M17681	*IL3* signaling pathway*	MSigDB C2 BIOCARTA (v5.1)	0.002062	0.03874	3	15
M11736	Cytokines can induce activation of matrix metalloproteinases, which degrade extracellular matrix*	MSigDB C2 BIOCARTA (v5.1)	0.002062	0.03874	3	15
P00006	Apoptosis signaling pathway	PantherDB	0.002073	0.03874	7	102
1269195	Antigen processing-Cross presentation*	BioSystems: REACTOME	0.002192	0.04034	7	103
137939	Direct *P53* effectors	BioSystems: Pathway Interaction Database	0.002234	0.04051	8	132
1269357	GPVI-mediated activation cascade*	BioSystems: REACTOME	0.002476	0.04291	5	54
M14775	G alpha s Pathway	MSigDB C2 BIOCARTA (v5.1)	0.002506	0.04291	3	16
1270299	*RIPK1*-mediated regulated necrosis	BioSystems: REACTOME	0.002506	0.04291	3	16
1270298	Regulated Necrosis	BioSystems: REACTOME	0.002506	0.04291	3	16
1269544	*GPCR* ligand binding	BioSystems: REACTOME	0.0027	0.04561	17	455
812256	*TNF* signaling pathway*	BioSystems: KEGG	0.002867	0.04778	7	108
M4891	Regulation of transcriptional activity by PML*	MSigDB C2 BIOCARTA (v5.1)	0.003003	0.04873	3	17
1270264	Ligand-dependent caspase activation*	BioSystems: REACTOME	0.003003	0.04873	3	17
194384	African trypanosomiasis*	BioSystems: KEGG	0.003129	0.04946	4	35
137944	*IL1*-mediated signaling events*	BioSystems: Pathway Interaction Database	0.003129	0.04946	4	35

*Pathways annotated with a * are immune related.*
FDR B&H, FDR using Benjamini–Hochberg method; Genes from input, number of significant genes included in given pathways; Genes in annotation, number of genes involved in functional pathway, MSigDB C2 BIOCARTA, Molecular Signatures Database curated gene set derived from BIOCARTA database; KEGG, Kyoto Encyclopedia of Genes and Genomes; PANTHER, Protein Analysis Through Evolutionary Relationships Classification system.

infection-associated PTB (58, 84). These findings are consistent with the literature and are more reflective of early rather than late PTB. The upregulated inflammatory-related pathways in this study may be in part attributed to clinical or sub-clinical infection. However, diagnosis of infection is often not available in population studies, which precludes further exploration of the contribution of infection in the observed signal.

Based on the maternal data, we found that genes and cell types associated with innate immunity were upregulated in PTB while those relevant to adaptive immunity were downregulated in PTB. Genes identified in the fetal CB analysis showed enrichment in pathways that were immune related but the signature was flipped; innate immunity was downregulated in babies born preterm. One hypothesis is that the immune systems of women who deliver preterm are less responsive to specific foreign antigens such as infections which themselves could lead to PTB, while mothers whose adaptive immunity was stronger were able to maintain the pregnancy due to better immune coping mechanisms. Previously, polymorphisms of genes pertaining to the innate immune system were found to have only moderate effects on subsequent PTB, although they played a functionally relevant role in host immune response (85). On the other hand, babies that were born preterm showed a downregulation of innate immunity, which suggests opposing signals in the maternal and fetal immune tolerance but also could be a result of the incomplete development of immune defense. Since innate immunity serves as the first defense of the human immune systems, weaker innate immunity signals could be indicative of vulnerability and susceptibility to life-threatening infections (86). There is some evidence that this homeostasis of the fetal–maternal immune tolerance can be perturbed during infection, resulting in immune activation and the observed opposing signals can be indicative of the breakdown of the tolerance mechanism leading up to PTB.

Specifically, there are several genes that are reversed in the maternal and fetal signatures. *TLR5* was one of the genes we found to have opposing differential gene expression when comparing mother and fetus. While *TLR5* showed lower expression in PTB CB samples, *TLR5* was upregulated in PTB maternal whole blood samples. *TLR5*, as well as other toll-like receptors, play an important role in pathogen recognition and subsequent activation of the inflammatory innate immune response. *TLR5* (along with *TLR2* and *TLR3*) has previously been implicated in regulation of pro-inflammatory and pro-labor responses in primary human myometrium cells (87). One of the downstream

targets of this gene in the *MyD88*-dependent pathway is *NF-kB*, a critical transcription factor in the activation of genes related to immune and inflammatory responses (88–90). *TLR5* has also been shown to increase production of various pro-inflammatory interleukins including *IL-6* and *IL-8* (87, 91).

Importance of *TLR5* in pregnancy and its association with PTB has been shown repeatedly. The *TLR5* (g.1174C>T) variant, which encodes a non-functional protein, is significantly associated with development of severe bronchopulmonary dysplasia in very low-birth weight infants born prematurely. This evidence shows that the non-functionality of *TLR5* in preterm infants results in an insufficient immune response to flagellated bacteria (92). Furthermore, *TLR5* mRNA expression has repeatedly been found to be increased in the placenta following spontaneous term labor (91, 93).

This study has several limitations that may be encountered in other similar studies. First, we were limited to the number of studies with publicly available data that could be aggregated together for our meta-analysis. In addition, a common shortcoming of using publicly available data is that samples lacked demographic information as well as detailed clinical annotations. Furthermore, samples included in our study are heterogeneous as they came from studies with different design (cohort or case–control), phenotype—late and early PTB, and different populations (dataset GSE46510 consisting samples from women with threatened preterm labor). Yet, the current comparison between sPTB cases and term birth controls were likely to be an underestimation of the underlying different gene expression profile between the two groups due to the inclusion of symptomatic women non-differentially as both cases and controls (increasing the baseline risk of sPTB among the controls) as well as confounding factors such as infection and other obstetric complications. In addition, although we propose several potential novel biomarkers, our data are limited in discerning whether the differential expression signatures observed reflect the membrane bound proteins or their secreted isoforms. However, despite these drawbacks, this paper presents novelty in being the largest published meta-analysis of PTB transcriptomics using publicly available data to date. Since PTB samples are difficult to obtain, the ability to aggregate data by using standardized methods to correct for heterogeneity is exciting since it increases our statistical power and, as a result, allows for the discovery of novel pathways and biomarkers. For example, the pathways associated with sPTB and potential biomarkers for indication of early switch to a pro-inflammatory state of the maternal immune system could have been missed in the single-study analysis due to not reaching statistical significance: only 26 significant genes were identified (FDR < 0.10) in the GSE59491 study and no significant genes were identified (FDR < 0.10) in the GSE73685 study (analysis of maternal blood sample only). Although additional validation is needed, we hope that this paper informs the design and interpretation of clinical biomarker studies. Furthermore, we hope that this meta-analysis incentivizes others to add their data to public repositories with the goal of creating a more comprehensive database for PTB.

This paper presents several future directions including validation of the observed cell type signals through methods such as flow cytometry and CyTOF (94) as well as further exploring the presented transcriptomic signatures for diagnostics and therapeutics. We may be able to validate *TFPI* and *IL-1R1* in additional datasets collected prospectively in combination with clinical data and direct analysis of cell types to correlate with findings in plasma. In addition, staining and imagining of these two proteins in preterm and term whole blood samples can elucidate their sub-cellular location and potential as a clinical biomarker. This could lead to additional large animal studies to identify pathways whose inhibition could be beneficial and efficacious, similar to *IL1* signaling blocked by Anakinra in rheumatoid arthritis. Furthermore, although we evaluated the effect of cell type proportions as a covariate for our differential expression analysis, future studies involving single cell or sorted cell analysis will be much more informative.

CONCLUSION

Overall, our comprehensive analysis using publicly available data was able to elucidate genetic signatures associated with sPTB as well as identify potential biomarkers that could be translated to clinical practice. The novel finding of the reversal of regulation in innate immunity in maternal blood samples relative to fetal blood samples in PTB brings to light potential mechanisms that may be at play, which may allow for the prediction of sPTB as well as the development of therapeutics to extend pregnancy to term. In addition, the identification of two potential biomarkers, such as *TFPI* and *IL-1R1*, which are differentially expressed starting at mid-gestation, allows the possibility for clinically diagnostic biomarkers which may identify women at risk for PTB.

MATERIALS AND METHODS

Study Design

The purpose of this study was to perform a cross-study meta-analysis using multiple independent datasets to identify differential expressed genes comparing mothers who deliver preterm to mothers who deliver at term using maternal whole blood samples. Additional analyses across different time points and various tissue types were also performed to investigate differential expression between these two groups (**Figure 1**).

We searched the NCBI GEO database for public human microarray genome-wide expression studies using search terms including PTB and premature (23, 24). Abstracts were screened and only studies that met the following criteria were included: (i) had both spontaneous preterm cases and term delivery controls in the same study, (ii) included samples collected before or at delivery, and (iii) had a sample size of 20 or more. We used samples from maternal blood for our main analysis as they have the most samples. Classification of samples as PTB or term birth was extracted from sample matrices downloaded from the GEO database.

Cross-Study Meta-Analysis

We implemented the meta-analysis pipeline by Hughey and Butte (21) for data processing and normalization. All microarray data were renormalized from raw data and merged based on genes

meeting two criteria: those with non-missing values and those which were mutually inclusive across all three studies. The merged dataset was subsequently corrected for study-specific effects using ComBat, which implements an empirical Bayes method to correct for study-specific biases and batch effects by performing cross-study normalization (29). An *F*-test was performed to test the equality of variance across the three studies. Differential gene expression analysis was performed on this normalized merged dataset of genes to obtain significance level (*p*-values) of each gene using the R package limma which fits linear models to expression data for each gene (95). We corrected for multiple hypothesis testing using the Benjamini and Hochberg's (i.e., FDR) method (96) with a pre-specified cutoff of 0.1 to identify more significant genes.

Effect size of each gene is expressed in FC, which was calculated for each study separately using the raw expression data before ComBat. Samples were divided with respect to preterm or term delivery and mean gene expression was calculated for each gene meeting the FDR < 0.1 cutoff. The logged (base 2) average expression values were used to calculate fold-change [$FC = 2^{(\text{average expression for preterm samples} - \text{average expression for term samples})}$]. We further filtered the significant genes that met the FDR < 0.1 criteria using a significance threshold at a FC > 1.3 for upregulated genes or <1/1.3 for downregulated genes (22). We obtained a list of genes that showed the largest fold-change and were most differentially expressed, when comparing preterm and term births in the respective study. A final gene set was compiled by combining the significant genes from all three studies; if a gene met the FC cutoff of 1.3 in at least one of the studies, it was included in the final gene data set.

To investigate the relevance of these results, we performed a gene list functional enrichment analysis using ToppFun (34) to identify the pathways our genes were involved in and met a cutoff of FDR < 0.05, evaluated connectivity using the STRING database (31–33), explored biomarker identification using the UniProtKB database (35), and executed a cell type enrichment analysis using xCell (37). We extracted the significant cell types from the xCell output by performing a Student's two-sided *t*-test and subsequent Benjamini and Hochberg's (96) multiple testing correction (FDR < 0.05). A more stringent cutoff was used for cell deconvolution to extract the cell types that were robustly, significantly different between the two groups.

We utilized samples collected at the second trimester from GSE59491 (28) and samples from multiple tissues from GSE73685 and performed (1) single-study analysis and (2) tissue-level analyses to further investigate the common differentially expressed gene signatures across time points and different tissue types.

Single-Study Second Trimester Analysis

To perform a single-study gene expression analysis on the second trimester samples from GSE59491, we merged the gene expression values across all studies, extracted the second trimester samples from GSE59491, and implemented a linear model on that subset of samples. After calculating the *p*-value for each gene, we filtered our list of genes using the output from the cross-study meta-analysis done prior; this resulted in a list of overlapping set of genes which were previously found to be significant in the third trimester. To determine whether these genes were significantly differentially expressed in the second trimester as well, we corrected the raw *p*-values for multiple hypothesis testing for this subset of genes using the Benjamini and Hochberg's method and imposed an FDR < 0.1 (96).

As an exploratory analysis, we input the resulting genes from the FDR < 0.1 cutoff into the UniProtKB database to determine which genes are secreted as proteins in humans (35).

Regulatory Network Analysis

Transcription factor regulation networks and cytokine networks were analyzed through the use of Upstream Regulator analytic in IPA (QIAGEN Inc., https://www.qiagenbioinformatics.com/products/ingenuitypathway-analysis). Only significant connections are included in our networks and all connections are based on prior knowledge in IPA Knowledge Base. The transcription factors were filtered and only those that were expressed in a specific trimester were kept, to allow as much accuracy in results as possible.

Tissue-Level Analyses

To evaluate differential gene expression at a higher resolution, we performed gene expression analyses at an individual tissue level. Combining all tissue data into a merged dataset with all eight tissue types based on mutual genes, we extracted each tissue and created eight tissue-specific datasets for linear model fitting using limma (95). After correcting the raw *p*-values from limma using the Benjamini–Hochberg method for multiple hypotheses testing, seven of the eight tissues did not show significantly differentially expressed genes after implementing an FDR < 0.05; however, one tissue type, CB, had an output of genes which met the FDR criteria (96). A more stringent FDR cutoff was used to delineate the genes with the strongest differential expression between the two groups since an FDR < 0.1 resulted in 1,035 differentially expressed genes. These genes were further filtered by imposing a fold-change cutoff of 1.3 (22) which resulted in a list of significantly differentially expressed genes; the relevance of these genes was explored by performing pathway analysis using ToppFun in which a FDR < 0.05 was used as a cutoff (34).

All the results and the data are available as an RShiny Application for the benefit of the research community: http://comphealth.ucsf.edu/preterm_transcriptomics/.

DATA AVAILABILITY STATEMENT

The datasets analyzed for this study can be found in the National Institute of Health Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/). All datasets as well as the results generated in this study are available on the RShiny application (http://comphealth.ucsf.edu/preterm_transcriptomics/) and the ImmPort database (accession: SDY1327).

AUTHOR CONTRIBUTIONS

BV, MS, and AW conceived of the study. BV, AW, and IK carried out data analysis. IP carried out data visualization and app development. All authors contributed to writing and editing the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.00993/full#supplementary-material.

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

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Progesterone Modulation of Pregnancy-Related Immune Responses

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Progesterone (P4) is an important steroid hormone for the establishment and maintenance of pregnancy and its functional withdrawal in reproductive tissue is linked with the onset of parturition. However, the effects of P4 on adaptive immune responses are poorly understood. In this study, we took a novel approach by comparing the effects of P4 supplementation longitudinally, with treatment using a P4 antagonist mifepristone (RU486) in mid-trimester pregnancies. Thus, we were able to demonstrate the immune-modulatory functions of P4. We show that, in pregnancy, the immune system is increasingly activated (CD38, CCR6) with greater antigen-specific cytotoxic T cell responses (granzyme B). Simultaneously, pregnancy promotes a tolerant immune environment (IL-10 and regulatory-T cells) that gradually reverses prior to the onset of labor. P4 suppresses and RU486 enhances antigen-specific CD4 and CD8 T cell inflammatory cytokine (IFN-γ) and cytotoxic molecule release (granzyme B). P4 and RU486 effectively modulate immune cell-mediated interactions, by regulating differentiated memory T cell subset sensitivity to antigen stimulation. Our results indicate that P4 and RU486, as immune modulators, share a reciprocal relationship. These data unveil key contributions of P4 to the modulation of the maternal immune system and suggests targets for future modulation of maternal immune function during pregnancy.

Keywords: pregnancy, progesterone, RU486, immune modulation, immune response

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INTRODUCTION

Progesterone (P4) plays a key role in the establishment and maintenance of pregnancy and its withdrawal causes the onset of labor (1, 2). In humans, the production of P4 gradually rises throughout pregnancy; the corpus luteum is the major source in early pregnancy, but after 8 weeks, the placenta takes over (1). P4 and its metabolites work through a series of nuclear P4 (PR) and glucocorticoid (GR) receptors, as well as membrane-bound P4 receptors (mPR), to exert their effects (3–5). The majority of P4 actions on immune function are mediated by its interactions with GR (6–9). Human PBMCs are thought to express a number of endocrine receptors including: mPR, GR, and estrogen receptors (4, 6, 7, 10). In murine models, GR engagement increases regulatory T cell (Treg) immune-suppressive function, and P4 binding to GR is thought to be responsible for this finding in pregnancy (8, 9). In humans, GR agonists have been shown to increase apoptosis of $CD4^+$ T cells *via* the GR (7). In contrast to most animal models of pregnancy, where there is a systemic withdrawal of P4 prior to the onset of labor, in the human, the literature suggests that there is a functional withdrawal at the level of the PR at the end of pregnancy (11). In particular, there are differences seen in the

level of expression of P4 receptor isoforms, P4 receptor gene polymorphisms in reproductive tissue, and a decline in secretion of a lymphocyte-derived immunomodulatory protein known as P4-induced blocking factor (PIBF) (1, 11). P4 acts, either directly, or indirectly through PIBF, to modulate the immune system to achieve a successful pregnancy. These include promoting a T_H2-dominant cytokine profile and upregulating HLA-G expression on trophoblast, which enables γδ T-cell activation and evasion of host defenses by acting as a ligand for inhibitory receptors on natural killer (NK) cells (1, 12–14). In fact, PIBF is a potent suppressor of cytotoxic immune cells and regulator of cytokine secretion (11, 15).

Pregnancy is associated with a series of immune adaptations that begin pre-implantation and span the length of the antenatal and postnatal period (16–18). As a result, maternal immune responses are different compared to non-pregnant women, and they fluctuate during the course of pregnancy (19, 20). In fact, some autoimmune conditions, such as rheumatoid arthritis, enter remission during pregnancy, but flare up in the postnatal period (21). Although clinical trials using HRT in women with rheumatoid arthritis have not shown the same effect as pregnancy, animal models using pregnancy-like levels of hormones have shown promising findings (22). In clinical practice, P4 supplementation is used in pregnancy as an effective treatment for the prevention of preterm birth (23). Its effects are likely to be a combination of immune modulation and a reversal of the functional withdrawal of P4 action in reproductive tissue. Spontaneous labor is associated with a loss of suppression of syngeneic and allogeneic T-cell responses (24). Interestingly, gestational changes are seen *ex vivo* where fetal-specific T effector memory (T_{EM}) cells and detectable fetal DNA are longitudinally increased in pregnancy (18). Peripheral blood IFN-γ, IL-4 spontaneous responses, and paternal antigen stimulated responses, measured using enzyme-linked immunospot (ELISpot), appear to peak at 35 weeks of pregnancy (19).

We investigated the hypothesis that in the peripheral circulation during pregnancy, P4 actively suppresses CD4 and CD8 T cell inflammatory cytokine and cytotoxic molecule production. In addition, we hypothesized that P4 alters T cell, NK cell, and dendritic cell (DC) phenotype to regulate immune responses. To determine the effects of P4, we began by determining the gestational changes in immune responses and leukocyte phenotype, longitudinally, in healthy uncomplicated pregnancies, which we expected may be affected by the use of P4. We then compared this cohort of patients to those supplemented with vaginal P4. Finally, to understand the potential effects of P4 antagonism in a clinical setting, we recruited patients receiving the most widely used P4 antagonist mifepristone (RU486) in second trimester pregnancies and analyzed its effects longitudinally. Our study takes a novel approach by comparing the effects of P4 supplementation and the use of RU486 in pregnancy. Importantly, this is the first study to investigate the immune-modulatory effects of RU486, *in vivo,* in the mid second trimester of pregnancy. We found that advancing pregnancy may be associated with an inherent loss of sensitivity to P4. More importantly, P4 reduces pro-inflammatory and cytotoxic T cell responses. It achieves this by a combination of effects to modulate immune cell-mediated interactions, including memory T cell antigen sensitivity and regulation of leukocyte migration.

MATERIALS AND METHODS

Ethics Statement

All subjects were recruited from Chelsea and Westminster Hospital, London, UK. This study was carried out in accordance with the recommendations of National Institute of Health Research (NIHR) Good Clinical Practice guidelines, and a NHS Research Ethics Committee. The protocol was approved by the National Research Ethics Service (NRES), London, UK committee as well as by Chelsea and Westminster NHS Trust, London, UK; Ref: 11/LO/0971. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

Study Design

Based on the premise that maternal peripheral blood will provide a window to the systemic effects of P4 in pregnancy, a combination of ELISpot, 9-parameter flow cytometry, and multiplex assays were developed to assess functional cellular responses and leukocyte phenotype. A standardized protocol was used for all samples to ensure consistency and comparability. Blood samples were processed within 2 h of collection and all phenotypic and functional work was performed on fresh samples.

Study Participants

For the control group, healthy pregnant patients ($N = 42$) were recruited from the antenatal clinic from April 2013 to September 2014 at Chelsea and Westminster Hospital during their first visit before 20 weeks of gestation. These patients had no previous history of premature delivery defined as less than 37 weeks of gestation. Women with a history of previous vaginal deliveries were not excluded so that the sample population was comparable to the P4 supplemented cohort, which were often started on P4 due to a previous history. Once recruited, these patients were asked to provide peripheral blood samples longitudinally. The time points for sample collection were: at recruitment (range 11–20 weeks of gestation), 28, 34 weeks, in labor or at delivery, 24 h postnatal, and 6–8 weeks postnatal.

Pregnant patients receiving P4 supplementation ($N = 15$) (once a day Cyclogest® 400 mg per vaginum/rectum, Actavis UK) were recruited from the preterm antenatal clinic at Chelsea and Westminster Hospital between April 2013 and September 2014, ideally prior to 20 weeks of gestation (range 17–20 weeks of gestation). These patients were commenced on P4 due to: a previous history of preterm labor and delivery, second trimester loss, or ultrasonographic evidence of cervical shortening of <25 mm, which carries a significantly increased risk of preterm delivery at ≤32 weeks of gestation (23). These patients continued P4 treatment until 34 weeks of gestation. For these patients, the time points for longitudinal sample collection were: at recruitment, 28, 34, and 36 weeks of gestation. Since exogenous vaginal P4 (100 mg tablets) has a terminal half-life of approximately 14 h, the effect of P4 was thought to have substantially declined by the 36 weeks of gestation (25). The effects of supplementary P4 should disappear

in the postnatal period. Since treatment stopped at 34 weeks of gestation, no differences were expected between the P4 treated and control subjects, and so these were not compared.

In order to understand the effects of P4 antagonism, pregnant patients ($N = 8$) undergoing medically indicated terminations of pregnancy due to fetal anomaly in an otherwise uncomplicated pregnancy were recruited from the fetal medicine unit at Chelsea and Westminster Hospital, from June 2013 to May 2015. As part of the termination process, these patients received RU486 (once only Mifegyne® 400 mg orally, Nordic Pharma UK) and then attended the hospital for prostaglandin-induced labor 2 days later. Once recruited, these patients were asked to provide peripheral blood samples prior to taking RU486, 48 and 72 h post RU486, which was 24 h post-delivery. The mean gestation at delivery was 19 weeks (SD ± 1.8).

Study inclusion criteria included: age at booking under 40 years as replicative senescence, immune exhaustion, and thymic output is age linked with the latter reduced beyond 40 years of age (26). Exclusion criteria included: failure to meet the inclusion criteria; the development of or past medical history of any pregnancy related or unrelated complications that affected the course of pregnancy, such as pre-eclampsia, gestational diabetes, or intrauterine growth restriction as well as any autoimmune, hypertensive, or renal conditions. Demographic data are summarized in Table S1 in Supplementary Material.

Non-pregnant female control samples were obtained from individuals working in the Centre for Immunology and Vaccinology, Imperial College London, UK and clinical staff working in the Maternity Department, Chelsea and Westminster Hospital.

Preparation of Cells

Approximately 35 ml of peripheral blood was obtained using a Vacutainer™ system and using 6 ml lithium heparin blood collection tubes (Becton Dickinson, Oxford, UK). PBMCs from adult peripheral blood were prepared by density gradient centrifuge on Histopaque (Sigma-Aldrich, Dorset, UK) as described previously (27). Viability was determined using a trypan blue exclusion test of cell viability and samples where this was greater than 80% were used. For functional work, the cells were suspended in TCM [RPMI-1640 with Penicillin and Streptomycin (Sigma-Aldrich), at final concentrations of 100 IU/ml and 100 μg/ml, and L-glutamine (Sigma-Aldrich) at a final concentration of 2 mM], and for phenotype the cells were suspended in Ca^{2+} and Mg^{2+} containing PBS (Sigma-Aldrich). All ELISpot and flow cytometry assays were performed on fresh samples. Processing was commenced within 2 h of obtaining peripheral blood. Plasma for multiplex immunoassay was collected, aliquoted, and stored at −80°C until use.

ELISpot Assay

IFN-γ, IL-10, IL-4, and Granzyme B ELISpot assays were performed in order to detect recall antigen/peptide specific T cell responses as previously described (28). Briefly, 1×10^5 PBMC/well were cultured in 10% (heat-inactivated) male AB plasma-RPMI (200 μl/well, Sigma-Aldrich) in 96-well polyvinylidene difluoride backed plates (Merck Millipore, Hertfordshire, UK), which were coated in antibodies for the cytokines and proteases of interest, namely IFN-γ, IL-10, Granzyme B, and IL-4 (MabTech, Nacka Strand, Sweden). PBMCs, in duplicate wells, were stimulated with 100 μl of an antigen/peptide pool obtained from NIBSC (NIBSC, Hertfordshire, UK) and Virion-Serion (Virion-Serion, Würzburg, Germany) at the manufacturer's recommended concentrations. These included: EBV, CMV, influenza A, measles, and HSV whole lysates; purified protein derivative (PPD) of *M. Tuberculosis* Tuberculin; purified tetanus toxoid (TTOX); and flu/EBV/influenza (FEC) peptide pool. Positive and negative controls were provided by phytohemagglutinin (5 μg/ml) and TCM. The plate was incubated at 37°C in 5% CO_2 for 48 h for anti IFN-γ, IL-10, and Granzyme B coated and 96 h for anti-IL-4 coated plates. Detection of spot forming cells (SFC) was carried out by the addition of biotinylated anti IFN-γ, IL-10, Granzyme B, or IL-4 (MabTech) and incubation, followed by the use of a concentrated streptavidin-alkaline phosphatase conjugate (MabTech). Finally, the development step required the use of a chromogen prepared from a premixed BCIP/NBT substrate kit (BioRad labouratories Ltd., Hertfordshire, UK). Spot reading and counting was performed using an AID ELISpot reader (Oxford Biosystems Cadama, Oxfordshire, UK).

Flow Cytometric Leukocyte Quantification

Multicolor color flow cytometry was used to phenotype CD4 and CD8 T-cell subsets, NK cells, and DC. In order to evaluate T cell subtypes, PBMCs were stained with the following murine, anti-human monoclonal antibodies according to the manufacturer's instructions: peridinin chlorophyll protein (PerCP) Cy5.5-labeled anti-CD3 (Biolegend, London, UK); allophyocyanin (APC)-H7-conjugated anti-CD8 (Biolegend); BD Horizon V450-labeled anti-CD38 (BD Biosciences, Oxford, UK), anti-CD127 (BD), and anti-CCR4 (BD); BD Horizon V500-labeled anti-HLA-DR (BD) and anti-CD4 (BD); fluorescein isothiocyanate (FITC)-labeled anti-CD31 (BD), anti-CD25 (BD), anti-CCR6 (R&D Systems, Abingdon, UK) and anti-PIBF (rabbit polyclonal; Biorbyt, Cambridge, UK); phycoerythrin (PE)-conjugated anti-CCR7 (R&D), anti-CCR5 (BD) and anti-CCR3 (BD); APC-labeled anti-CD28 (BD), anti-HLA-G (eBioscience, Cheshire, UK), anti-CXCR3 (BD); PECy7-labeled anti-CD45RA (BD), anti-CD45RO (BD) and anti-CXCR4 (BD). NK subsets were phenotyped with: BD Horizon V450-labeled anti-CD56 (BD); FITC-labeled anti-PIBF; PE-labeled anti-iNKT (BD); APC-labeled anti-TCR-γδ (BD); PE-Cy7-labeled anti-CD16 (BD); and PerCP-Cy 5.5-labeled anti-CD3. Approximately 2×10^6 cells were stained per tube, incubated in the dark at room temperature for 30 min, washed with PBS, and fixed with BD stabilizing fixative (BD Biosciences), before acquisition within 24 h. At least 100,000 events were acquired on a 3-laser flow cytometer (BD Biosciences LSR II) and subsequently gated according to respective isotype controls.

Dendritic cell of myeloid and plasmacytoid lineage, as well as HLA-G expressing tolerant variants were identified using the antibodies: Qdot® 605-labeled anti-CD3 and anti-CD19 (Invitrogen, Paisley, UK); BD Horizon V450-labeled anti-CD11c (BD); BD Horizon V500-labeled HLA-DR; FITC-labeled anti-CD16 (BD); PE-labeled anti-ILT4 (eBioscience); APC-labeled anti-HLA-G (eBioscience); PE-Cy7-labeled anti-CD83 (BD); PerCP-Cy 5.5-labeled CD123 (BD); and APC-H7-labeled anti-CD14 (BD). A minimum of 500,000 events were acquired according to the

description detailed above. Analysis of flow cytometric data was performed using FlowJo version 7.65 (Tree Star Inc., Ashland, OR, USA).

Multiplex Immunoassay

A human 17-plex Bio-Plex Pro® Multiplex immunoassay kit (BioRad) was used to determine plasma cytokine and chemokine concentrations. The kit contained the following cytokines: G-CSF, GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p70), IL-13, IL-17, MCP-1 (MCAF), MIP-1β, TNF-α. The multiplex immunoassay was performed according to the manufacturer's instructions and assays were read using a Bio-Plex© MAGPIX© reader (BioRad).

Statistics

Longitudinal analysis of un-supplemented pregnancies was undertaken using mixed-effects modeling to avoid a loss of statistical power by omitting patients with incomplete data. For normally distributed data, a linear mixed effects model was used, and pairwise multiple comparisons of estimated marginal means with sequential Bonferroni correction was performed where the main effect was significant. Where data did not follow a normal distribution, a generalized linear mixed effects model with gamma log-link was used. If the main effect was significant, pairwise multiple comparisons of estimated marginal means with sequential Bonferroni correction was performed. Data were analyzed following the methods outlined by Duricki et al. and using IBM© SPSS Version 21.0 (Armonk, New York, NY, USA) for mixed effects modeling (29). In addition, longitudinal ELISpot data presented as a heatmap was produced and the analysis and presentation of distributions was performed using SPICE version 5.1, downloaded from http://exon.niaid.nih.gov. Comparison of distributions was performed using a Student's *t*-test and a partial permutation test as described (30).

P4 supplemented and un-supplemented pregnancies were compared using a Students *t*-test where the data were continuous and parametric, and for non-parametric data, Mann–Whitney *U* test was used. For longitudinal analysis of P4 and RU486 treated pregnancies with repeated measures and parametric data, a one-way analysis of variance (ANOVA) with multiple group comparisons and a Tukey correction was used to compare group means. For non-parametric longitudinal repeated measures, data analysis was undertaken using a Friedman test with Dunn's correction. Statistical analysis was performed on GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA, USA).

Data are presented as means ± SEM or medians ± interquartile range (IQR) as appropriate for the distribution normality. All *P*-values were two-tailed and significance was defined as $P < 0.05$.

RESULTS

Pregnancy Promotes a Tolerant Immune Environment in the Peripheral Circulation That Gradually Reverses Prior to the Onset of Delivery

Before investigating the effects of P4, leukocyte functional and phenotypic profiles were analyzed longitudinally in un-supplemented pregnancies. This established the effects of pregnancy and advancing gestation as well as providing a control group. Baseline results from pregnant participants were compared with non-pregnant volunteers. Where longitudinal analysis in pregnancy was significant, pairwise analysis comparing with baseline, 34 weeks and labor were reported. These co-responded to recruitment, prior to the onset of labor and labor/delivery.

T cell antigen-specific responses were determined using ELISpot and the results are summarized using the heat-map in **Figure 1A**. The majority of pregnant patients at baseline produced robust IFN-γ responses that were comparable to non-pregnant volunteers (**Figures 1A–C**; Figure S1 in Supplementary Material). Irrespective of the baseline response, labor and delivery was associated with a significant peak in cellular responses to CMV (**Figure 1C**) and HSV (**Figure 1A**). IL-10 cytokine responses behaved in a similar manner to IFN-γ. Our results showed that labor and delivery was characterized by a peak in responses to measles and CMV (**Figures 1B,C**) whole lysates, as well as TTOX (Figure S1 in Supplementary Material), PPD antigens, and FEC peptide pool (**Figure 1A**). Both IFN-γ and IL-10 responses returned to non-pregnant levels in the postnatal period. In contrast to IFN-γ, pregnancy was associated with improved IL-10 cellular responses throughout its course. The majority of IL-4 responses were below the positive threshold. When measurable, IL-4 response patterns were associated with an improved response to influenza A, PPD, TTOX, FEC peptide pool, and herpesvirus antigen (**Figure 1A**; Figure S1 in Supplementary Material). However, the peak response in pregnancy was at 34 weeks of gestation and the majority of these returned to non-pregnant levels in labor or 24 h post-delivery (**Figure 1A**). Most pregnant patients had positive cytotoxic granzyme B responses at baseline, but this was not true for all non-pregnant patients where some responses fell below the threshold of 20 per 10^6 SFC. When compared to baseline, 34 weeks of pregnancy showed a significant peak in SFC and a positive response to measles whole lysate and TTOX antigen (**Figure 1B**; Figure S1 in Supplementary Material).

The changes in functional responses were reflected in the leukocyte phenotype observed. CD38 is a surface glycoprotein and enzyme that is important for cell adhesion and regulation of T cell functions including proliferation (31). CD38 expression on CD4 and CD8 T cells was reduced at recruitment and then rose with advancing gestation (**Figures 2A,D**). This occurred without a similar increase in HLA-DR expression on CD4 or CD8 T cells (not shown). In addition, we observed longitudinal increases in CCR6 expression on CD4 T cells (**Figures 2B,D**), and increased CD83 expression on both mDC and pDC (**Figures 3A,D**). CCR6 is important for mucosal immunity. As well as being expressed on T_H17 cells, the expression of CCR6 on memory T cells enables their migration in response to CCL20 to sites of inflammation (32,33). Proportions of CD28 expressing effector memory subtypes fell longitudinally during pregnancy (**Figures 2C,D**). CD28 is an important co-stimulatory marker for T cell activation. However, on memory T cells, the loss of expression correlates with greater peripheral homing and effector function (34). Concurrently, T_H17 proportions, defined using chemokine markers CCR4 and CCR6 as previously described (35), were reduced in pregnancy compared to non-pregnant controls but saw a longitudinal increase

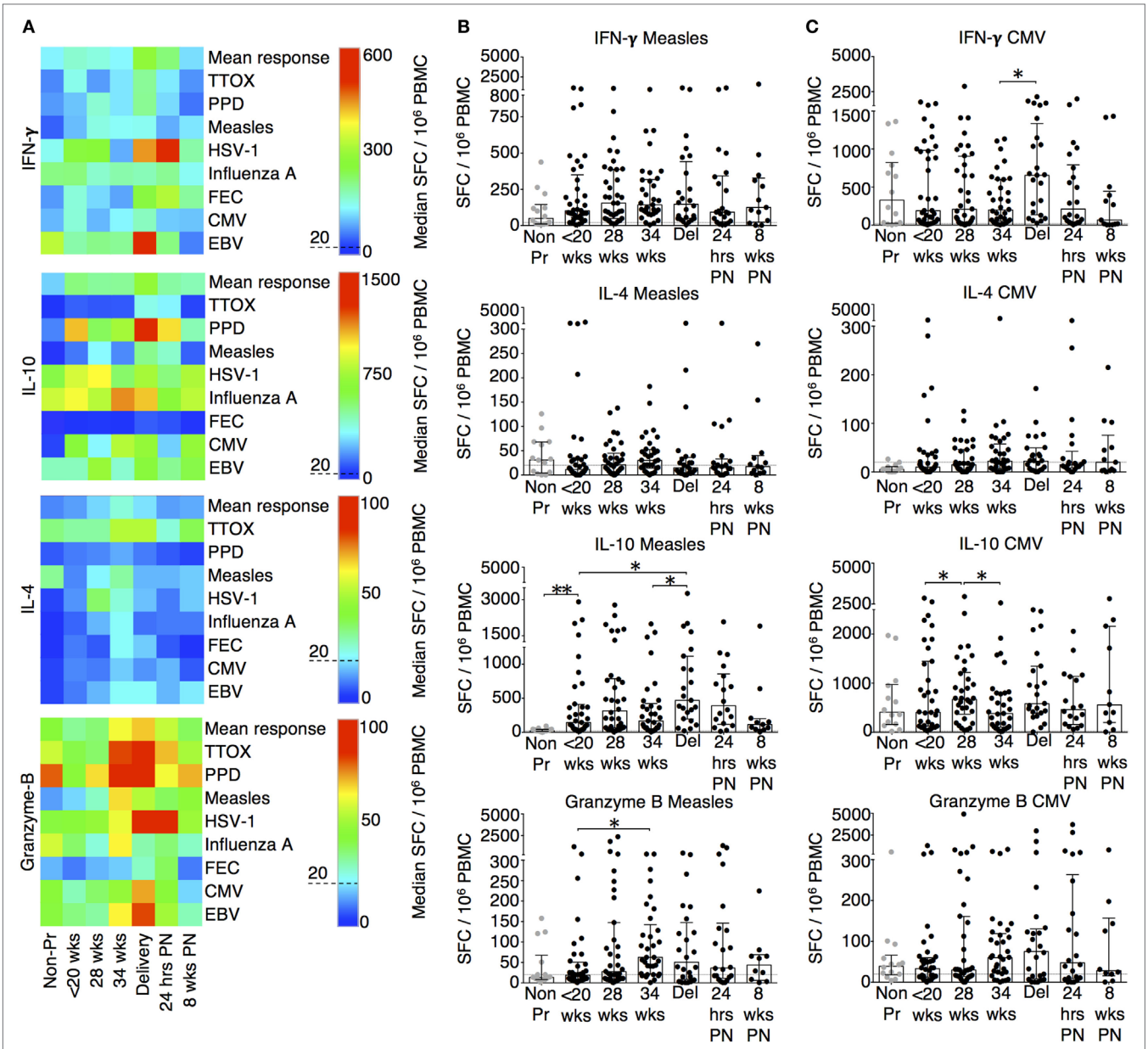


FIGURE 1 | Advancing pregnancy is associated with greater IFN-γ, IL-10, IL-4, and granzyme B ELISpot responses. **(A)** Summary heat-map of gestation specific median ELISpot responses to antigens/peptides as well as overall mean response for each analyte. The individual scales used for each cytokine is shown with the threshold of 20 spot forming cells (SFC)/10^6 PBMC indicated using a dashed line. The heat-map was generated and analyzed using SPICE (30). **(B)** ELISpot responses to measles and **(C)** CMV whole lysates. Columns indicate median and interquartile range. Gestation at sampling is indicated in pregnancy (●): < 20^{+0} weeks (*N* = *42*), 28 weeks (*N* = *35*), 34 weeks (*N* = *33*), at delivery (*N* = *24*), 24 h post-delivery (*N* = *22*), 6–8 weeks postnatal (*N* = *13*). Non-pregnant controls are depicted as ● (*N* = *14*). A dashed line represents <20 SFC/10^6. *P* values are two tailed and significance is defined as $^*P < 0.05$ and $^{**}P < 0.01$. Non-pregnant and baseline pregnant data analyzed by Mann–Whitney *U* test. Longitudinal data analyzed by generalized linear mixed effects model with gamma log-link and pairwise multiple comparisons of estimated marginal means with sequential Bonferroni correction.

that peaked at 34 weeks (**Figures 3B,E**). In contrast, CD4 Treg proportions were increased in pregnancy at baseline compared to controls (**Figures 3C,F**). Collectively, these results were consistent with an initial immune suppression and its subsequent reversal during pregnancy. The perforin/granzyme pathway for target cell killing is used by both cytotoxic T lymphocytes and NK cells (36). Although CD38 expression on CD8 T cells showed a longitudinal increase in pregnancy (**Figure 2A**), the more cytotoxic NK cell subtype with the phenotype $CD16^+CD56^{lo}$ (**Figure 4A**) were reduced and showed an increase in PIBF expression at 34 weeks (**Figure 4B**). Furthermore, the 9mer FEC peptide pool, which contains CD8 T cell epitopes, predominantly stimulated IL-4 and IL-10 responses (**Figure 1A**). This suggested that although we demonstrated functionally greater

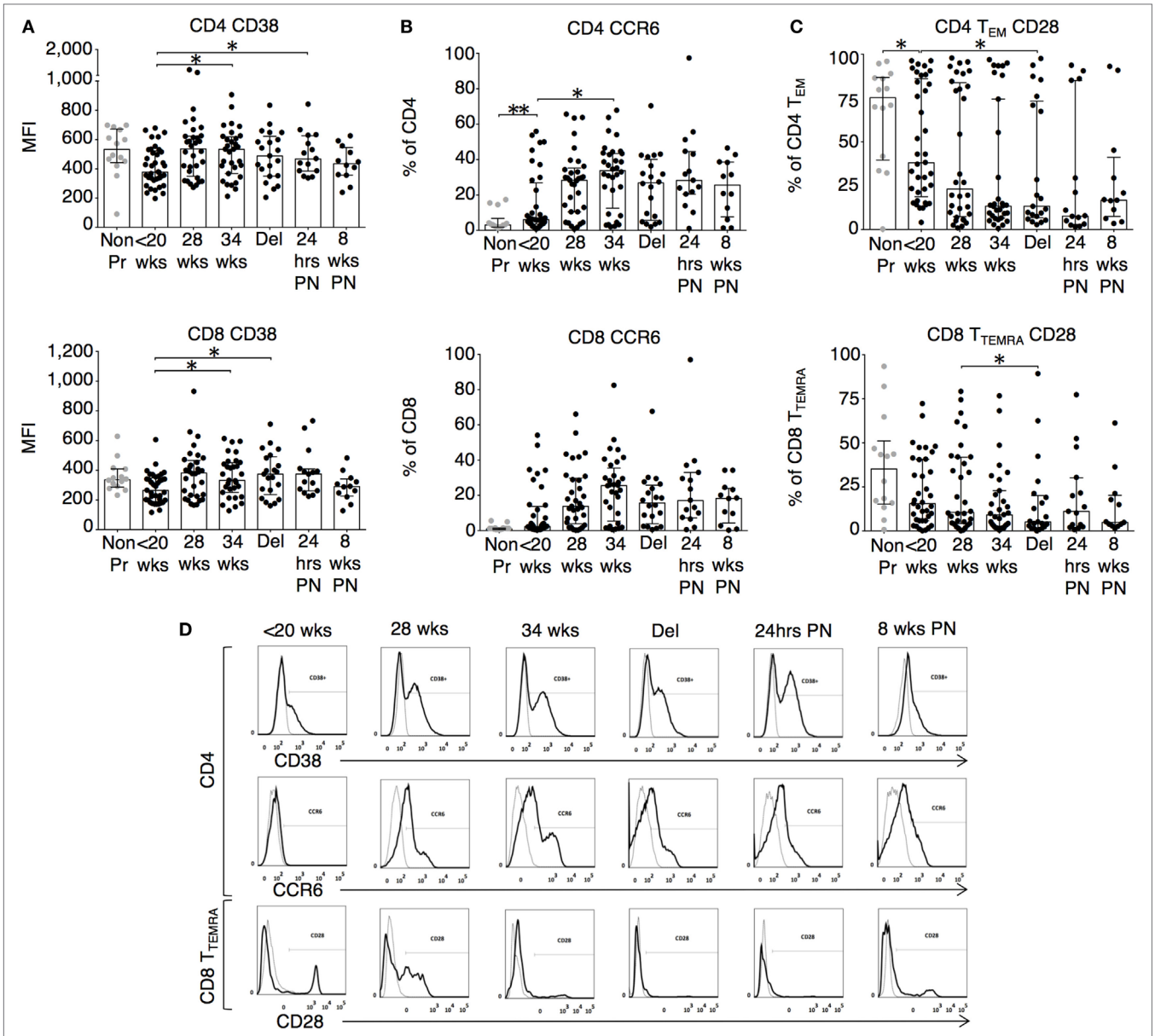


FIGURE 2 | Leukocyte phenotype during pregnancy shows increased activation and migratory potential. **(A)** CD38 mean fluorescence intensity and the proportion of expression of **(B)** CCR6 on CD4 and CD8 T cells; **(C)** CD28 expression on CD4 T_{EM} ($CCR7^{-}CD45RA^{-}$) and CD8 T_{TEMRA} ($CCR7^{-}CD45RA^{+}$) T cell subtypes analyzed longitudinally with gestation and compared to controls. Gestation at sampling is indicated in pregnancy (●): $<20^{+0}$ weeks (*N* = *42*), 28 weeks (*N* = *35*), 34 weeks (*N* = *33*), at delivery (*N* = *24*), 24 h post-delivery (*N* = *22*), 6–8 weeks postnatal (*N* = *13*). Non-pregnant controls are depicted as ● (*N* = *14*). *P* values are two tailed and significance is defined as $^{*}P < 0.05$ and $^{**}P < 0.01$. Non-pregnant and baseline pregnant data analyzed by Mann–Whitney *U* test. Longitudinal data analyzed by generalized linear mixed effects model with gamma log-link and pairwise multiple comparisons of estimated marginal means with sequential Bonferroni correction. **(D)** Shows representative flow cytometry histograms across gestations with matched isotype controls for CD38 and CCR6 expression on CD4 T cells as well as CD28 expression on CD8 T_{TEMRA} and HLA-DR expression on CD8 T cells.

granzyme B responses with gestation, these were concurrently being modulated.

P4 Suppresses IFN-γ and Granzyme B Responses in Pregnancy

To determine the effects of P4, we recruited patients receiving either P4 supplementation or P4 antagonism with RU486 for clinically relevant indications as outlined in the Section "Materials and Methods." Longitudinal differences in IFN-g responses with P4 treatment were not significantly different (**Figure 5A**). However, when compared to untreated patients, P4 treatment significantly reduced pro-inflammatory IFN-γ responses to measles whole lysate at 34 weeks and TTOX antigen at 28 weeks of gestation (**Figure 5B**; Figure S2 in Supplementary Material). An opposite effect was seen in the RU486-treated group at 72 h where IFN-γ responses to EBV, influenza A, HSV, measles,

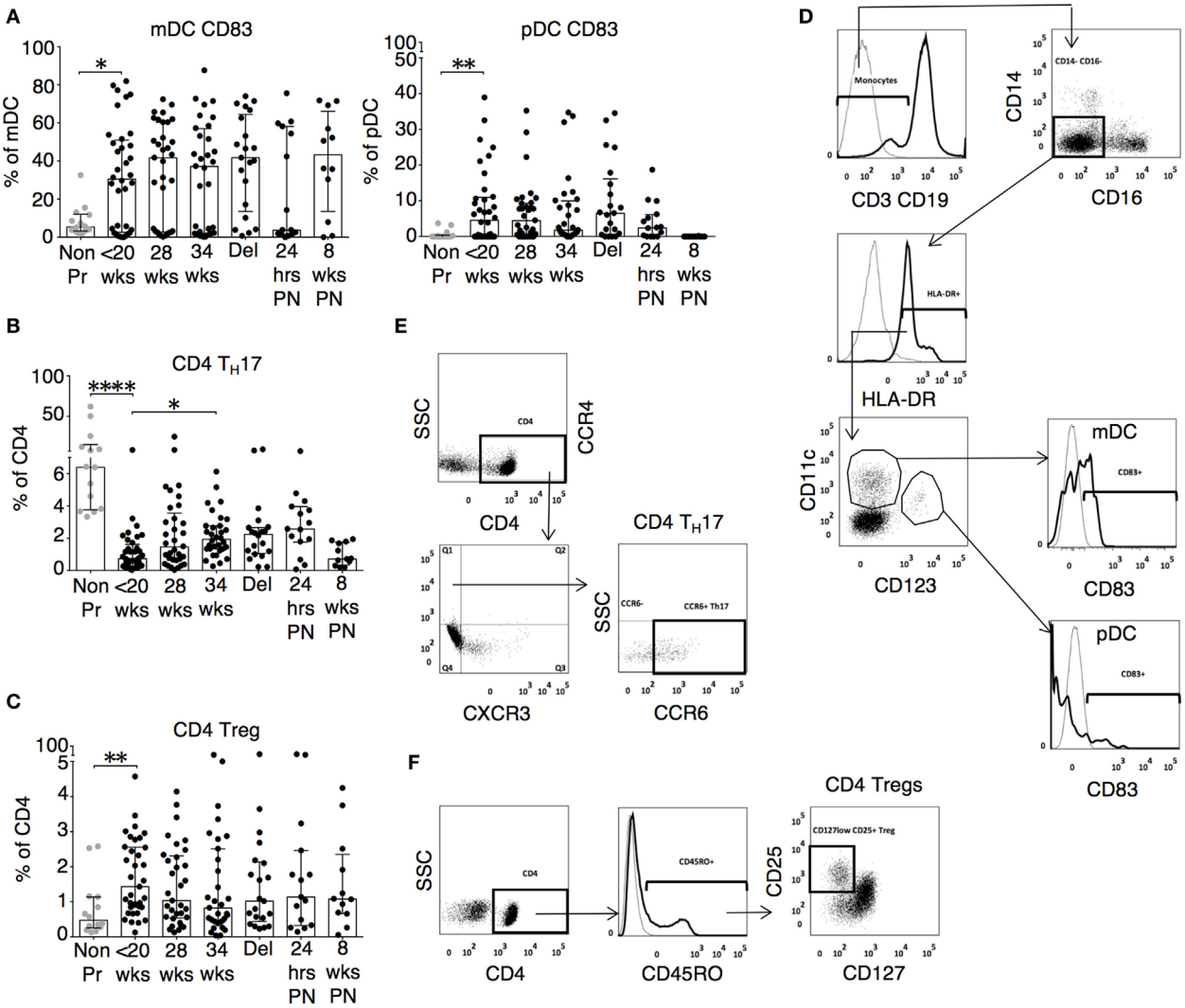


FIGURE 3 | Inflammatory, activated, and cytotoxic leukocyte subsets show gestational variation in pregnancy. **(A)** Proportions of activated mDC ($CD3^-CD19^-CD14^-CD16^-HLA\text{-}DR^+CD11c^+CD123^-CD83^+$) and pDC ($CD3^-CD19^-CD14^-CD16^-HLA\text{-}DR^+CD11c^+CD123^+CD83^+$) during pregnancy and compared with gestation matched controls. **(B)** Longitudinal analysis of T_H17 cells ($CCR4^+CXCR3^-CCR6^+$) and **(C)** regulatory T cell (Tregs) with phenotype $CD4^+CD45RO^+CD25^+CD127^{lo}$ in pregnancy and compared with gestation matched controls. Gestation at sampling is indicated in pregnancy (●): $<20^{+0}$ weeks (*N* = *42*), 28 weeks (*N* = *35*), 34 weeks (*N* = *33*), at delivery (*N* = *24*), 24 h post-delivery (*N* = *22*), 6–8 weeks postnatal (*N* = *13*). Non-pregnant controls are depicted as ● (*N* = *14*). *P* values are two tailed and significance is defined as $^*P < 0.05$ and $^{**}P < 0.01$ $^{****}P < 0.0001$. Non-pregnant and baseline pregnant data analyzed by Mann–Whitney *U* test. Longitudinal data analyzed by generalized linear mixed effects model with gamma log-link and pairwise multiple comparisons of estimated marginal means with sequential Bonferroni correction. Representative flow cytometry plots of **(D)** dendritic cell subtypes mDC and pDC, **(E)** T_H17 CD4 T cells, and **(F)** $CD4^+$ Tregs and, and their respective gating strategies.

and PPD were significantly increased compared to baseline (**Figure 5C**; Figure S2 in Supplementary Material). Longitudinal and gestation matched analysis of P4 supplemented pregnancies did not show significant changes in antigen-specific IL-10 and IL-4 responses (**Figures 5D–F** and **6A–C**). However, longitudinal analysis of RU486 treated patients showed a significant increase in IL-10 response to measles whole lysate (**Figure 5F**). Cellular IL-4 production in response to measles whole lysate at 2 weeks post P4 treatment was reduced but remained largely unmeasurable (**Figure 6B**).

Granzyme B responses showed the same pattern as the IFN-γ responses (**Figures 6D,E**). P4 treatment was associated with a significantly reduced granzyme B response at 34 weeks of gestation to measles, influenza A and TTOX (**Figure 6E**; Figure S3 in Supplementary Material). An opposite effect was seen in the RU486 treated group. Although granzyme B production at baseline was below 20 per 10^6 SFCs, when measurable, PBMC from RU486 treated patients produced a greater number of granzyme B SFCs in response to measles and influenza A whole lysate, and FEC peptide pool measured 72 h post RU486 (**Figure 6F**; Figure S3 in Supplementary Material).

A representative sample of six patients, from the P4 and RU486 treated groups, with plasma samples taken longitudinally were analyzed to measure cytokine concentrations without cell

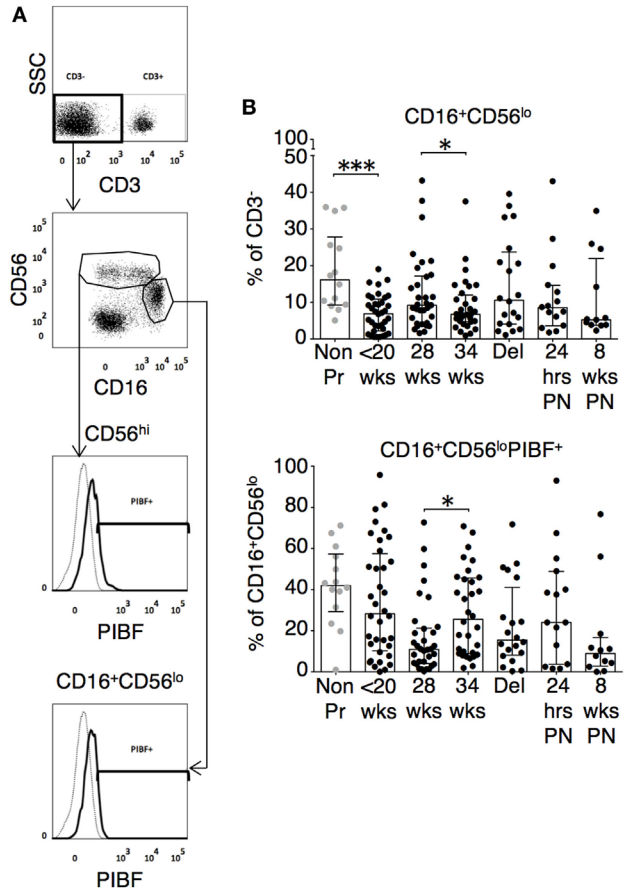


FIGURE 4 | Cytotoxic natural killer (NK) cell subtype with the phenotype $CD16^+CD56^{lo}$ are reduced and show an increase in P4-induced blocking factor (PIBF) expression in late pregnancy. **(A)** Representative flow cytometry plots are shown for NK cell subsets. **(B)** Longitudinal analysis of $CD16^+CD56^{lo}$ and their PIBF expression during pregnancy and compared with gestation matched controls. Gestation at sampling is indicated in pregnancy (●): $<20^{+0}$ weeks (*N = 42*), 28 weeks (*N = 35*), 34 weeks (*N = 33*), at delivery (*N = 24*), 24 h post-delivery (*N = 22*), 6–8 weeks postnatal (*N = 13*). Non-pregnant controls are depicted as ● (*N = 14*). *P* values are two tailed and significance is defined as $^*P < 0.05$ and $^{***}P < 0.001$. Non-pregnant and baseline pregnant data analyzed by Mann—Whitney *U* test. Longitudinal data analyzed by generalized linear mixed effects model with gamma log-link and pairwise multiple comparisons of estimated marginal means with sequential Bonferroni correction.

culture or stimulation. Longitudinally, cytokine concentrations in P4 treated patients remained stable (**Figure 7A**). However, when compared to gestation-matched controls, baseline IFN-γ, IL-7, and IL-1β were significantly reduced in P4 treated patients (**Figure 7B** and not shown). This was sustained for IFN-γ and IL-7 at 28 weeks of gestation (**Figure 7B**). Despite these observations, longitudinal cytokine concentrations in the RU486-treated cohort remained stable (**Figure 7C**).

P4 Antagonism Results in Activation of CD4 and Memory T cell Subtypes

The longitudinal changes seen in the expression of CD38 and CCR6 on both CD4 and CD8 T cells in the un-supplemented group were reproduced in the P4 treated (**Figures 8A,D**). P4 had no impact on CD38 or CCR6 expression on CD4 T cells when compared to gestation-matched controls (**Figures 8B,E**). However, with P4 supplementation, CD38 expression on CD8 T cells at 34 weeks of gestation was increased (**Figure 8B**). Nonetheless, CD38 and CCR6 expression was unaffected by RU486 (**Figure 8C,F**). Supplemental P4 appeared to have no impact on CD4 and CD8 HLA-DR expression either longitudinally nor when compared to gestation-matched controls (**Figures 8G,H** and data not shown). However, antagonism with RU486 resulted in increased expression of HLA-DR on CD4 but not on CD8 T cells (**Figure 8I** and data not shown).

Memory T cells provide quantitatively enhanced responses to secondary or recall antigen challenge. In our study, these subsets were defined as previously described (37). Their activated and expansive potential was determined by their expression of HLA-DR and CD28 as these are associated with peptide presentation and co-stimulation (34). Proportions of CD4 terminally differentiated effector memory (T_{TEMRA}) T cells and CD8 T_{EM} and T_{TEMRA} were reduced 72 h post RU486 treatment (**Figures 9A–E**). CD4 T_{CM} expressing HLA-DR and CD28 (**Figures 10A,E** and not shown) showed an increase 72 h post RU486 and proportions of CD4 T_{EM} expressing HLA-DR increased 48 h following RU486 (**Figures 10B,E**). HLA-DR^+ CD8 T_{CM} proportions increased 72 h post RU486 but HLA-DR^+ CD8 T_{TEMRA} decreased 48 h post RU486 and returned to baseline 72 h post RU486 (**Figures 10C–E**).

P4 Was Associated With a Fall in Proportions of Tregs, but Had No Impact on T-Helper Subtypes or PIBF-Expressing T Cells

Although P4 has previously been shown to be able to generate induced Tregs *in vitro*, in our study, proportions of CD4 Tregs decreased longitudinally with P4 treatment (**Figure 11A**). However, when proportions of Tregs with P4 supplementation were compared to gestation matched controls, there were no differences (**Figure 11B**). In addition, the longitudinal P4 effects were not reciprocated with the use of RU486 (**Figure 11C**). This led us to assume *in vivo*, P4 supplementation has little effect on the induction of Tregs in the periphery. Similarly, proportions of T-helper subtypes T_H1, T_H2, and T_H17 were unaffected by the use of P4 or RU486 (not shown). There were no longitudinal effects of P4 supplementation on CD4 PIBF expression. However, when compared to controls (**Figure 11D**), PIBF expression on CD4 T cells was decreased at 34 weeks of gestation, but the reverse was not observed with RU486 treatment (**Figures 11E,F**). PIBF expression on CD3 γδ or CD8 T cells was unaffected by P4 or RU486 (not shown).

P4 Does Not Appear to Affect NK or DC Phenotype

Overall, P4 had no longitudinal effects on NK cell expression of PIBF (Figures S4A,D,E in Supplementary Material). $CD16^+CD56^{lo}$ NK cells expressing PIBF represented a smaller proportion when compared to un-supplemented pregnancies at 34 weeks (Figure S4B in Supplementary Material). However,

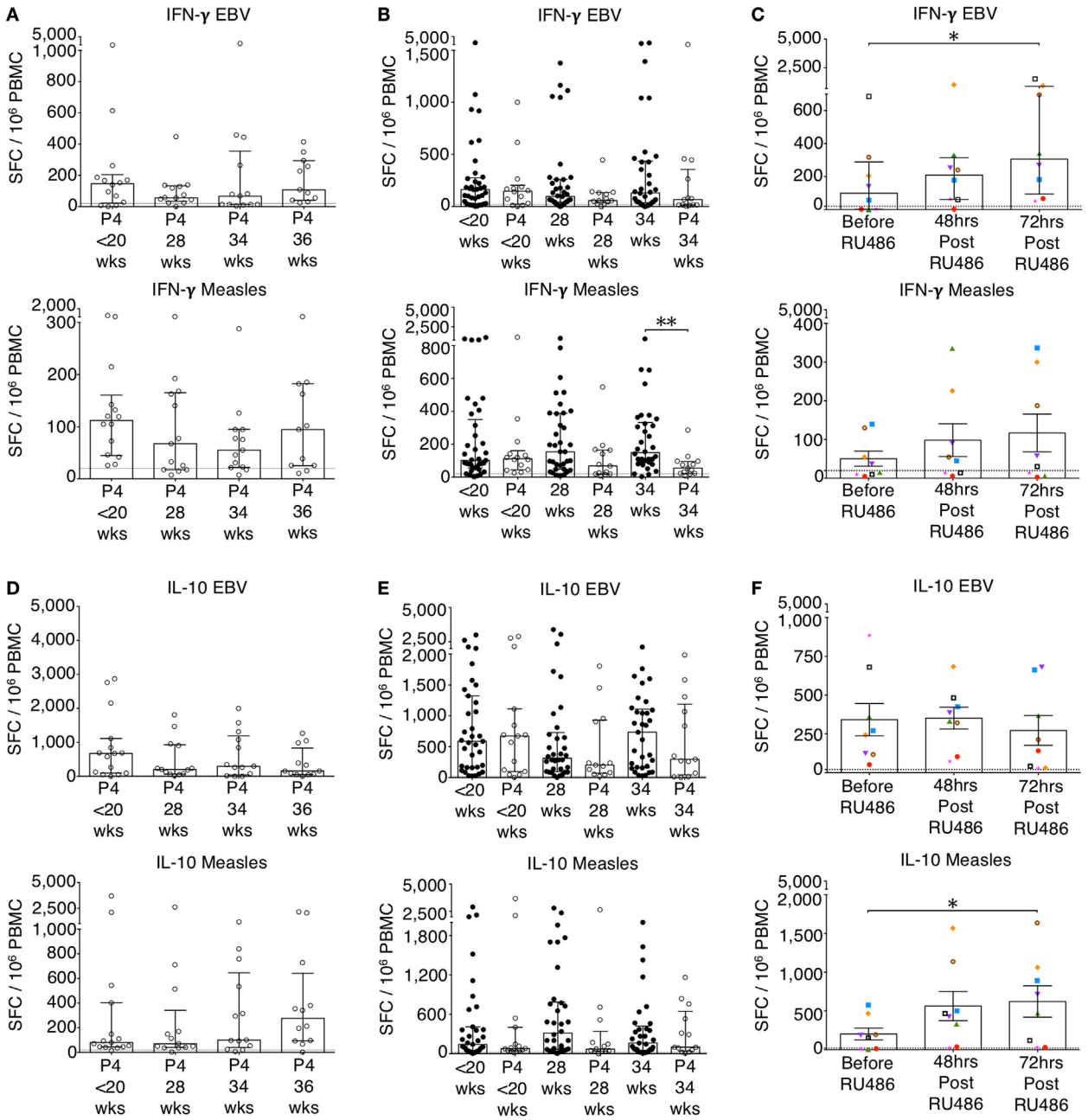


FIGURE 5 | P4 supresses IFN-γ and IL-10 enzyme-linked immunospot (ELISpot) responses. EBV and measles responses are shown. **(A)** Longitudinal analysis of IFN-γ responses in P4 treated. **(B)** IFN-γ responses in P4 treated compared to controls. Unpaired Mann–Whitney *U* test. **(C)** Longitudinal analysis of IFN-γ responses in RU486 treated. **(D)** Longitudinal analysis of IL-10 responses in P4 treated. **(E)** IL-10 responses in P4 treated compared to controls. Unpaired Mann–Whitney *U* test. **(F)** Longitudinal analysis of IL-10 responses in RU486 treated. Gestation at sampling is indicated: in pregnant controls (●) at $<20^{+0}$ weeks (*N* = *42*), 28 weeks (*N* = *35*), 34 weeks (*N* = *33*); and P4 treated pregnancies (○) at $<20^{+0}$ weeks (*N* = *15*), 28 weeks (*N* = *13*), 34 weeks (*N* = *13*), 36 weeks (*N* = *11*). For RU486 treated (*N* = *8*) symbols represent individual patients. A dashed line represents <20 spot forming cells/10^6. Longitudinal data were analyzed with either one-way analysis of variance with Tukey's *post hoc* correction or Freidman test with Dunn's *post hoc* correction depending on the data distribution. *P* values are two tailed and significance is defined as $^*P < 0.05$ and $^{**}P < 0.01$.

RU486 had no effect (Figure S4C in Supplementary Material). Likewise, proportions of $CD56^{hi}$ and iNKT did not change with P4 supplementation or RU486 treatment (Figures S4E,F,H,I in Supplementary Material).

Despite the previously mentioned changes seen on memory T cells, proportions of activated mDC and pDC expressing co-stimulatory markers, and HLA-G expressing $CD14^+$ DC-10 (38) remained un-affected by P4 or RU486 (not shown).

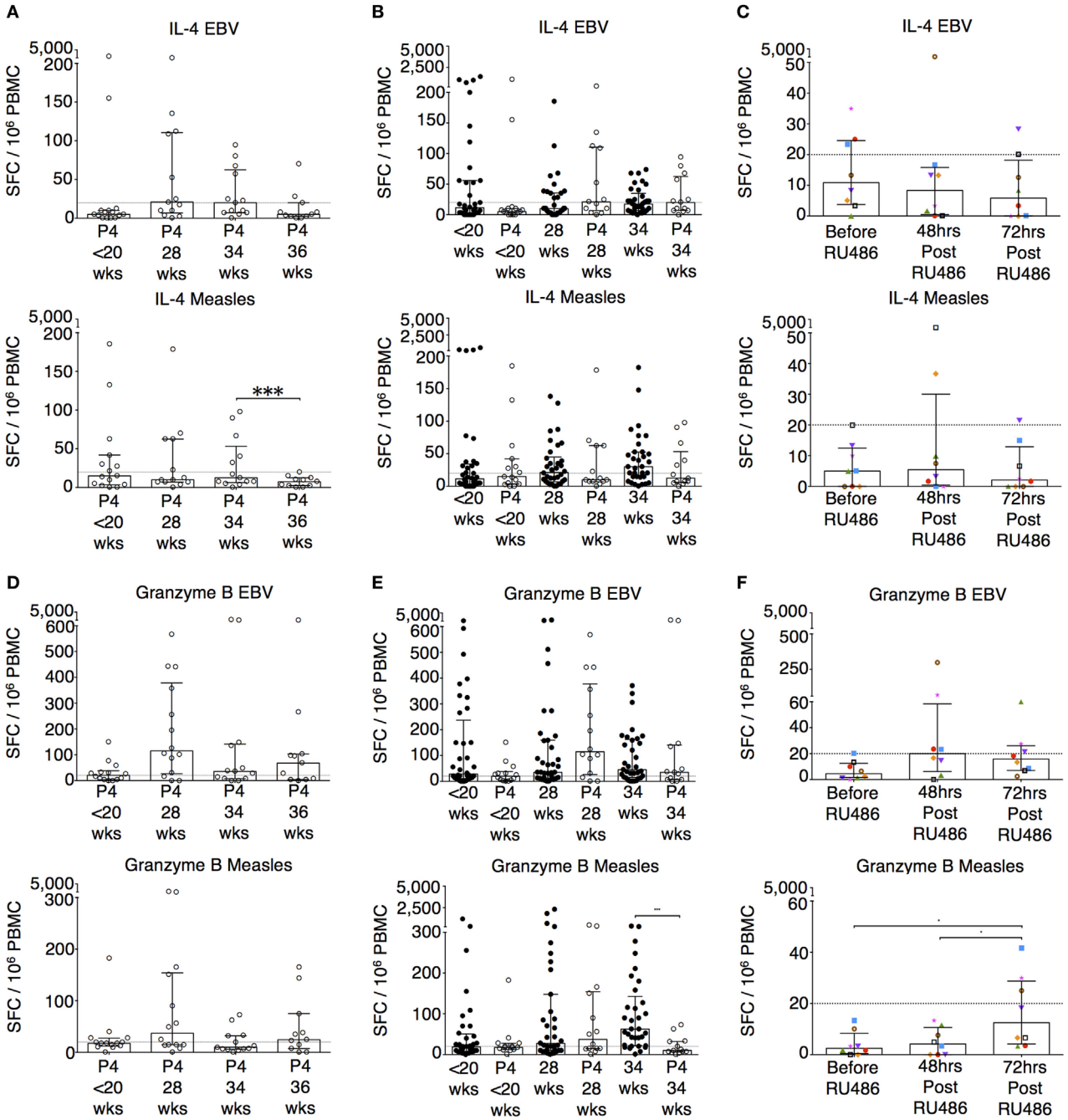


FIGURE 6 | P4 and RU486 have little impact on IL-4 B ELISpot responses but P4 suppresses and RU486 enhances Granzyme B ELISpot responses. EBV and measles responses are shown. **(A)** Longitudinal changes in IL-4 responses with P4 treatment. **(B)** Comparison of IL-4 responses in P4 treated and controls, and **(C)** longitudinal analysis in RU486 treated. **(D)** Longitudinal changes in granzyme B responses with P4 treatment. **(E)** Comparison of granzyme B responses in P4 treated and controls, and **(F)** longitudinal analysis in RU486 treated. Gestation at sampling is indicated: in pregnant controls (●) at $<20^{+0}$ weeks ($N = 42$), 28 weeks ($N = 35$), 34 weeks ($N = 33$); and P4 treated pregnancies (○) at $<20^{+0}$ weeks ($N = 15$), 28 weeks ($N = 13$), 34 weeks ($N = 13$), 36 weeks ($N = 11$). For RU486 treated ($N = 8$) symbols represent individual patients. A dashed line represents <20 spot forming cells/10^6. Longitudinal data were analyzed with either one-way analysis of variance with Tukey's *post hoc* correction or Freidman test with Dunn's *post hoc* correction depending on the data distribution. *P* values are two tailed and significance is defined as $^{***}P < 0.001$.

DISCUSSION

In this study, we investigated the effect of P4 on the peripheral maternal immune system. We used a novel approach by recruiting pregnant women taking P4 or RU486 using clinically effective regimens specifically designed to either reduce their risk of preterm labor or induce a functional P4 withdrawal with the aim of initiating labor. Furthermore, we are the first group to

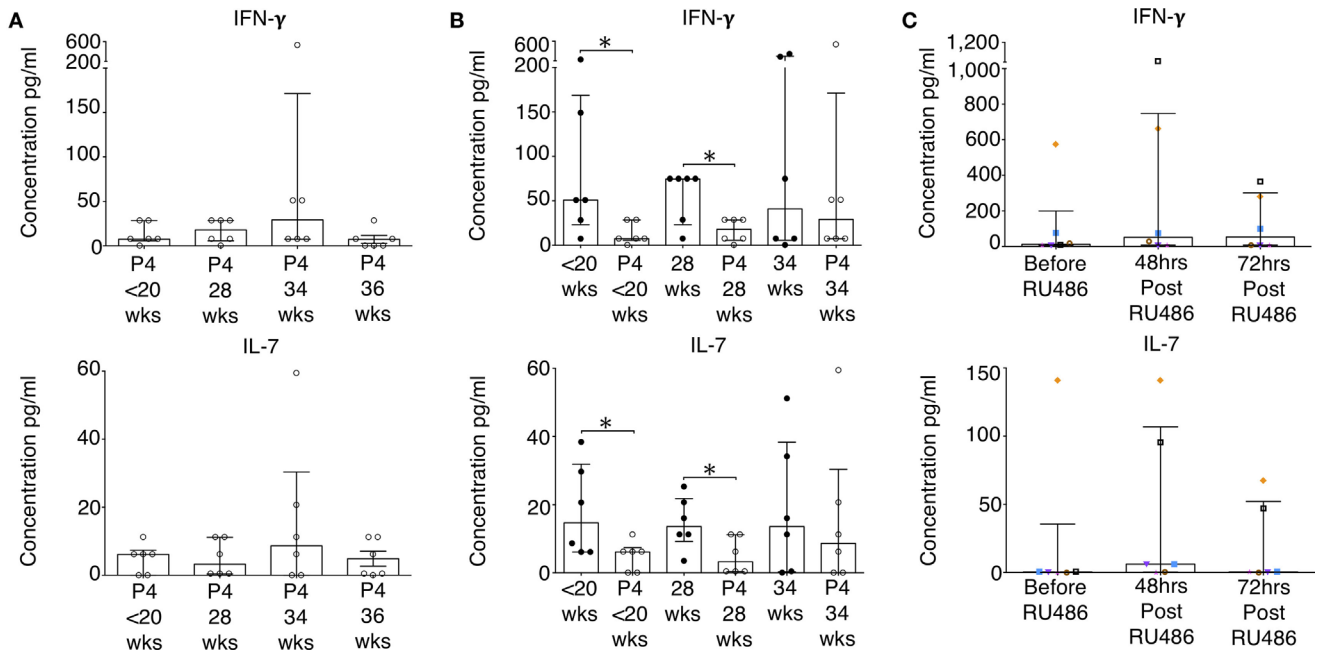


FIGURE 7 | Baseline concentrations of pro-inflammatory cytokines in the P4-treated group are reduced. Plasma concentrations of cytokines measured by 17-plex multiplex assay were determined. **(A)** Longitudinal analysis of IFN-γ and IL-7 concentrations of noted in the P4 treated group. **(B)** IFN-γ and IL-7 concentrations in P4 treated compared to gestation-matched controls. Unpaired Mann–Whitney *U* test. **(C)** Longitudinal analysis of RU486 effect on plasma cytokine concentrations. A representative sample of patients (*N* = *6*) were assessed. Gestation at sampling is indicated: in pregnant controls (●) at $<20^{+0}$ weeks (*N* = *42*), 28 weeks (*N* = *35*), 34 weeks (*N* = *33*); and P4 treated pregnancies (○) at $<20^{+0}$ weeks (*N* = *15*), 28 weeks (*N* = *11*), 34 weeks (*N* = *11*), 36 weeks (*N* = *10*). For RU486 treated (*N* = *6*) symbols represent individual patients. Longitudinal data were analyzed with Freidman test with Dunn's *post hoc* correction. *P* values are two tailed and significance is defined as $^{*}P < 0.05$.

examine the *in vivo* effects of RU486 on leukocyte function in the mid second trimester of pregnancy. Our data demonstrate that, in pregnancy, P4 reduces both pro-inflammatory and cytotoxic T cell responses, and that this suppressive effect is reversed with the use of RU486.

We have previously shown that, in normal pregnancy, IFN-γ and IL-10 responses to recall antigens are elevated in the third trimester (39). This occurs despite an increase in CD4 T_{EM} in the second trimester, suggesting that IL-10 may restrict the activation of the immune system by suppressing antigen specific T cell responses (39, 40). In the current study, our findings supported this concept. Both IFN-γ and IL-10 peripheral recall responses were largely elevated at delivery. Notably though, IL-10 responses showed marked increases in pregnancy across gestation despite a leukocyte phenotype that seemed to suggest a gradual decline of IL-10 antigen-induced immune tolerance with a longitudinal increase in T_H17 proportions, and the loss of any baseline pregnancy-related bias in CD4 Treg proportions. This occurred alongside an increasingly activated immune system, suggested by longitudinal increases in activated (CD38) and migratory (CCR6) T cells, mature memory T cells (CD28) with greater homing and effector function (34), and activated DCs (CD83). This gradual decline of immune-modulation was also evident in humoral and cytotoxic responses suggested by more frequent IL-4 SFCs in pregnancy, as well as increased granzyme B cytotoxic T cell activity, and IFN-γ and IL-10 responses to CD8 epitopes at delivery. In contrast, other studies have suggested that both humoral and cell-mediated immune responses are largely reduced in the third trimester, but innate responses to bacteria are increased (20, 41, 42). Our IL-4 findings likely reflect heightened humoral immune function in pregnancy, but the change in cytotoxic responses are suggestive of a loss of modulation of antigen specific memory CTL functions. We sought to determine how influential P4 may be as a driver of these changes.

Of note in our non-pregnant controls, we did not control for different phases in their menstrual cycle. In non-human primates, in peripheral blood, the luteal phase of the menstrual cycle, when P4 is at peak concentration, is associated with tolerant immune responses that favor successful pregnancy (43). It is assumed these changes transfer to humans. However, the majority of studies investigating the effects of menstruation show conflicting data. For example, although some researchers have shown Tregs initially expand during the follicular phase and then decrease mid-luteal, and that the luteal phase is associated with a decline in PBMC proliferation and IFN-γ production, T cell PHA responses are unchanged, and variations in Th1/Th2 cytokines have not been shown consistently in the literature (44–49). In contrast, NK cytotoxicity may be influenced by menstruation (44, 50). It is also important to appreciate that previous exposure to paternal antigens induces tolerance and so parity and sexual activity may enhance the effects of P4 (45, 51, 52). Furthermore, when compared to pregnant patients, the peak serum P4 concentration

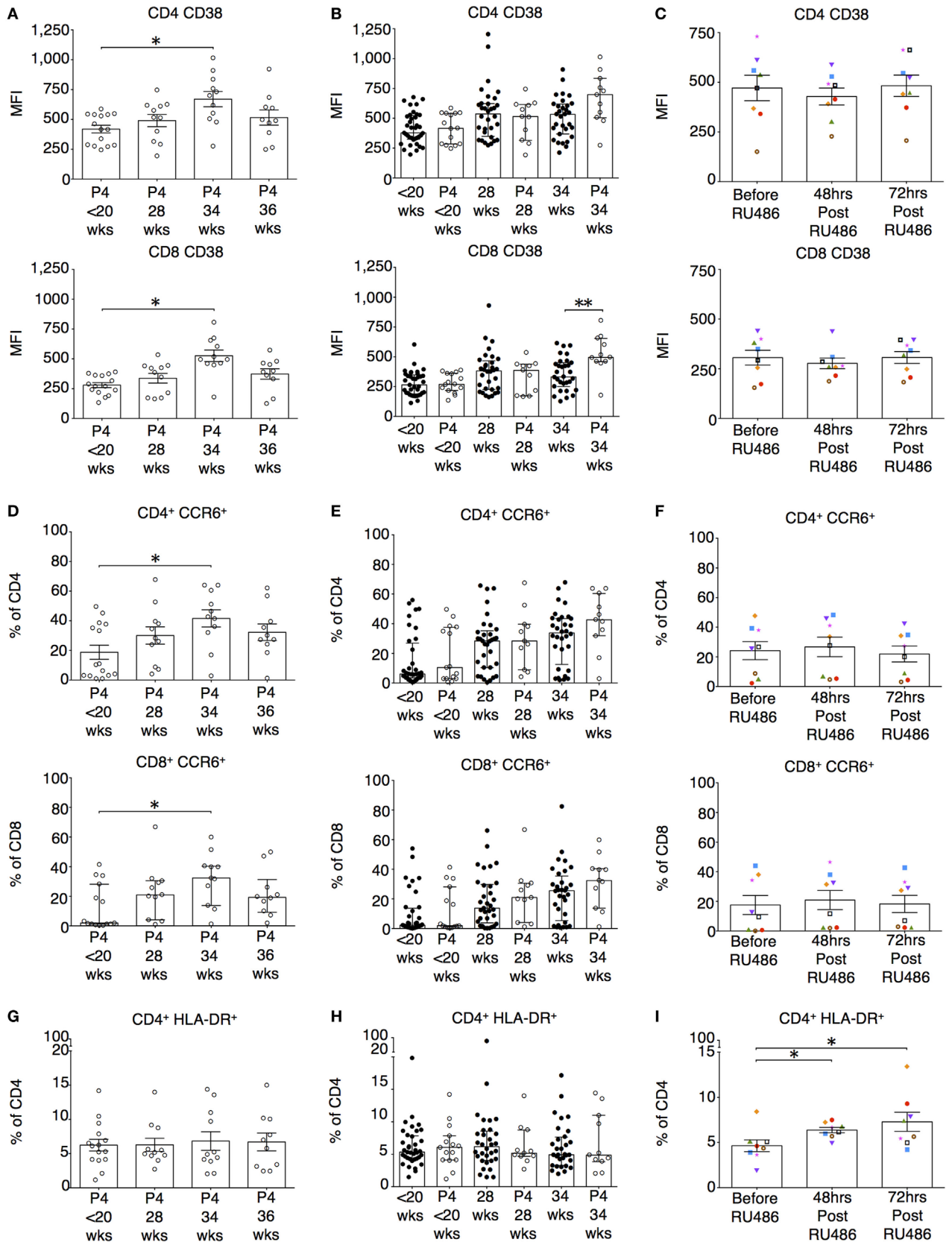


FIGURE 8 | Continued

FIGURE 8 | P4 does not suppress CD38 or CCR6 expression on T cells. However, RU486 increases HLA-DR expression on CD4 T cells. **(A)** Longitudinal analysis of CD38 expression measured by mean fluorescence intensity (MFI) on CD4 and CD8 T cells. **(B)** Comparing CD38 expression on CD4 and CD8 T cells in P4 treated with gestation matched controls. Unpaired Mann–Whitney *U* test. **(C)** Longitudinal analysis of the effect of RU486 on CD4 and CD8 T cell CD38 expression. **(D)** Longitudinal analysis of CCR6 expression measured by MFI on CD4 and CD8 T cells (Freidman test with Dunn's *post hoc* correction). **(E)** Gestation matched comparison of CCR6 expression on CD4 T cells between P4 treated and controls. **(F)** Longitudinal analysis of CCR6 expression on CD4 T cells from the RU486 group. **(G)** Longitudinal variation in HLA-DR expression on CD4 T cells in the P4-treated group. **(H)** Gestation-matched comparison of HLA-DR expression on CD4 T cells between P4 treated and controls. **(I)** Longitudinal analysis of HLA-DR expression on CD4 T cells from the RU486 group. Gestation at sampling is indicated: in pregnant controls (●) at $<20^{+0}$ weeks ($N = 42$), 28 weeks ($N = 35$), 34 weeks ($N = 33$); and P4 treated pregnancies (○) at $<20^{+0}$ weeks ($N = 15$), 28 weeks ($N = 11$), 34 weeks ($N = 11$), 36 weeks ($N = 10$). For RU486 treated ($N = 8$) symbols represent individual patients. Longitudinal data was analyzed with either one-way analysis of variance with Tukey's *post hoc* correction or Freidman test with Dunn's *post hoc* correction depending on the data distribution. *P* values are two tailed and significance is defined as $*P < 0.05$ and $**P < 0.01$.

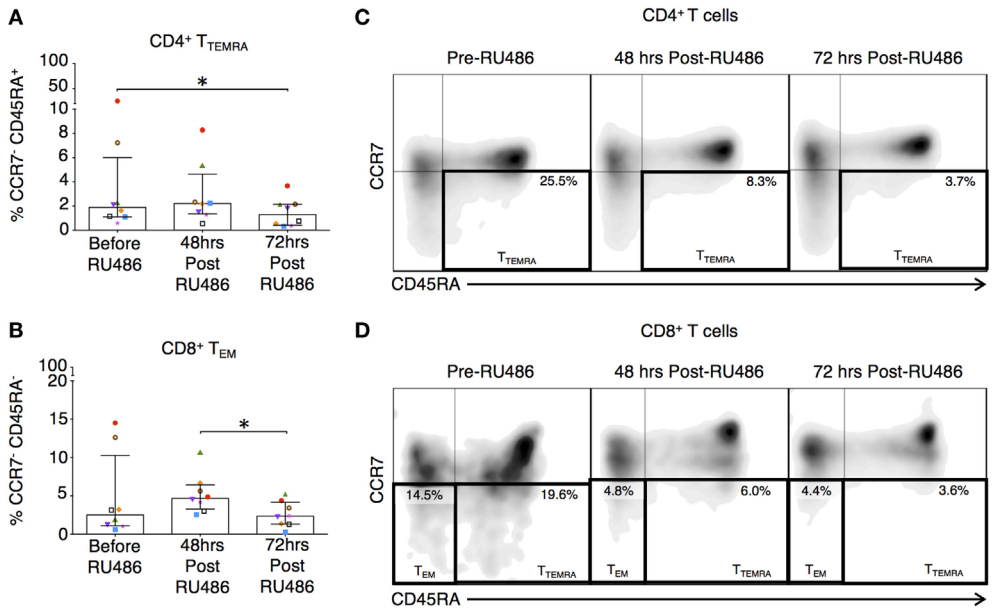


FIGURE 9 | RU486 treatment is associated with reduced proportions of CD4 and CD8 differentiated effector memory subtypes. **(A)** Longitudinal analysis of proportions of $CD4^+CCR7^-CD45RA^-$ T_{EM} and **(B)** CD8 T_{EM} ($CCR7^-CD45RA^-$) in RU486-treated ($N = 8$) symbols represent individual patients. *P* values are two tailed and significance is defined as $*P < 0.05$, by Freidman test with Dunn's *post hoc* correction. Representative flow cytometry plots of **(C)** singlet $CD4^+$ lymphocytes co-expressing CCR7 and CD45RA is shown with percentages of T_{EM} cell, and **(D)** singlet $CD8^+$ lymphocytes co-expressing CCR7 and CD45RA is shown with percentages of T_{EM} and T_{TEMRA} cells. Populations were gated using isotype controls.

in non-pregnant women is approximately 15 versus 60 ng/ml in early and 180 ng/ml in late pregnancy, which represent a 4- and 12-fold increase, respectively (53, 54). This suggests that any changes due to menstrual variation in serum P4 are unlikely to compare to changes in pregnancy. In fact, in our previous work, we found that despite *in vitro* culture with P4, leukocyte PIBF expression in controls, at different stages in their menstrual cycle, did not equal those seen in pregnancy suggesting that neuro-endocrine differences are not the sole determinant of leukocyte phenotype in pregnancy (39). Therefore, in our study, peripheral blood samples were not controlled for phases of the menstrual cycle.

Serum concentrations of endogenous P4 increase throughout pregnancy and reach 175–636 nmol/l in the maternal circulation during the third trimester (53). Yet, as a potent endogenous suppressor of cytotoxic immune responses and regulator of cytokine secretion, our results suggest its effects decline after 34 weeks of pregnancy. Therefore, we expected the addition of exogenous P4, as well as modeling its withdrawal using RU486, would be noticeable until the end of the second trimester pregnancy and thereafter become less obvious. Previous studies have shown that, outside of pregnancy, P4 is antiproliferative and suppresses the production of the pro-inflammatory cytokines IFN-γ, TNF-α, while upregulating IL-4 in PBMC cultures with PHA, and that these effects are reversed by RU486 (1, 14). In addition, P4 has been shown to suppress cytotoxic T cell activity (12). Conversely, RU486 has been shown to increase uterine NK cytotoxicity (12, 55, 56). In human and animal models, P4 also has systemic and tissue specific anti-inflammatory effects, acting predominantly *via* the inhibition of NFκB and MAPK/AP-1 pathways (3, 57–59). However, in some disease states, P4 is less effective once pro-inflammatory processes are established, and this may be associated with a differential tissue expression of PR isoforms (60, 61). In fact, in myometrial tissue, this is thought to be one of

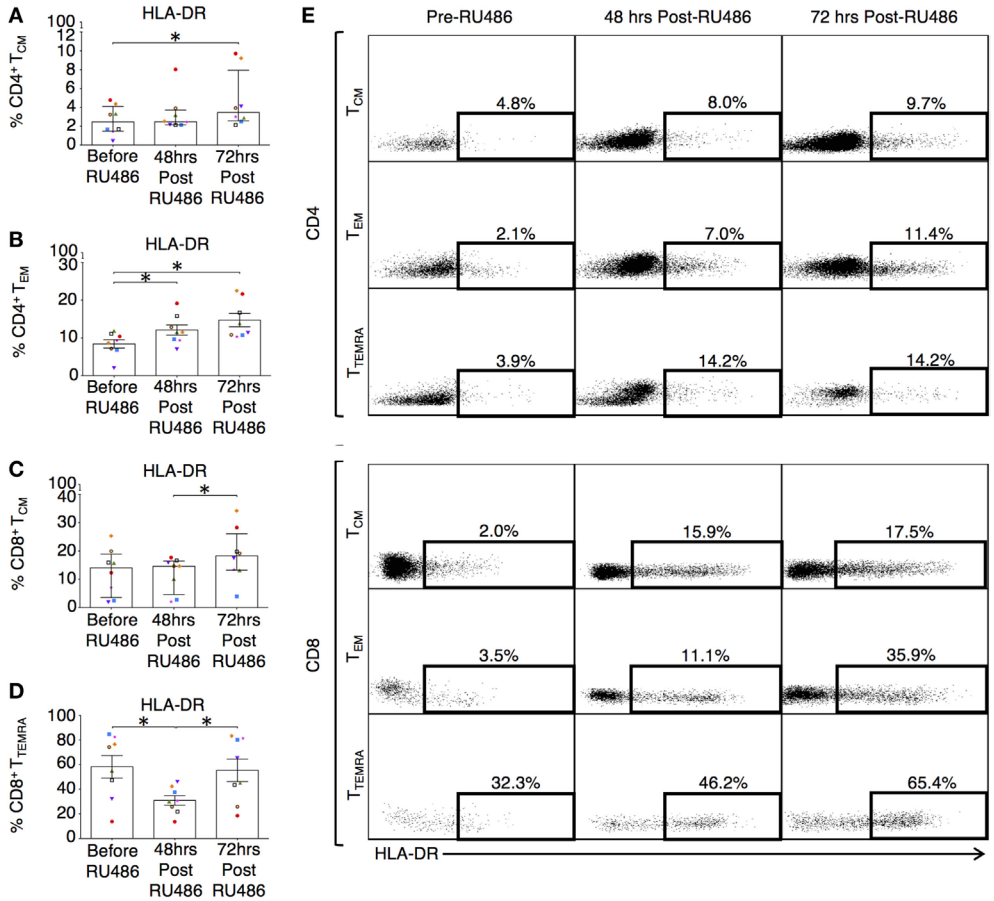


FIGURE 10 | Differentiated effector memory subtypes in RU486 treated have an increased expression of HLA-DR. **(A)** Longitudinal change in percentage expression of HLA-DR on: CD4 T_{CM} (Freidman test with Dunn's *post hoc* correction), **(B)** CD4 T_{EM} [one-way analysis of variance (ANOVA) with Tukey's *post hoc* correction], **(C)** CD8 T_{CM} (Freidman test with Dunn's *post hoc* correction), and **(D)** CD8 T_{TEMRA} (one-way ANOVA with Tukey's *post hoc* correction). *P* values are two tailed and significance is defined as $^*P < 0.05$. **(E)** Representative flow cytometry plots showing the percentage expression of HLA-DR on both CD4 and CD8 ($CCR7^+CD45RA^-$) T_{CM}, ($CCR7^-CD45RA^-$) T_{EM}, and ($CCR7^-CD45RA^+$) T_{TEMRA} subsets in PBMC from subjects receiving RU486 treatment. Memory $CD4^+$ and $CD8^+$ subset HLA-DR expression, gated with SSC on the vertical axis is shown. Populations were gated using isotype controls and percentages of HLA-DR expressing cells is indicated.

the contributors to the onset of term labor (62). We found that, in contrast to control pregnancies, P4 supplementation appeared to encourage a stable IFN-γ response that was significantly reduced when compared to gestation-matched controls. This occurred without any negative effect on longitudinal CD38 and CCR6 T-cell expression, which continued to increase despite exogenous P4 supplementation. Both receptors are important for inflammatory responses and cell migration. CD38 is characteristically raised in chronic inflammation and is associated with an enhanced ability to produce IFN-γ (63, 64). CCR6 is expressed on effector memory populations that contribute to recall responses (33). RU486 treatment had the opposite effect, with significant increases in IFN-γ responses posttreatment. These changes were statistically significant 72 h post RU486, which was post-delivery, rather than 48 h post treatment. However, altogether, our findings point to a P4 mediated effect rather than a consequence of delivery. P4 is known to negatively effect polyfunctional cytokine production from CD8 T cells and suppresses decidual lymphocyte cytotoxicity (12, 14). Granzyme B is primarily produced by activated CTLs and NKs cells and is, therefore, a useful surrogate marker for the functional activity of CTLs. Similar to the IFN-γ data, granzyme responses in the P4 treated group, when compared to gestation-matched controls at 34 weeks of pregnancy, were reduced; conversely, in the RU486 treated group, the response was increased, and these included responses against FEC peptide pool that incorperates CD8 specific epitopes. Although the latter results were significant post delivery, the control pregnancies did not show delivery was associated with any changes in the granzyme B response, suggesting that the differences are are most likely to be RU486 related.

Interestingly, although IL-10 and IL-4 responses in the P4 as well as in the RU486 treated groups were, on the whole, unaffected, suggesting the altered antigen-specific responses are not T_H2 or IL-10 driven. Our findings contradict murine models that have previously shown that in pregnancy, systemic and uterine Treg proportions as well as their suppressive activity is increased with P4 and blocked by RU486, but this increase does

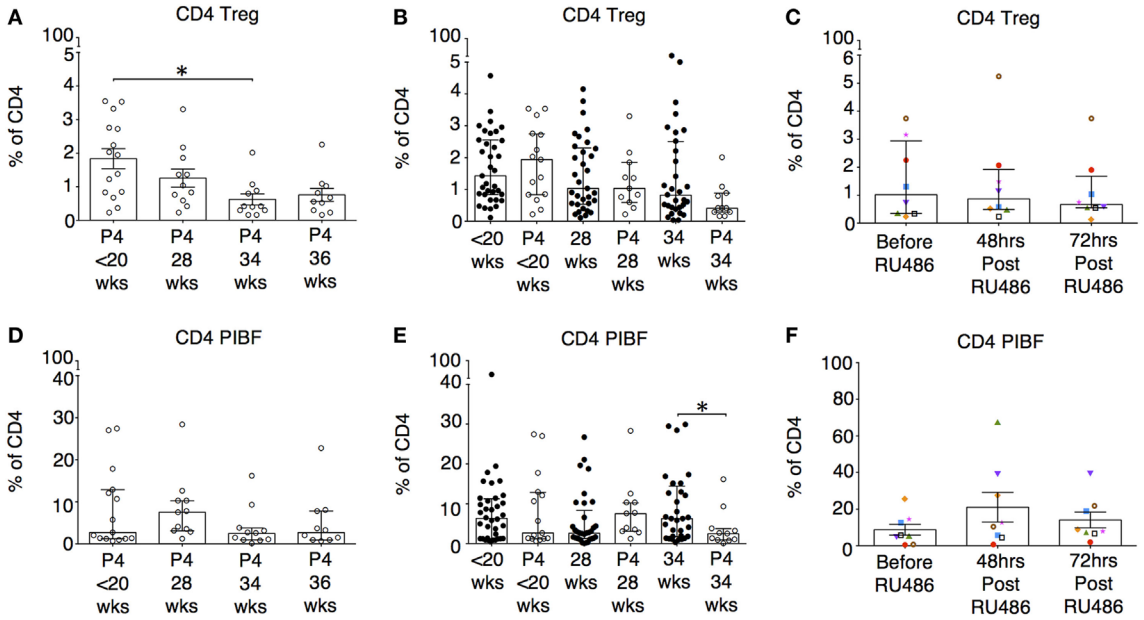


FIGURE 11 | The influence of P4 and RU486 on CD4+ regulatory T cell (Tregs) and P4-induced blocking factor (PIBF) expressing CD4+ T cells. **(A)** Longitudinal analysis of CD4 Treg proportions ($CD4^+CD45RO^+CD25^+CD127^{lo}$) in peripheral blood obtained from P4 treated patients. **(B)** Gestation-matched paired comparisons of Treg proportions in peripheral blood obtained from P4 treated versus untreated pregnant controls that were previously analyzed longitudinally in **Figure 3C**. **(C)** Longitudinal analysis of Treg proportions in peripheral blood obtained from RU486 treated pregnant patients. **(D)** Longitudinal analysis of PIBF expressing CD4 T cells in peripheral blood obtained from P4-treated patients. **(E)** Gestation matched paired comparisons of PIBF expressing CD4 T cells in peripheral blood obtained from P4 treated versus untreated pregnant controls (Unpaired Mann–Whitney *U* test). **(F)** Longitudinal analysis of PIBF expressing CD4 T cells in peripheral blood obtained from RU486-treated pregnant patients. Gestation at sampling is indicated: in pregnant controls (●) at $< 20^{+0}$ weeks (*N* = *42*), 28 weeks (*N* = *35*), 34 weeks (*N* = *33*); and P4 treated pregnancies (') at $<20^{+0}$ weeks (*N* = *15*), 28 weeks (*N* = *11*), 34 weeks (*N* = *11*), 36 weeks (*N* = *10*). For RU486 treated (*N* = *8*) symbols represent individual patients. Longitudinal data were analyzed with either one-way ANOVA with Tukey's *post hoc* correction or Freidman test with Dunn's *post hoc* correction depending on the data distribution. *P* values are two tailed and significance is defined as $^*P < 0.05$.

not necessarily prevent spontaneous fetal loss (65). Schumacher et al. showed that in abortion-prone mice, although decidual Treg numbers increased following intra-peritoneal P4 administration, this did not result in greater fetal survival (66). It is possible that these Tregs may have been poorly functioning or that other pathways are more influential at improving fetal outcomes. Of note, the human equivalent doses (HED) of P4 used in both of these studies were either comparable or lower than the 400 mg P4 dose used in our study, and the RU486 HED (30 mg) used by Mao et al. was far less than that used in our study (200 mg) (65–67). Therefore, the different findings in their work compared to ours are surprising. All of the aforementioned murine studies analyzed the effects of P4 in early and mid pregnancy in mice, when the maternal response to the initial surge in fetal antigen is vital for ongoing pregnancy success. Therefore, expansion of potentially poorly functioning Tregs may not be sufficient to improve tolerance to fetal antigen. Interestingly, intravenous adoptive transfer of Tregs from normal pregnant to abortion prone mice at days 0–4 of pregnancy improves placentation and reduces reabsorption rates (68–70). However, miscarriage rates were not completely abrogated in these studies, and the same positive findings were not reproduced when the Tregs were isolated from non-pregnant mice (68–70). Our own results in pregnancy compared to non-pregnant controls showed an increased proportion of Tregs at baseline. However, we also found a longitudinal fall in Treg proportions with P4 supplementation. Mjösberg et al. have previously predicted such an effect, where, *in vitro,* P4 and 17β-estradiol reduced functionally suppressive CD4 $Foxp3^+$ Tregs (71). Unfortunately, we did not investigate the suppressive activity of Tregs in this study, but the fall in number likely reflects the systemic anti-inflammatory effect of P4 and suggests pathways other than those involving Tregs are at play.

It is also worth noting that our unstimulated sera concentrations of IFN-γ, IL-1β, and IL-7 were significantly reduced pretreatment in the P4 group, and IFN-γ and IL-7 were also comparatively reduced at 28 weeks, suggesting that the effects were P4 related. These findings were not reciprocated in the RU486 group. The most likely confounder is that the P4-treated cohort represents patients at risk of premature delivery and so may have inherent immune differences. However, there were no other baseline differences between groups despite two of the patients delivering preterm at 34+ weeks of gestation immediately after stopping P4 treatment.

Our ELISpot results suggest that P4 may disrupt pro-inflammatory cell-mediated responses and its prolonged use is associated with suppressed antigen specific memory CTL responses, which are not fetal antigen specific. From our data, the latter effect does not seem to be driven by PIBF unlike in previous published reports (12, 72). In the P4-treated group, despite proportions of PIBF expressing CD4 T cells and cytotoxic

$CD16^{+}CD56^{lo}$ NK cells being reduced at 34 weeks of gestation, our RU486 data did not show any reciprocal changes. This is unusual as P4 has been shown to promote the production of PIBF from lymphocytes *in vitro via* PR (72). PIBF has a known anti-cytotoxic effect on NK cells, and this is thought to be by co-localizing in cytoplasmic granules and blocking degranulation (73, 74). Therefore, our results suggest that P4 supplementation in pregnancy has a limited effect on cellular PIBF expression, and consequently, that its effect on gestational length is independent of PIBF synthesis. It is likely that this is because P4 is acting *via* GR and thus the lack of opposite effects by RU486 may be due to different receptor-binding affinity (75, 76). Furthermore, GR binding may not always produce an immune suppressed affect. In transgenic rats, endogenous GR engagement is associated with an increase in activated and memory T cells (77). Regardless, our results showed suppressed IFN-γ responses, but we did not find any differences in memory T cell activation marker expression. This is in contrast to *in vitro* studies in animals, which have shown that in addition to inhibiting DC stimulation of naïve T cells in rats and memory CD8 IFN-γ production in mice, P4 reduces murine memory CD8 T cells during heterosubtypic influenza virus challenge (78–80). However, in the RU486 group, we found changes in MHC class II molecule expression on memory T cells that was highly suggestive of a reversal of pregnancy associated immune-modulation. The expression of HLA-DR increased on CD4 T_{EM} cells post RU486 treatment, and although the expression of HLA-DR was reduced on CD8 T_{TEMRA} cells, it subsequently increased 72 h post RU486. It is possible that CD8 T cells have a greater threshold for antigen exposure, and that the contributions of T_{EM} and T_{CM} cells can vary (81, 82). Furthermore, we show delivery in the presence of RU486 was associated with a fall in T_{TEMRA} and T_{EM} proportions, but with concurrent increases in HLA-DR expression. Surface MHC class II molecule expression on CD4 T cells corresponds to an improved ability for these cells to present antigen and regulate immune responses (83). Previously, murine data suggested that RU486 inhibits apoptosis of mature DC *via* GR and promotes HLA-DR expression on monocytes in humans (84, 85). Therefore, the effects of RU486 may be to initiate a rapid recovery of immune function as demonstrated by the ELISpot data, since, classically, memory T cells are highly responsive to recall antigens.

In conclusion, this study describes a novel method of examining the effects of P4 on the maternal immune system. Our data suggest that exogenous P4 reduces pro-inflammatory and cytotoxic T cell responses in pregnancy. It achieves this by a combination of effects on cell-mediated interactions, including altering memory T cell antigen sensitivity and regulating leukocyte migration. These effects are, in part, reversed with the use of RU486. Our results have identified which aspects of the maternal immune response are P4-regulated, as such modulation of these pathways may have potential as future therapeutic targets with the aim of modulating the maternal immune response to pregnancy. Future *in vivo* human work will help to key establish the cellular interactions at play during human pregnancy.

ETHICS STATEMENT

All subjects were recruited from Chelsea and Westminster Hospital, London, UK. This study was carried out in accordance with the recommendations of National Institute of Health Research (NIHR) Good Clinical Practise guidelines, and a NHS Research Ethics Committee. The protocol was approved by the National Research Ethics Service (NRES), London, UK committee as well as by Chelsea and Westminster NHS Trust, London, UK; Ref: 11/LO/0971. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

NS, NI, and MJ had a substantial contribution to the conception and design of the project and its interpretation; were responsible for the acquisition, analysis, and interpretation of the data, and drafted the work. All authors contributed to revising of the manuscript and have approved the final version. All authors agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01293/full#supplementary-material.

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REVIEW
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Disruption in the Regulation of Immune Responses in the Placental Subtype of Preeclampsia

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Preeclampsia is a pregnancy-specific disorder, of which one of its major subtypes, the placental subtype is considered a response to an ischemic placental environment, impacting fetal growth and pregnancy outcome. Inflammatory immune responses have been linked to metabolic and inflammatory disorders as well as reproductive failures. In healthy pregnancy, immune regulatory mechanisms prevent excessive systemic inflammation. However, in preeclampsia, the regulation of immune responses is disrupted as a result of aberrant activation of innate immune cells and imbalanced differentiation of T-helper cell subsets creating a cytotoxic environment *in utero*. Recognition events that facilitate immune interaction between maternal decidual T cells, NK cells, and cytotrophoblasts are considered an indirect cause of the incomplete remodeling of spiral arteries in preeclampsia. The mechanisms involved include the activation of immune cells and the subsequent secretion of cytokines and placental growth factors affecting trophoblast invasion, angiogenesis, and eventually placentation. In this review, we focus on the role of excessive systemic inflammation as the result of a dysregulated immune system in the development of preeclampsia. These include insufficient control of inflammation, failure of tolerance toward paternal antigens at the fetal–maternal interface, and subsequent over- or insufficient activation of immune mediators. It is also possible that external stimuli, such as bacterial endotoxin, may contribute to the excessive systemic inflammation in preeclampsia by stimulating the release of pro-inflammatory cytokines. In conclusion, a disrupted immune system might be a predisposing factor or result of placental oxidative stress or excessive inflammation in preeclampsia. Preeclampsia can thus be considered a hyperinflammatory state associated with defective regulation of the immune system proposed as a key element in the pathological events of the placental subtype of this disorder.

Keywords: preeclampsia, trophoblast invasion, immune regulation, pregnancy, monocytes, inflammation

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INTRODUCTION

Preeclampsia is a multisystem heterogeneous disorder unique to pregnancy and is considered a leading cause of maternal as well as fetal/neonatal morbidity and mortality (1). The World Health Organization has estimated 50,000–60,000 preeclampsia-related maternal deaths per year worldwide (2–4). Preeclampsia is a syndrome defined as *de novo* gestational hypertension [systolic- and

diastolic blood pressure (BP) of more than 140/90 mmHg respectively] plus new-onset proteinuria (minimal of 300 mg), or one or more signs of systemic involvement (5, 6). The clinical spectrum includes manifestations of the maternal and fetal component associated with reduced blood flow and abnormal oxygenation in the placenta, intrauterine growth restriction (IUGR), and prematurity (7, 8). Adverse clinical conditions and maternal organ dysfunction associated with preeclampsia include renal insufficiency, liver involvement, neurological or hematological complications, and uteroplacental dysfunction (9). These conditions can progress to eclampsia, stroke, uncontrolled severe hypertension, acute kidney injury, liver hematoma, liver rupture, and cardiac failure as well as severe complications also involving the fetus by possible abruption of placental membranes and stillbirth (5). A 12-fold increase in the risk of cardiovascular disease has been found in women with a history of preeclampsia and metabolic disease, highlighting a relationship between preeclampsia and cardiovascular disease (10).

Preeclampsia is characterized by two major subtypes: the maternal subtype also known as the metabolic immunologic subtype and the placental subtype that entails placental ischemic–hypoxic stress followed by systemic maternal inflammation. Although immune dysregulation plays a substantial role in both subtypes, the two subtypes have different etiologies and phenotypes, while the placental subtype refers to early-onset preeclampsia with an etiology of abnormal placentation under hypoxic conditions (11). The pathogenesis of preeclampsia was originally ascribed to endothelial dysfunction (12), which also plays a central role in the development of cardiovascular disease. In fact, preeclampsia shares many risk factors with cardiovascular disease, such as obesity, hypertension, insulin resistance, and dyslipidemia, all conditions, which are characterized by inflammation (13–16). It is now believed that adverse immune responses generate the endothelial dysfunction that can lead to hypertension in pregnant women (17). Pregnancy already imposes an immunological challenge on the host, since direct contact of circulating and uterine immune cells with placental tissue requires adaptations by the maternal immune system to maintain tolerance to the fetus (18). The exact pathogenesis of preeclampsia is, however, still unclear and has resulted in multiple hypotheses about the underlying mechanisms (19). One such hypothesis is that the etiology of preeclampsia is primarily immunological, since immune mechanisms are the interconnection between placental ischemia and maternal cardiovascular disease (17, 20). The placenta is a major etiological factor in the pathogenesis of preeclampsia and other etiological factors such as placental cells, angiogenic and antiangiogenic proteins involved in the complex pathology of preeclampsia are described later in the widely accepted two-stage model. Briefly, poor placentation results in an oxidatively damaged placenta (21, 22) that releases several placental factors into the maternal circulation, eliciting a maternal systemic inflammatory response and endothelial dysfunction (23). This review briefly discusses the aspects associated with the placental subtype of preeclampsia with special focus on the dysregulation of immune responses.

THE PATHOLOGICAL EVENTS OF PREECLAMPSIA

The placental cells involved in the pathological events of preeclampsia are specialized extravillous cytotrophoblasts and villous syncytiotrophoblasts, which have distinctive proliferative and invasive properties (19). These separate subgroups originate from two different villous cytotrophoblast precursors (24). Extravillous cytotrophoblasts are differentiated into an invasive phenotype, with high migratory, proliferative, and invasive properties (25). During weeks 8–18 of normal pregnancy, cytotrophoblasts invade the decidua to induce extensive remodeling of the uteroplacental spiral arteries (21). Remodeling of these spiral arteries is important for reducing resistance to maternal blood flow to enable efficient blood supply to the fetal compartment (26). This process is called placentation and effectively modifies the quality of maternal blood flow to be non-pulsatile and ensures a low-pressure state in the placenta (21). An optimal uterine environment is established to meet the metabolic demands and the required rate of the physiological exchange of nutrients and oxygen between the maternal and fetal systems (27). In the initial stage of preeclampsia, cytotrophoblasts fail to invade the decidua and restrict the subsequent modification of the uteroplacental spiral arteries (8). However, poor placentation is not the only cause of the placental subtype of preeclampsia, but acts as a predisposing factor to the development of a maternal syndrome with immunological involvement (7, 21). The complex pathology of preeclampsia can be explained according to a widely accepted two-stage model (21).

The Pathological Events of Preeclampsia in a Two-Stage Model

The pathophysiology of preeclampsia has been formalized in a two-stage model that delineates the evolution from poor placentation to the maternal clinical syndrome (22). As described above, poor placentation is due to the incomplete remodeling of the spiral arteries, resulting in high BP flow and irregular delivery of fully oxygenated arterial blood to the placenta (21). The latter is termed placental ischemia/hypoxia and is associated with distortion of the placental villous architecture and increased oxidative stress (21). The subsequent uteroplacental insufficiency results in compromised blood flow to the uterus, resulting in pregnancies with increased perinatal morbidity and mortality (1). However, incomplete remodeling of the spiral arteries is not only associated with preeclampsia but is also found in IUGR and gestational hypertension (22, 28), suggesting that it is not the only cause of preeclampsia. During the initial stage of preeclampsia, poor placentation occurs with no clinical features, followed by a second stage that involves a maternal clinical syndrome with cardiovascular manifestations and renal features (17, 21, 29).

Inadequate invasion of cytotrophoblasts following the incomplete remodeling of spiral arteries exposes the placenta to oxidative stress (22). The second stage of the disorder ensues because the ischemic placenta and increased placental oxidative stress cause excessive systemic inflammation and endothelial dysfunction that manifest as new-onset hypertension and proteinuria

(21, 22, 29, 30). The end result of oxidative stress in preeclampsia is exaggerated placental necrosis or apoptosis, which are common histologic features of the preeclamptic placenta (21, 31). Excessive shedding of syncytiotrophoblast microparticles, reflecting placental ischemia and apoptosis of placental cells, into the maternal circulation, creates an inflammatory burden and indirectly affects endothelial function (32–35). This role of a diseased placenta in preeclampsia is part of the emerging theory classifying preeclampsia as two different diseases, either placentogenic or maternogenic (36). The different phenotypes of preeclampsia can thus either be placentogenic, which usually occurs in early pregnancy with association of poor placentation and different severities of IUGR, or maternogenic, which occurs late in pregnancy with no relation to placental insufficiency or IUGR (37). The metabolic syndrome (defined as the occurrence of hypertension, ischemic heart disease, type 2 diabetes mellitus, obesity, and insulin resistance) has also been associated with increased circulating microparticles and subsequent systemic inflammation (38).

The exact stimuli inducing inflammation in preeclamptic patients are, however, incompletely understood and various other possibilities exist (32, 39). For instance, an injured endothelium may release several factors thought to play a role in this systemic inflammatory response (40). These factors include pro-inflammatory cytokines, markers of oxidative stress, thrombomodulin, fibronectin, endothelin-1, and Von Willebrand factor (40, 41). Other placental factors, such as anti-angiogenic [soluble fms-like tyrosine kinase (sFlt-1)] factors secreted by placental cells can also be considered as possible stimuli, since these factors affect the endothelium, a component of the inflammatory system (22, 29, 34). Protein toxic aggregates such as aggregated transthyretin which, is a placental toxin elevated in preeclampsia, have been suggested to contribute to the pathogenesis of this disorder. Elevated levels of aggregated transthyretin are formed in preeclampsia and transported through the secretion of placental extracellular vesicles by syncytiotrophoblasts (42). Aggregated transthyretin in these vesicles may also allow targeted delivery of these toxic proteins to other maternal organs, conducting a signal of cellular stress from the diseased placenta and contributing to the pathogenesis of preeclampsia (42). Protein toxic aggregates is a novel approach, they have been considered to be pathogenic in other diseases, thus the possibility of contributing to excessive inflammation in preeclampsia through creating cellular stress should also be considered (42–44).

The role of inflammatory markers with elevated levels in preeclampsia has also been investigated, including the role of procalcitonin (PCT) and C-reactive protein (CRP) (45). PCT is a precursor of calcitonin and is used as a marker of bacterial infection and resultant systemic inflammation (45, 46). The exact role of PCT and CRP in preeclampsia remains controversial, with the literature revealing conflicting accounts of their usefulness in predicting preeclampsia and its severity (45).

It has been well established that the human microbiota plays a fundamental role in the functioning of the immune system and the regulation of immune responses (47). Microbiota–immune interaction is mediated through microbial-associated molecular patterns; for instance, the bacterial endotoxin lipopolysaccharide (LPS) is recognized by cellular toll-like receptors (TLRs) (47). Researchers have established LPS to be an external stimulus, which is able to induce hypertension and proteinuria identical to preeclampsia in pregnant rats (48). In this particular animal model of human preeclampsia, lipid A/LPS, a known pro-inflammatory stimulus, provoked systemic inflammation and was associated with the clinical manifestations of preeclampsia (48). In a study by Cotechini et al. (49) performed on pregnant rats, the administration of LPS induced a systemic and local inflammatory response, fetal growth restriction (FGR), and an increase in mean arterial pressure, through a mechanism mediated by tumor necrosis factor (TNF). In addition, the authors observed deficient trophoblast invasion and impaired spiral artery remodeling characteristic of preeclampsia, to be linked to LPS-induced FGR (49). Similar results were found by Xue et al. (50) who demonstrated that a single administration of LPS to pregnant rats induced inflammation specifically by binding to its receptor, the TLR-4 in the placenta. TLR-4 signaling in the placenta was associated with deficient trophoblast invasion and spiral artery remodeling contributing to poor placentation that may result in a preeclampsia-like syndrome (50). Ultimately, all of these secreted placental factors and stimuli, including bacterial endotoxin, are capable of disrupting the fine-tuned balance of the immune system and inducing a systemic inflammatory response.

Angiogenesis as Part of Placentation and Biomarkers Present in Preeclampsia

The extensive angiogenesis that is characteristic of successful placentation is indirectly disrupted by placental ischemia/hypoxia in preeclampsia (1, 27, 51). Angiogenesis is critical for the improvement of placental circulation and blood flow by the formation of new vascular beds to enable vascular growth and development of the placenta (27). Placental factors, specifically pro-angiogenic [vascular endothelial growth factor (VEGF) and placental growth factor (PIGF)] and anti-angiogenic proteins, are responsible for placental growth, vascularization, and maintenance of vessel health (8, 27). These factors act as biomarkers that reflect unique patterns in preeclampsia according to the severity as well as the gestational age at the onset of the disorder (52). Preeclampsia is associated with decreased levels of vasodilators, such as nitric oxide (NO) and prostacyclin, as a result of the disproportionate increase in sFlt-1 and decrease in VEGF (**Figure 1**) and PIGF (53–55).

The growth factor VEGF and its homolog, PIGF, are essential for angiogenesis because of their pro-angiogenic activity that induces vascular permeability and promotes the proliferation and survival of epithelial cells (40). VEGF may cause vasodilation by stimulating the NO-cyclic guanosine monophosphate vascular relaxation pathway as well as by increasing the production of the calcium ion $(Ca^{2+})i$ (53). An increase in $(Ca^{2+})i$ promotes the binding to endothelial NO (eNOS) and subsequently increases eNOS activity and stimulates NO production (**Figure 1**) (53). Transforming growth factor (TGF)-β1 is involved in angiogenesis by regulating the expression of VEGF by means of intracellular signaling (40, 56). The inhibition of TGF-β1 signaling will lead to reduced endothelium-dependent vasodilation and increased

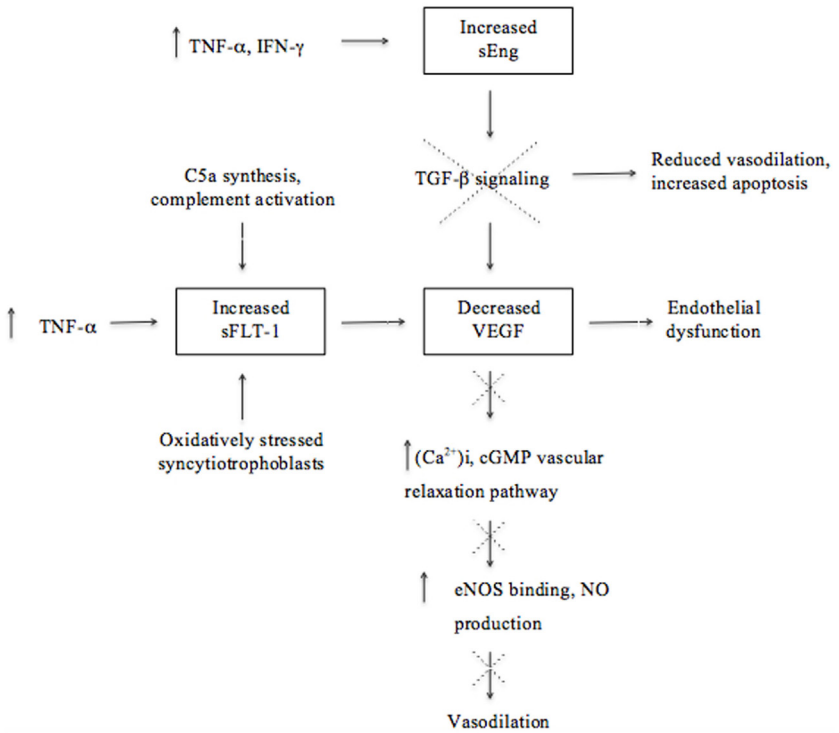


FIGURE 1 | Molecular mechanisms involved in the upregulation and downregulation of antiangiogenic and angiogenic factors ultimately leading to endothelial dysfunction and reduced vasodilation in the pathogenesis of preeclampsia. Although there is a lack of proof that soluble fms-like tyrosine kinase (sFlt-1) is associated with excessive systemic inflammation, this illustration emphasizes the effect of the immune system on angiogenesis and that dysregulation thereof could contribute to the pathogenesis of preeclampsia.

endothelial cell apoptosis, suggesting that dysregulated TGF-β1 signaling may be involved in the pathogenesis of preeclampsia (40). A decline of VEGF results in glomerular endotheliosis, a specific endothelial renal lesion presenting with proteinuria (21). In addition to low VEGF concentrations, a decline in NO, PIGF, and TGF-β1 levels, in concert with an increase of anti-angiogenic factors, deprives endothelial cells of support leading to endothelial dysfunction (8, 17, 22). Thus, endothelial dysfunction is a result of an antiangiogenic state in the placenta and contributes to the pathology of preeclampsia (**Figure 1**) (9, 52, 57).

Immunological factors also play a role in the secretion of antiangiogenic factors. For instance, complement activation has been shown to stimulate monocytes to release antiangiogenic factors (20). Studies in murine models of experimental pregnancy loss demonstrated that conditions of hypoxia and inflammation lead to the release of increased amounts of antiangiogenic factors and are therefore strong triggers of angiogenic dysregulation (20, 58). These studies and those in humans demonstrate that pregnancy complications, such as recurrent pregnancy loss (RPL), preterm birth, and preeclampsia, are associated with excessive complement activation, especially enhanced C5a synthesis, that promotes the secretion of the antiangiogenic factor sFlt-1 (20, 59). Activation of C5 contributes to fetal loss and adverse pregnancy outcomes by inducing a dysregulation of angiogenic factors and initiating a complex series of events, as demonstrated in the abortion-prone mouse mating combination CBA/J and DBA/2 (20). The importance of activation of complement through the lectin pathway was further illustrated with the administration of inhibitory factors, such as Polyman2, which neutralizes mannose-binding lectins and anti-C5, a neutralizing recombinant antibody that prevented fetal loss in this mating combination (20, 60). The important role of disruption in the regulation of complement activation in preeclampsia is also demonstrated by the findings of C4A deficiencies and C4b deposits in preeclamptic placentas (61, 62).

Soluble fms-like tyrosine kinase (also referred to as sVEGFR-1) is a soluble form of the VEGF/PIGF receptor Flt-1 and is a potent inhibitor of VEGF and PIGF angiogenic activity (51, 53–55). Elevated levels of sFlt-1 are associated with preeclampsia and have been found to induce hypertension, proteinuria, and glomerular capillary endotheliosis when administered to pregnant animals (63). Increased levels of sFlt-1 are more pronounced at an early stage before the onset of preeclampsia and are associated with several associated risk factors, including diabetes mellitus and gestational hypertension (52, 64, 65). Excess levels of sFlt-1 inhibit cytotrophoblast differentiation and invasion and subsequently contribute to poor placentation (20). A chronic increase in the production of pro-inflammatory cytokine, TNF-α, was found to induce hypertension in rats and stimulate the excessive secretion of sFlt-1, further supporting the effect of the immune system on inadequate angiogenesis in preeclampsia (17). Excessive secretion of sFlt-1 is also suggested to be induced by hypoxic conditions and reflect the response of oxidatively stressed syncytiotrophoblasts during poor placentation. Therefore,

possible sources contributing to the higher concentrations of sFlt-1 observed during preeclampsia are the syncytiotrophoblasts in the placenta in the presence of oxidative stress, as well as peripheral blood mononuclear cells, macrophages, and endothelial- and vascular smooth muscle cells (34, 66).

Increased concentrations of another antiangiogenic factor, soluble endoglin (sEng), in preeclampsia contributes to endothelial dysfunction through its inhibition of TGF-β1 signaling (35, 67). Elevated levels of sEng could be attributed to oxidative stress and hypoxic conditions in the placenta as well as to stimulation by inflammatory cytokines, such as TNF-α and interferon (IFN)-γ, from endothelial or placental cells during non-hypoxic conditions (29, 68). The combined action of increased sFlt-1 and sEng concentrations is suggested to characterize preeclampsia better than a single analyte, emphasizing the role of several antiangiogenic factors in clinical preeclampsia (40, 69). Considering that the endothelium is a component of the systemic inflammatory system, elevated levels of antiangiogenic factors (sFlt-1 and sEng) secreted by a diseased placenta may contribute to systemic inflammation (**Figure 1**) (29).

In contrast to the pattern of the antiangiogenic factors, pro-angiogenic PIGF and VEGF are present in decreased concentrations in preeclampsia even before clinical manifestation occurs (52). The patterns of these biomarkers are considered relevant and an altered ratio of pro-angiogenic to anti-angiogenic factors is correlated with the severity of the disorder (70). Increased concentrations of sFlt-1 lead to PIGF and VEGF being sequestered (35, 52); hence, an imbalance in the expression of angiogenic and antiangiogenic factors leads to inadequate angiogenesis and disruption of the maternal endothelium and immunological balance advancing to the clinical signs of preeclampsia (**Figure 1**) (9, 17, 52).

Stimulation of the Immune System at the Fetal–Maternal Interface Through the Recognition of Antigens

The systemic inflammatory response associated with preeclampsia involves immunological conflict originating in the decidua where maternal immune cells are in close contact with trophoblasts at the feto-maternal interface operative in placental tissue and the secondary (peripheral) lymphoid organs (28, 29, 34, 71, 72). In pregnancy, the maternal immune system is immunologically naïve to the tissue-specific antigens from the fetus and placenta as well as foreign antigens from the paternal genome (72). The maternal immune system relies on immune regulatory mechanisms to avoid rejection of the semi-allogeneic fetus while protecting the host against infections (73). Primary contact with maternal immune cells occurs as cytotrophoblasts invade the decidua (34, 71). Close contact between cytotrophoblasts and decidual immune cells will influence maternal immune cells to either cause tolerance, and subsequently facilitate trophoblast invasion, or elimination at the feto-maternal interfaces (28, 34, 74, 75). In addition, the syncytiotrophoblast is in contact with immune cells in maternal blood flowing through the intervillous space (71, 76). This contact can be direct or indirect through the release of soluble factors (e.g., cytokines), immunosuppressants (e.g., progesterone and prostaglandins), specific suppressor molecules [e.g., soluble isoforms of human leukocyte antigen (HLA)-G and HLA-E], tolerogenic molecules [e.g., TGF-β1 and interleukin (IL)-10], and immunomodulatory products [e.g., indoleamine 2,3-dioxygenase (IDO), Fas ligand (FasL), and TNF-related apoptosis-inducing ligand (TRAIL)] (71, 72, 77).

Paternally derived antigens are expressed on trophoblasts and recognition events by the maternal immune system stimulate an immune response from innate and adaptive immune cells, which controls trophoblast invasion and placentation through the release of trophic substances such as cytokines and PIGFs (21). Interaction of cytotrophoblasts with target cells facilitates maternal–fetal immune recognition through receptors that recognize HLA ligands (78, 79). The classes of HLA antigens differ in their preference for specific immune cell interactions to facilitate their important role in immune regulation (71). In humans, unique combinations of the genotypes of class I major histocompatibility complex (MHC) antigens, the classic (polymorphic) HLA-C and non-classic (non-polymorphic) HLA-G are expressed on extravillous cytotrophoblasts (71). The lack of expression of the highly polymorphic classical MHC class I antigens (HLA-A and -B), the principal stimulators of graft rejection, and class II antigens is an important mechanism to escape immune rejection of the fetus (71, 80).

Human leukocyte antigen C is recognized by decidual T cells and decidually derived $CD16^{-}CD56^{bright}$ natural killer (dNK) cells (79, 81). These dNK cells are phenotypically and functionally distinct from NK cells in the peripheral circulation and have unique functions, including the production of chemokines, cytokines, and growth factors, in addition to reduced cytotoxicity; qualities that make their presence acceptable at the feto-maternal interfaces (81, 82). These functional differences may indicate a distinct NK cell lineage, or alternatively, that these changes are induced by the unique decidual microenvironment that is characterized by physiological stress hypoxia in the placenta and regulation by estrogen, progesterone, and trophoblast-derived soluble factors, such as soluble HLA-G (81, 82). While the origin of dNK cells is still unclear, Carlino et al. (83) have suggested that these cells are recruited from peripheral NK cells able to migrate through endothelial and stromal decidual cells, partly due to its higher migratory ability during the first trimester of pregnancy (83). Once peripheral NK cells come into contact with stromal cells, they acquire a chemokine receptor profile similar to that of dNK cells, indicating that this communication between NK cells modulated the NK cell phenotype (83). Decidual NK and T-cell subsets constitutively express killer immunoglobulin-like receptors (KIRs) at the fetal–maternal interface, composed of a series of inhibitory and activating receptors that interact with HLA ligands (84). Polymorphic KIRs, consisting of haplotype A and B, are distinguished based on their activating or inhibitory properties (28, 76).

The maternal KIR B haplotype has between one and five activating receptors while the KIR A haplotype contains only inhibitory receptors (85). Decidual NK cells display a specific KIR repertoire that is biased toward recognizing HLA-C, the dominant KIR ligand found on fetal extravillous trophoblasts (86). The specific ligand–receptor interaction of KIR BB and

HLA-C ligand genotypes favors proper activation of dNK cells for the production of immunoregulatory cytokines and angiogenic factors (PIGF and VEGF, TGF-β1, and angiopoietin 1 and 2) that promote placental development (25, 81, 87–89). Adequate activation of dNK cells will thereby supply sufficient dNK-derived growth factors and chemokines to allow for adequate trophoblast invasion and vascular remodeling (81). Generally, weaker inhibitory interactions are associated with better protection against viral infection, or greater susceptibility to autoimmune conditions, secondary to greater NK cell activation (90). The HLA-G interacts best with KIR2DL4 receptors on dNK cells to stimulate the production of angiogenic factors as well as pro-inflammatory and immunoregulatory cytokines (71, 91, 92). Soluble HLA-G may also contribute to immune tolerance by upregulating immunosuppressive NK subsets (84). Although the principal interaction of these antigens is with dNK cells, HLA-G also interacts with the CD8 receptor on T cells. The latter interaction subsequently activates the Fas/FasL pathway, promoting the apoptosis of activated $CD8^+$ T cells, and protecting trophoblasts from a T cell-mediated attack (71, 92).

Immune interaction with KIRs resulting in too strong inhibition prevents NK cell activation and this is postulated to play a role in preeclampsia where NK cell activation is needed to stimulate vascularization (90). Some have shown that insufficient activation of dNK cells contribute to the development of preeclampsia by promoting the lysis of trophoblast cells lacking HLA-G and the loss of trophoblast cells that should invade the developing spiral arteries, resulting in the insufficient supply of oxygen and nutrients to a developing placenta (93). Therefore, dNK cells stop the process of placental development and spiral artery remodeling by the extravillous cytotrophoblasts (93, 94). Protection against the development of preeclampsia therefore relies on adequate activation of dNK cells by receptor–ligand interactions that favor activating KIR receptors (haplotype BB) above ligation to inhibitory receptors (haplotype AA) (23, 81, 95). When maternal activating receptors are, however, absent as in the KIR AA genotype, binding to fetal HLA-C2 increases susceptibility to preeclampsia (79, 85). In preeclampsia, the activation of inhibitory KIR receptors through HLA-C2 binding is associated with inadequate immune recognition and trophoblast invasion (28, 71, 75). Altered dNK cell activation also results in inadequate angiogenesis. For instance, KIR-AA and HLA-C receptor–ligand interaction is responsible for the defective secretion of angiogenic factors by dNK cells and the increased expression of antiangiogenic factors, sFlt-1 and sEng, previously described to cause endothelial dysfunction (59, 87, 96).

Reduced levels of expressed HLA-G and its soluble concentration in maternal plasma were observed in cases of miscarriage and preeclampsia, suggesting that inadequate immune recognition and tolerance contributed to pregnancy complications in these women (97, 98). In addition, preeclamptic pregnancies have reduced levels of progesterone along with reduced levels of immunomodulators, such as IDO and TRAIL, and soluble CD30, a member of the tumor necrosis superfamily of receptors and a marker of T-helper (Th)2 polarization (99–101). These findings, in addition to those of epidemiological studies, support the hypothesis that immune maladaptation play a role in the pathophysiology of preeclampsia by means of an inappropriate release of cytokines and placental factors that cause both shallow invasion of trophoblasts and endothelial dysfunction (102, 103).

THE KEY ROLE PLAYERS IN THE DISRUPTION OF THE IMMUNE SYSTEM IN PREECLAMPSIA

The innate and adaptive components of the immune system play an important role in immune regulation to ensure a successful pregnancy (104). Various immune cells most notably dNK cells, decidual macrophages, T cells, and dendritic cells (DCs) are involved in this process (105). Decidual NK cells, the most abundant decidual leukocytes in the first trimester, are only weakly cytotoxic and are an important source of immunoregulatory cytokines, matrix metalloproteinases (MMPs), and angiogenic factors that promote key processes in placentation, such as extracellular matrix remodeling, trophoblast invasion, and angiogenesis (106–108). Decidual macrophages are present throughout pregnancy and similarly produce factors associated with tissue remodeling and angiogenesis, e.g., MMP9 and VEGF (109). These macrophages are mostly of the immunomodulatory phenotype (M2) and contribute to the creation of a tolerogenic immune environment by producing immunosuppressive cytokines, such as IL-10 and IL-35, inducing expression of regulatory T cells (Tregs), phagocytosing apoptotic trophoblast cells to prevent the release of pro-inflammatory substances, and inhibiting the cytotoxic function of dNK cells (110). Decidual T cells are predominantly of the $CD8^+$ phenotype and play a role in the regulation of trophoblast invasion, while $CD4^+$ Tregs promote tolerance to the fetus (105). Finally, DCs, even though occurring in very low numbers in the decidua, are believed to play a role in driving the differentiation of naïve $CD4^+$ T cells into a Th2 phenotype and to regulate dNK proliferation and activation (105).

Regulation of immune responses through adequate activation of dNK cells, macrophages, and Tregs plays a role in the processes of implantation, placentation, and progression of pregnancy (75, 88). In the absence of immune regulation, continuous activation of monocyte phenotypes results in excessive immune activation that leads to a generalized inflammatory response (111). Regulatory products, cytokines, and other pro-inflammatory factors released from the placenta by syncytiotrophoblasts play a key role in signaling between cells of the inflammatory network (29, 109). Preeclampsia has been associated with disruption of many of these physiological processes. An ischemic placenta results in aberrant activation of immune cells by the continuous release of pro-inflammatory factors into the maternal circulation (111). Specifically, an imbalance in Th subsets, aberrant activation of dNK cells, and excessive recruitment of innate immune cells are mainly responsible for disrupting the regulation of immunological balance in pregnancy (111). These will now be discussed in turn.

Imbalances Between Th Cell Subsets

The regulation of pro- and anti-inflammatory immune responses lies in the interplay between different Th cell subsets, especially

between Th1 and Th2 cells, characterized by the release of pro-inflammatory (e.g., IL-2, IL-6, IL-8, IFN-γ, and TNF-α) and anti-inflammatory cytokines (IL-4, IL-10, and IL-13), respectively (112, 113). Th2 cytokines are involved in antibody production and take part in the regulation of inflammation in cooperation with IL-10-producing Tregs (88). During pregnancy, modulation of immune responses occurs, with distinct immunological features, observed at different stages (114). Implantation and placentation in the first and early second trimester of pregnancy are characterized by a pro-inflammatory environment that ensures adequate repair of the uterine epithelium and removal of cellular debris that accumulates secondary to embryo implantation (114). The second and third trimester are times of rapid fetal growth and development and the predominant immunological profile in the feto-maternal interface is anti-inflammatory (114). Progesterone, estradiol, prostaglandin D2, and leukemic inhibitory factor all promote the development of this Th2 profile (104, 105). The immunological effect of progesterone is mediated through a protein called P-induced blocking factor, which induces dominant Th2 cytokine production and immune modulation in aid of maternal tolerance toward the fetus (115). A Th2 bias in pregnancy modulates cell-mediated immunity, which is responsible for the increased susceptibility of pregnant women to bacterial LPS and intracellular pathogenesis of, for instance, *Listeria monocytogenes* (75, 104). Finally, during parturition, the immune profile reverts back to a pro-inflammatory state, which is necessary for the processes of delivery, such as contraction of the uterus and expulsion of the fetus and the placenta (104).

It has been proposed that a shift toward a Th2 profile in the second trimester does not occur in preeclampsia or, alternatively, that it is reversed in the early stages of the disease (71). This has the consequences that Th1 responses are not downregulated and cytokines exhibit mostly a pro-inflammatory profile, with elevated levels of IFN-γ and reduced levels of IL-4 and IL-10 reported (71, 116). Increased levels of Th1 type pro-inflammatory cytokines have also been associated with other pregnancy complications, such as preterm birth and IUGR (117). This pro-inflammatory systemic environment contributes to the failure of immune tolerance and results in immune dysregulation (**Figure 2**) (59, 118). The mediators that regulate this Th1/Th2 shift are not completely understood and are speculated to involve NK cells and IFN-γ, but also other cytokines, such as IL-12, IL-8, and the TGF-β family (117, 118). Interleukin-12 induces Th1 responses and stimulates the release of IFN-γ by NK and naïve T cells (7). In turn, IFN-γ primes monocytes to release more IL-12, increasing the production of IFN-γ. It has been suggested that this positive feedback cycle contributes to rapid deterioration in severe preeclampsia (7). Another key aspect in this paradigm is the negative feedback of IFN-γ on Th2 cells, in so doing suppressing immune regulation (71).

Possible mechanisms by which dominant Th1 immune responses induce the clinical aspects of preeclampsia are not completely known, but many of the respective Th1 cytokines (TNF-α, IL-2, IL-12, IL-18, and IFN-γ) have been reported to induce apoptosis of trophoblasts (25, 118). In addition, IL-12 and IFN-γ inhibit angiogenesis, whereas TNF-α activates endothelial cells and

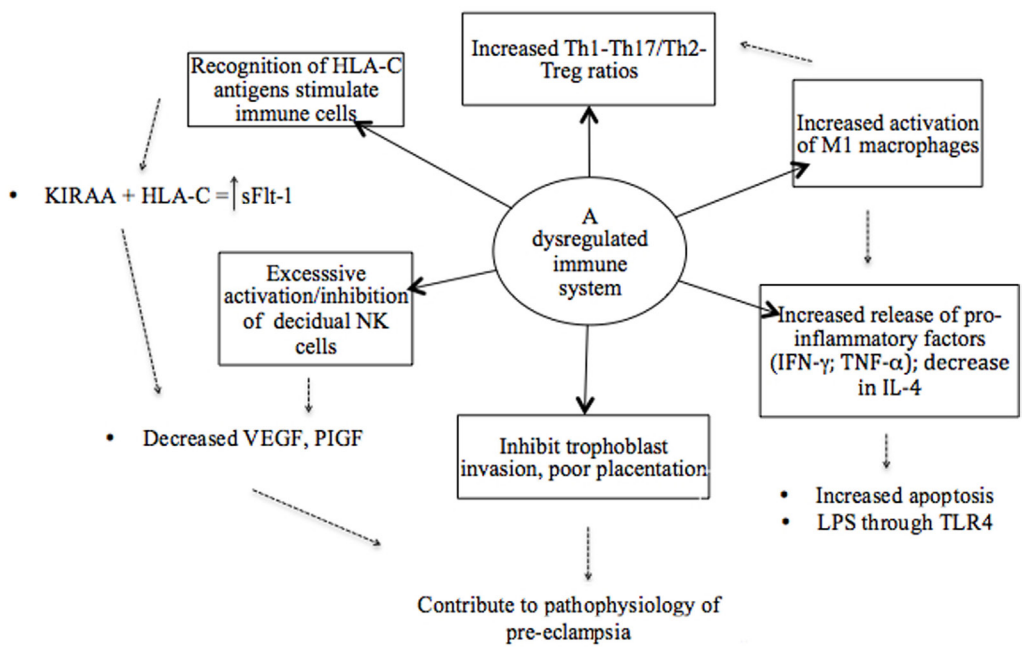


FIGURE 2 | An illustrative model of the various components of a dysregulated immune system in preeclampsia. The recognition of antigens is considered a starting point where immune cells are stimulated based on the interaction between specific killer immunoglobulin-like receptor (KIR) and human leukocyte antigen (HLA) ligand genotypes. Subsequently, a dysregulated immune system in preeclampsia is attributed to an imbalance of pro- and anti-inflammatory cytokines as result of the overactivation of immune cells and pro-inflammatory stimuli that promote inflammation. Therefore, a hyperinflammatory environment in preeclampsia is established by the failure of regulatory mechanisms.

induces glomerular endothelial damage (7, 117). Tumor necrosis factor-α also inhibits the production of NO and thus influences the circulating levels of angiogenic factors in the second stage of preeclampsia (**Figure 1**) (118). Studies have, however, reported highly variable results of the circulating levels of TNF-α in preeclampsia, with some showing increased, unchanged, or even insignificantly decreased levels, possibly due to differences in timing and study method used (119–126). Nonetheless, serum TNF-α concentrations have been positively and significantly correlated with diastolic BP and uric acid levels, and it has hence been proposed as a potential marker of the severity of preeclampsia (125). TNF-α plays a critical role in maternal inflammation. Cotechini et al. (49) provided evidence to highlight the importance of this cytokine by using a mouse model where it was observed to induce abnormal inflammation in pregnant rats leading to FGR and features characteristic of preeclampsia (49). Elevated levels of IFN-γ are thought to play a central role in endothelial dysfunction and the exaggerated systemic inflammatory response in preeclampsia (**Figure 2**) (71). Overproduction of TNF-α and IFN-γ has also been reported in other adverse pregnancy outcomes, such as preterm birth (127) and IUGR, and could be suggested viewed as a primary target in preeclampsia pathology also because of their ability to inhibit sufficient angiogenesis, as previously mentioned (**Figure 2**) (117).

This one-dimensional Th1/Th2 paradigm can, however, not fully explain the immunological changes observed during preeclampsia and recent work has expanded the dichotomy to include the Th17 phenotype and a subset of $CD4^+$ T cells known as $CD25^+$ Forkhead box P3 gene ($Foxp3^+$) Tregs (128). The latter are responsible for the development and regulation of immune responses and hence, the maintenance of immunological tolerance to the fetus (25, 112). Extrathymic and peripherally generated Tregs (pTregs) induce tolerance by suppressing the immune response generated by T-effector cells to foreign antigens, whereas thymic Tregs (tTregs) act to prevent autoimmunity by maintaining MHC class II antigen-specific tolerance (59).

Peripherally generated Tregs are produced within the decidualized endometrium during early pregnancy and are believed to play an essential role in pregnancy (129–131). Others have proposed that both tTregs and pTregs are indispensable, with tTregs potentially initiating a tolerant state and pTregs maintaining tolerance (132). Tregs have to be exposed to antigens presented by "tolerogenic" DCs in an appropriate cytokine environment for them to proliferate, mature, and eventually exert suppressive effects (128). Although HLA II is not expressed in either villous or extravillous cytotrophoblasts, antigenic stimulation is believed to come from the trophoblastic cell debris that contains intracellular fetal HLA-DR (59). It has also been suggested that Tregs recognize paternal HLA-C and can therefore downregulate anti-paternal responses, although the stability of this memory is still unknown and needs further investigation (79).

Regulatory T cells are differentiated according to the expression of surface markers, most notably the IL-2 receptor, CD25, and the transcription factor, forkhead box P3 ($Foxp3^+$) (25, 59, 133). It has been suggested that the decidual $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs subsets specifically play a role in pregnancy by mediating immune tolerance to fetal antigens and suppressing inflammatory responses (134).

Regulatory T cells have many suppressive functions and targets, including suppression of activation, proliferation and cytokine release of $CD4^+$ and $CD8^+$ T cells, suppressing B-cell proliferation and immunoglobulin production, inhibiting the cytotoxic function of NK cells, and inhibiting maturation and function of antigen-presenting cells, such as DCs and macrophages (129). The molecular mechanisms by which Tregs exert their control are, however, incompletely understood. Some propose that their main action is through suppression of specific NK phenotypes and pro-inflammatory cytokines (71) or through the production of immunoregulatory cytokines, such as IL-10 and TGF-β that in turn promote and inhibit the differentiation of Tregs and Th17 cells, respectively (135, 136). Others believe the major mechanism to be through the expression of the membrane glycoprotein, cytotoxic T lymphocyte antigen (CTLA)-4 (137). The co-ligation of CTLA-4 with the T cell receptor results in increased expression of IDO, which effectively starves T cells by restricting the accessibility of the essential amino acid, tryptophan (137, 138). The interaction between Tregs and IDO is bidirectional, and the presence of IDO promotes the differentiation of T cells into Tregs, while in its absence, Tregs are reprogrammed to acquire a Th17 pro-inflammatory phenotype (79).

T-helper 17 cells are a $CD4^+$ lymphocyte subpopulation, characterized by the production of the pro-inflammatory cytokine, IL-17 (112, 128). The Th17 cells induce a protective immune response against bacterial or fungal infections and the excessive upregulation of this subpopulation has been associated with the development and progression of autoimmune and chronic inflammatory diseases, allergies, and graft rejection (128, 139). A portion of IL-17A-producing cells have been found to also produce the Th1 cytokines, such as IFN-γ, and are hence termed Th17/Th1. Conversely, a small proportion of cells that have the ability to produce both IL-17A and IL-4 are termed Th17/Th2 cells. The latter clones originate in the presence of IL-4, suggesting that a microenvironment rich in IL-4 may induce a shift from Th17 to Th17/Th2 cells (140). IL-4 and HLA-G5 in the uterine environment is considered at least partly responsible for the development of specifically Th17/Th2 cells, which is crucial for successful implantation of the embryo in pregnancy (140, 141). Lombardelli et al. (141) reported that in successful pregnancy, a large number of $CD4^+$ T cells produce both IL-17 and IL-4, whereas pathogenic decidual Th17/Th1 cells are commonly found in patients with unexplained recurrent abortion (141). The same study conducted by Lombardelli et al. (141) propose that the potentially detrimental effects of IL-17 may be counteracted when Th17 cells produce IL-17 together with IL-4 in the first trimester of pregnancy (141). IL-17/IL-4-producing $CD4^+$ T cells promote an adequate response to extracellular pathogens, whereas IL-4 may induce tolerance toward the paternal HLA-C antigens and IL-17 could promote the proliferation and invasion of extravillous cytotrophoblasts (141).

A similar developmental lineage exists between Th17/Treg subsets, and the linked evolution of these subsets enables a balanced regulation of inflammation and autoimmunity (**Figure 2**) (84, 113, 142). Tregs and IL-17^+ T cells are both involved in the establishment and maintenance of pregnancy, where these cells function as regulatory and effector cells, respectively (84, 143).

Tregs act as regulators of Th17 cells and other immune cells involved in tolerance at the feto-maternal interfaces (25, 128).

Normal pregnancy is proposed to have a bias away from a Th17 response, while an increased ratio of IL-17$^+$/Tregs has been demonstrated in cases of RPL, unexplained miscarriage, preterm birth, and preeclampsia (**Figure 2**) (59, 79, 113, 128, 139, 144). It has been shown that IL-17-producing lymphocytes are increased in the peripheral blood of preeclamptic patients in the third trimester of pregnancy and a significant correlation has been found between levels of Th17, IL-2-, and IFN-γ-producing T cells and the development of preeclampsia (145).

In preeclampsia, the uterine microenvironment acts as a contributing factor by influencing the differentiation of T cell subsets, especially Th17 cells (146). Therefore, further differentiation of Th17 cells could be related to the continuous release of pro-inflammatory cytokines and a dominant Th1 environment in preeclampsia (118, 146). The increased production of IL-1β and IL-6 by activated monocytes that induce an inflammatory microenvironment, as seen in recurrent abortions, can also participate in the expansion of Th17 and their conversion from Tregs (74, 147).

In addition to the regulatory action of Tregs, dNK cells are present in elevated levels in normal pregnancy and inhibit local inflammation at the feto-maternal interface and maintain tolerance by suppressing the development of pro-inflammatory Th17 cells (74, 136). The ratio of dNK/Th17 cells is decreased in cases of RPL abortion, indicating impaired regulatory activity and increased decidual inflammation (74). Deficient levels of Tregs and the absence of dNK cells lead to a prominent Th17 response, extensive local inflammation, and the failure of maternal–fetal tolerance, which could be possible mechanisms that play a role in the development of preeclampsia (74, 136).

Tolerance at the maternal–fetal interface is achieved through the production of the immunoregulatory cytokines, such as IL-10 and TGF-β1, that promote and inhibit the differentiation of Tregs and Th17 cells, respectively (136). Tregs also suppress innate and adaptive immune responses and play a major role in modulating the activity of self-reactive cells (112). The importance of Tregs in pregnancy was confirmed when the administration of these cells was found to prevent spontaneous abortion in pregnant mice (25). It is suggested that Tregs do not directly suppress Th1 responses in normal pregnancy, but regulate immune responses through the suppression of specific NK phenotypes and pro-inflammatory cytokine production (71). Although Tregs cannot reverse the shift toward a Th1 response by promoting Th2, these cells promote a tolerant microenvironment at the feto-maternal interface (148).

Preeclampsia has been associated with an imbalance in the two subpopulations of Tregs (25, 149). In comparison to normal pregnancy, preeclampsia is associated with increased numbers of CD4$^+$CD25^{high}Foxp3$^+$ and decreased numbers and functional activity of CD4$^+$CD25$^+$Foxp3^{high+} cells (149). The stability of Tregs is suggested to be influenced by inflammatory environments, as has been demonstrated in inflammatory bowel disease (150). Pro-inflammatory cytokines, such as TNF-α, have been shown to have a direct negative effect on the function of Tregs, especially in cases of rheumatoid arthritis, and this may be an explanation for the altered levels of Tregs observed in preeclampsia (59, 150, 151). Furthermore, it has been suggested that a pro-inflammatory environment tends to suppress the generation of Tregs (79). In the pro-inflammatory environment of preeclampsia, IDO has been shown to be less active in late stages, possibly due to underactive Treg responses (79, 152). A decline in the level of decidual Tregs consequently leads to failed regulatory mechanisms, resulting in the immunological rejection of the fetus (25, 153).

Aberrant Activation of Decidual NK Cells

In the first trimester of pregnancy, a subset of peripheral blood NK cells (decidual CD16$^-$CD56^{bright} NK cells—dNK cells) differentiates to constitute the major immune cell population in the decidua (59, 75). Peripheral NK cells (CD16$^+$CD56^{dim}) are cytotoxic, express high levels of KIRs and CD57 (a marker of cell maturation and potent cytotoxic potential). and do not secrete cytokines (154, 155). By contrast, CD16$^-$CD56^{bright} cells do not induce antibody-mediated cell toxicity and express only low levels of perforin, but high levels of the inhibitory receptor CD94/NKG2 (154). Although CD16$^-$CD56^{bright} NK cells in the decidua possess cytotoxic properties, these cells are an important source of immunoregulatory cytokines, such as IL-10 and TGF-β1, which play an important role in tolerance and successful pregnancy by directly inhibiting the proliferation of Th17 cells and suppressing inflammatory responses, as mentioned earlier (7, 25, 74, 81, 87, 136). While dNK cells also secrete their classic, pro-inflammatory cytokine, IFN-γ, its effect is mainly exerted through inducing spiral artery remodeling by initiating vessel instability (74, 156). The secretion of chemokines, IL-8, and the IFN-inducible-protein (IP)-10 by dNK cells facilitates the migration of the extravillous cytotrophoblast into the *decidua basalis* and results in the invasion of spiral arteries, further contributing to uterine vascular remodeling crucial for placental development (87).

The phenotype of dNK cells can be altered by severe inflammation in the uterine microenvironment caused by severe stress, viral infection, and autoimmune diseases (74). The expression of the marker CD27 on NK cells, which correlates with an increased ability to proliferate and produce IFN-γ and with lower cytotoxic potential (157), has been suggested to be an important marker in distinguishing different subsets of NK cells (74). A large number of CD27$^+$ NK cells have been found in the decidua, where this subset of NK cells is an important source of cytokines and shows limited cytotoxicity (74). Studies of RPL as a model pathogenic state demonstrated an altered ratio of CD56^{bright} CD27$^+$ to Th17 cells, impaired release of the immunoregulatory cytokines, such as IL-10 and IFN-γ, as well as the failed inhibition of inflammation (74, 158). A possible explanation for these findings is that dNK cells may have increased cytotoxicity toward inflammatory cells together with impaired regulatory capacity, and that prolonged overactivation of dNK cells may result in them becoming immunodepleted and adopting an abnormal phenotype (74).

Preeclampsia is associated with a shift in NK cells toward a cytotoxic phenotype with increased production of IFN-γ, which causes apoptosis of cytotrophoblasts, thus inhibiting trophoblast invasion (25, 159). In addition, the lower expression of the natural cytotoxicity receptor, NKp46, on NK cells in women with preeclampsia has been suggested to increase the production of

the pro-inflammatory cytokine, TNF-α, which further drives a shift in NK cells (160). Accordingly, inappropriate recruitment of peripheral blood NK cells to specialized decidual cells may lead to the development of a cytotoxic environment *in utero* (113). Hence, successful implantation and placentation depend on a fine balance between decidual NK cell infiltration and activation of regulatory phenotypes (113).

Excessive Recruitment of Innate Immune Cells

In preeclampsia, monocytes, neutrophils, and macrophages are continuously activated due to the exposure to elevated levels of pro-inflammatory factors in the uterine microenvironment (109). Monocytes invade tissues upon an inflammatory stimulus and subsequently develop into macrophages that produce factors associated with tissue remodeling and angiogenesis in pregnancy (109, 161). In the decidua, macrophages are broadly distinguished into two phenotypes: (i) M1 macrophages with an inflammatory and microbicidal nature and (ii) M2 macrophages with immunosuppressive properties that maintain immunological homeostasis during pregnancy (75). The latter phenotype mainly represents decidual macrophages in pregnancy and contributes to immune regulation by producing immunosuppressive cytokines, such as IL-10 and TGF-β1, to regulate inflammation and suppress Th1 cell polarization (7, 75, 88). Decidual macrophages secrete angiogenic factors to promote placentation and the regulatory properties of these macrophages enable them to remove apoptotic cells to prevent the harmful effects of these pro-inflammatory factors (75, 127, 161). The decrease of immunosuppressive cytokines observed in preeclamptic patients indicates altered M2 macrophage polarization as reduced M2 numbers in the decidua contribute to failure of immune regulation (75, 162). This is expected because the polarization of M2 macrophages relies on exposure of type 2 immunosuppressive cytokines such as IL-10 that is present in low concentrations in preeclampsia (161).

When immune regulatory mechanisms are not intact, augmented inflammation will induce M1 macrophages and apoptosis of cytotrophoblasts, contributing to poor placentation (88). Increased apoptosis of cytotrophoblasts and syncytiotrophoblasts is attributed to elevated levels of pro-inflammatory TNF-α and IFN-γ that inhibit trophoblast invasion and spiral artery remodeling (127, 163, 164). In preeclampsia, excessive inflammation or oxidative stress in the placenta leads to increased cell death of syncytiotrophoblasts (7, 19). Increased placental apoptotic debris in preeclampsia is suggested to participate in the pathogenesis of the disorder by enhancing the inflammatory stimulus with or without specific immune recognition (7). The phagocytosis of these large numbers of necrotic or apo-necrotic trophoblasts by macrophages and neutrophils may lead to increased production of pro-inflammatory type 1 cytokines, such as TNF-α, IL-12, and IFN-γ (7, 117). Excessive apoptosis creates a danger signal and activates macrophages toward a pro-inflammatory cytokine profile (7). Furthermore, the activation of M1 macrophages by pro-inflammatory stimuli (TNF-α, IFN-γ, and bacterial LPS) inhibits trophoblast invasion that further contributes to the imbalance between Th1 and Th2 cytokines and the exaggerated inflammatory response observed in preeclampsia (**Figure 2**) (59, 127).

THE IMPACT OF A DISRUPTED IMMUNE SYSTEM ON THE METABOLIC CHANGES ASSOCIATED WITH PREECLAMPSIA

In preeclampsia, a dysregulated immune system is a result of dominant Th1 subsets, the elevated release of pro-inflammatory cytokines from the placenta, aberrant activation of macrophages and dNK phenotypes that continuously promote a pro-inflammatory environment, which further activates other immune cells (26). Elevated levels of inflammatory cytokines, specifically TNF-α and IL-6, generate widespread dysfunction of the maternal vascular endothelium that could result in hypertension (17). Increased pro-inflammatory cytokines also affect metabolic changes in preeclampsia. For instance, elevated levels of TNF-α induce insulin resistance and stimulate the adipocytes to release more free fatty acids (FFAs) (21, 165). Circulating levels of FFAs are increased in preeclampsia, contributing to insulin resistance and altered lipid metabolism in this disorder (165, 166). Several metabolic disorders are also associated with systemic inflammation and, as discussed previously, there is pathological resemblance between preeclampsia and the components of the metabolic syndrome (21). Obesity is a metabolic disorder characterized by systemic inflammation and an increased risk of developing preeclampsia. In obese individuals, adipose tissue is suggested to be a potent source of pro-inflammatory stimuli because of the ability of adipocytes to secrete the pro-inflammatory cytokines, such as TNF-α and IL-6 (21). Besides promoting inflammation, these cytokines also advance atherogenesis, the underlying pathological process in atherosclerosis (167). Atherosclerosis is an inflammatory disorder associated with endothelial dysfunction, similar to preeclampsia, and supports the hypothesis that a disrupted immune system, specifically a pro-inflammatory bias, is associated with metabolic disorders (167).

Obese individuals thus have increased susceptibility to preeclampsia during pregnancy because of an already increased inflammatory response (21). Interestingly, endotoxin (bacterial LPS) of Gram-negative bacteria such as *Escherichia coli* has been shown to trigger and maintain a low-grade inflammatory state (168). In a mouse model, injection of LPS induced increased expression of pro-inflammatory cytokines, such as TNF-α, monocyte chemoattractant protein-1, and IL-6 (169). In addition, endotoxin stimulates the expression of IL-1 and TNF-α in endothelial- and vascular smooth muscle cells (167, 170). IL-1 causes alterations in endothelial function, such as the induction of procoagulant activity that promotes blood clotting, that may play an important role in the vascular effects of inflammation and the pathogenesis of vascular diseases (170).

Innate recognition of LPS occurs through a receptor complex consisting of CD14, TLR-4, and myeloid differentiation factor 2 that recognizes pathogenic structures and subsequently induces an inflammatory response. Continuous activation of the innate immune system through TLRs by bacterial stimuli may disrupt immune homeostasis by the overproduction of pro-inflammatory

cytokines that can lead to a systemic inflammatory response (171). In preeclampsia, the increased expression of TLR-4 by placental trophoblasts increases the secretion of trophoblast chemokines, thus attracting more monocytes to the decidua (59). The increased recruitment of innate immune cells and subsequent increased expression of inflammatory mediators at the fetal–maternal interface caused by bacterial stimuli can also trigger preterm birth (172).

Previous studies suggested TLR-4 activation to be associated with inflammation in preeclampsia (173–175). Supporting this suggestion, a later study found a direct correlation between TLR-4 activation, inflammation, and plasma and placental oxidative damage in preeclampsia (176). These results suggest that the upregulation of TLR-4 activation in preeclampsia could be contributing to the systemic inflammatory response following local and systemic oxidative damage (176). Therefore, through the activation of TLR-4, bacterial stimuli may contribute to the oxidative, inflammatory, and metabolic stresses in preeclampsia (21).

In this review, we strongly support the role of a dysregulated immune system in preeclampsia. Pro-inflammatory stimuli may be responsible for disrupting immune homeostasis in this disorder and we therefore suggest that preexisting systemic inflammation, likely caused by multiple factors, including bacterial endotoxin, to cause preeclampsia. In addition to increased pro-inflammatory factors released by the placenta and activated immune cells contributing to excessive systemic inflammation, we suggest that bacterial endotoxin could also induce inflammatory responses as a predisposing factor to the oxidative damage and systemic inflammation in preeclampsia.

CONCLUSION

The precise mechanisms operative in the development of preeclampsia are unknown; however, evidence suggests that chronic inflammation, inadequate invasion of trophoblasts, poor angiogenesis, and the failure of immunological tolerance are co-influencing factors in the placental subtype of this disorder (146). Dysregulation of the immune system during pregnancy can lead to abnormal activation of innate immune responses that result in pregnancy complications, such as preeclampsia and IUGR (75). In pregnancy, emphasis is on the direct and indirect control of inflammation to maintain tolerance at the fetal–maternal interface, while still protecting the host. Trophoblast invasion, angiogenesis, and placentation are regulated by the secretion of angiogenesis-regulating molecules, chemokines and cytokines by phenotypically activated NK cells, T cell subsets, and macrophages (81). The unbalanced activation of the main immune mediators causes disruption of the regulation of immune mechanisms contributing to the failure of immune tolerance by inducing a cytotoxic environment that leads to endothelial dysfunction (17). However, in addition to the pro-inflammatory factors of the placenta, bacterial endotoxin could also act as a stimulus to disrupt the pregnancy-specific immune balance and cause the pathological events of preeclampsia. Future research needs to investigate the role of infectious agents as alternatives to placental factors to be responsible for the disruption of the immune system in preeclampsia.

AUTHOR CONTRIBUTIONS

JG was the project leader and involved in the writing of the manuscript. TR, HL, MK, and ME were involved in the review of the manuscript. All the authors read and approved the manuscript.

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Integrated Systems Biology Approach Identifies Novel Maternal and Placental Pathways of Preeclampsia

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Preeclampsia is a disease of the mother, fetus, and placenta, and the gaps in our understanding of the complex interactions among their respective disease pathways preclude successful treatment and prevention. The placenta has a key role in the pathogenesis of the terminal pathway characterized by exaggerated maternal systemic inflammation, generalized endothelial damage, hypertension, and proteinuria. This *sine qua non* of preeclampsia may be triggered by distinct underlying mechanisms that occur at early stages of pregnancy and induce different phenotypes. To gain insights into these molecular

pathways, we employed a systems biology approach and integrated different "omics," clinical, placental, and functional data from patients with distinct phenotypes of preeclampsia. First trimester maternal blood proteomics uncovered an altered abundance of proteins of the renin-angiotensin and immune systems, complement, and coagulation cascades in patients with term or preterm preeclampsia. Moreover, first trimester maternal blood from preterm preeclamptic patients *in vitro* dysregulated trophoblastic gene expression. Placental transcriptomics of women with preterm preeclampsia identified distinct gene modules associated with maternal or fetal disease. Placental "virtual" liquid biopsy showed that the dysregulation of these disease gene modules originates during the first trimester. *In vitro* experiments on hub transcription factors of these gene modules demonstrated that DNA hypermethylation in the regulatory region of *ZNF554* leads to gene down-regulation and impaired trophoblast invasion, while *BCL6* and *ARNT2* up-regulation sensitizes the trophoblast to ischemia, hallmarks of preterm preeclampsia. In summary, our data suggest that there are distinct maternal and placental disease pathways, and their interaction influences the clinical presentation of preeclampsia. The activation of maternal disease pathways can be detected in all phenotypes of preeclampsia earlier and upstream of placental dysfunction, not only downstream as described before, and distinct placental disease pathways are superimposed on these maternal pathways. This is a paradigm shift, which, in agreement with epidemiological studies, warrants for the central pathologic role of preexisting maternal diseases or perturbed maternal–fetal–placental immune interactions in preeclampsia. The description of these novel pathways in the "molecular phase" of preeclampsia and the identification of their hub molecules may enable timely molecular characterization of patients with distinct preeclampsia phenotypes.

Keywords: inflammation, ischemia, liquid biopsy, omics, placenta, pregnancy, systems biology, trophoblast invasion

INTRODUCTION

Preeclampsia, one of the most severe obstetrical complications affecting 5–8% of pregnant women (1–5), is a leading cause of maternal (4–15) and perinatal morbidity and mortality (6, 16–18). In addition, pathologic changes in the affected mothers and fetuses lead to a higher risk of subsequent metabolic and cardiovascular diseases later in life (8, 9, 11, 13, 19–23), further increasing healthcare costs. In spite of the severity of the problem, there is yet no early diagnosis of all forms of preeclampsia, and the current therapy is still based on the delivery of the placenta (2, 24), given the complexity of the disease and the lack of insight into the early perturbed molecular pathways.

Indeed, preeclampsia is a syndrome with heterogeneous etiology and a spectrum of phenotypes (**Figure 1**). It may affect women at varying gestational ages with different degrees of severity and consequences for the fetus (2, 25–31). The current classifications of preeclampsia are based upon its severity and the timing of clinical presentation, mostly dividing preeclampsia into preterm (<37 weeks) or term (≥37 weeks) and early-onset (<34 weeks) or late-onset (≥34 weeks) phenotypes (24, 26, 32–36). Preterm preeclampsia has a more severe clinical presentation and is often accompanied by fetal growth restriction compared to term preeclampsia (2, 25, 26, 29). However, severe maternal disease and fetal growth restriction may be observed in both term and preterm preeclampsia, and their presentation may not be associated with each other, suggesting that the clinical phenotype is the result of an interplay between various factors and disease pathways, also supported by observations in animal models (37).

A growing body of evidence offers support for the conclusion that the maldevelopment and/or dysfunction of distinct trophoblast lineages of the placenta have a central role in the pathogenesis of preeclampsia, and that the severity of the placental disease is subsequently reflected in the clinical phenotype of this syndrome. In the preclinical stage, extravillous trophoblast (EVT) development may be impaired, leading to EVT dysfunction, shallow trophoblast invasion, failure of the physiological transformation of the maternal spiral arteries, abnormal blood-flow to the placenta, and histological changes consistent with maternal vascular malperfusion (28, 30, 38–43). The frequency and severity of these lesions decrease from the preterm toward the term phenotype of preeclampsia (28, 33, 44–46) (**Figure 1**), mirrored by the decreasing prevalence of fetal growth restriction or the delivery of small-for-gestational age (SGA) neonates (47–53). Thus, EVT development is less frequently and extensively affected in late-onset preeclampsia, which constitutes about 90% of all cases (50, 54, 55).

In preterm preeclampsia, failure of the remodeling of the spiral arteries may lead to abnormal blood flow to the placenta and subsequently to placental structural damage and ischemic stress, villous trophoblast (VT) dysfunction and the release of

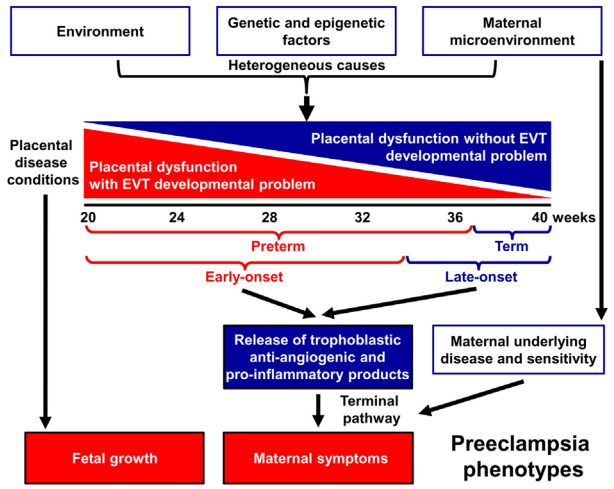


FIGURE 1 | Pathogenesis of preeclampsia. Preeclampsia is a syndrome with heterogeneous etiology and a spectrum of phenotypes. It may appear at varying gestational ages with different degrees of severity and involvement of the fetus. Preterm, especially early-onset preeclampsia generally has a more severe clinical presentation in the mother and is more often associated with the delivery of a growth-restricted neonate than term or late-onset preeclampsia. It is a multi-stage disease with the maldevelopment and/or dysfunction of distinct trophoblast lineages of the placenta at the center of the disease. Villous and extravillous trophoblast (EVT) development and/or function may be impaired in the preclinical stage, most extensively in preterm preeclampsia associated with fetal growth restriction. The resulting abnormal maternal spiral artery remodeling, fluctuating blood-flow, and ischemic stress lead to placental histological changes and the release of harmful substances from the placenta. As a consequence, the terminal pathway of preeclampsia, an exaggerated maternal systemic inflammatory and anti-angiogenic condition, occurs. The frequency and severity of placental developmental problems continuously decrease with advancing gestational age. In term forms, other stressors than maternal vascular malperfusion and placental ischemia may trigger placental stress, trophoblastic dysfunction, and the induction of the terminal pathway. Alternatively, the maternal endothelium may have an exaggerated sensitivity to factors released from a relatively normal placenta.

detrimental placental substances (e.g. anti-angiogenic factors, pro-inflammatory cytokines, and syncytiotrophoblast debris) into the maternal circulation (40, 41, 43, 56–66). As a consequence, the terminal pathway of preeclampsia, featuring an anti-angiogenic state and exaggerated maternal systemic inflammation, occurs in most cases, and its intensity correlates with the severity of preeclampsia, which may be coupled with damage to the maternal endothelium and to the kidneys, liver, and central nervous system during the clinical phase (21, 42, 43, 64, 67–72). VT development can also be impaired in preeclampsia (73–75), especially in the preterm form associated with SGA, where VT turnover is affected together with morphometric features (73, 76).

Term preeclampsia is characterized by a lesser magnitude of maternal systemic inflammatory and anti-angiogenic states (30, 37, 55–57, 61, 62, 77–92). This phenotype may result from different stressors other than maternal vascular malperfusion and ischemia of the placenta, which include various preexisting maternal disorders, such as obesity, chronic hypertension, diabetes, and metabolic, kidney, and autoimmune diseases (25, 93, 94). These stressors may still trigger placental stress and VT dysfunction (31, 91) and induce a maternal pro-inflammatory milieu. Alternatively, maternal endothelial dysfunction may result from an exaggerated sensitivity to factors released from the placenta (21, 25, 31, 95), which increases the risk of preeclampsia upon maternal genetic predisposition for cardiovascular disease (96, 97). Indeed, preeclampsia has a genetic predisposition with high heritability of both phenotypes, and it shares common risk alleles with coronary artery disease (98–104).

In spite of extensive research efforts, our understanding of the early pathologic pathways of preeclampsia has been limited given several obstacles. First, the complexity of the disease pathways and the heterogeneity of the syndrome have not been investigated in an integrative manner in both maternal and placental compartments throughout pregnancy. Second, it has been impossible to investigate the early placental disease pathways because of the invasive nature of placental biopsy and the limited information on placental functions obtained non-invasively. Consequently, an increasing number of high-dimensional studies aiming to detect molecular signatures of preeclampsia either in the placenta or in maternal blood have mostly targeted later stages of pregnancy, at a more advanced stage of placental development and pathology (105–143). Third, animal models of preeclampsia fail to mimic early placental pathways of preeclampsia due to the anatomical and physiological uniqueness of deep placentation in humans (144–147). Fourth, *in vitro* studies on human placental development and trophoblast functions are hindered by the lack of self-replicating trophoblast stem cells with the ability to differentiate into both VTs and EVTs (148–150).

Here, we used a systems biology approach that integrated various omics and targeted methods to investigate both placental and maternal compartments and most aspects of preeclampsia, including placental disease; fetal and maternal outcomes; environmental, maternal, microenvironmental, and epigenetic factors; and trophoblastic functions. In the first placental study, we performed extensive investigations of the placenta at histologic, transcriptomic, epigenetic, and protein levels to target molecular pathways at the center of the disease. Molecular changes were correlated with maternal and neonatal morbidities associated with preeclampsia to uncover placental pathways affecting either maternal or fetal wellbeing. In the second, maternal study, we employed maternal blood proteomics and "virtual" liquid biopsy of the placenta to reveal blood factors of maternal or placental origin that can reflect disease conditions in early pregnancy. In the third, trophoblast study, we utilized *in vitro* functional assays on the trophoblast to investigate hub transcription factors at the center of placental disease gene modules and to model their *in vivo* involvement in placental pathways associated with maternal or placental/fetal disease (**Figure 2**).

RESULTS

Placental Study

Alterations in the Placental Transcriptome in Preterm Preeclampsia

Given that the pathogenesis of preeclampsia has been implicated to originate from the placenta, we first aimed to investigate placental transcriptomic changes leading to placental dysfunction

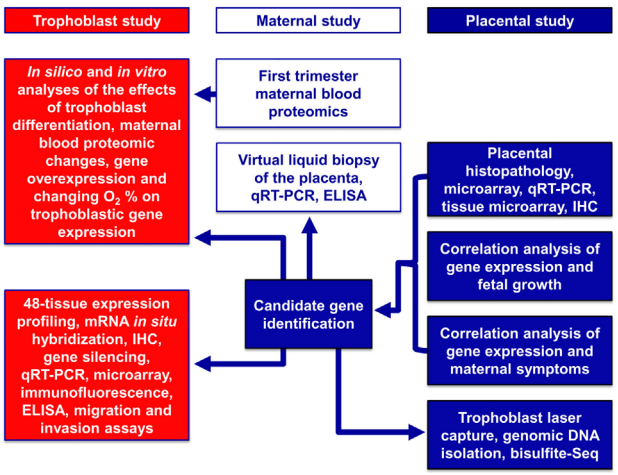


FIGURE 2 | Flow-chart of experimental procedures. The placental study included extensive histologic, transcriptomic, epigenetic, and protein level investigations of the placenta to target the molecular pathways in the center of disease. Molecular changes were correlated with disease outcomes in both mothers and babies to uncover placental pathways affecting either maternal or fetal wellbeing. The maternal study included first trimester maternal blood proteomics and "virtual" liquid biopsy of the placenta to reveal blood factors of maternal or placental origin that can reflect disease conditions in early pregnancy. In the trophoblast study, hub transcription factors in placental gene modules separately associated with maternal or placental/fetal disease were investigated with various *in vitro* methods to model *in vivo* disease pathways.

TABLE 1 | Demographics of Hungarian women included in the placental microarray study.

Groups	Controls for preterm PE ($n = 5$)	Preterm PE ($n = 12$)
Maternal age (years)[b]	31.6 (31.5–34.3)	30.3 (26.1–35)
Primiparity[a]	40	66.7
Gestational age (weeks)[b]	31.0 (30.9–34.0)	31.2 (29.3–33.2)
Race[a]		
Caucasian	100	100
African-American	0	0
Other	0	0
Systolic BP (mmHg)[b]	120 (120–120)	163 (160–170)[c]
Diastolic BP (mmHg)[b]	80 (70–80)	100 (100–101)[c]
Proteinuria[a]	0	100[c]
Birthweight (g)[b]	1,990 (1,640–2,210)	1,065 (990–1,420)
Cesarean delivery[a]	100	100

BP, blood pressure; PE, preeclampsia.
[a]*Percentage.*
[b]*Median (interquartile range).*
[c]$p < 0.01$.

as well as regulatory networks involved in the pathologic pathways. The combined analysis of preterm preeclampsia cases and gestational age-matched controls ($n = 17$) in a Hungarian patient population (**Table 1**) in our placental microarray data (132) revealed 1,409 differentially expressed (DE) genes (Data S1 in Supplementary Material), which are involved in fundamental cellular processes, including blood pressure (BP) regulation, apoptosis, development, hormone secretion, metabolism, homeostasis, and signaling (**Figure 3A**). DE genes included 137 transcription regulatory genes and 38 predominantly placenta-expressed genes. This latter set of genes ($n = 164$, Data S2 in Supplementary Material), which was defined by BioGPS microarray data ($n = 153$) or expression data from separate studies in the lack of BioGPS data ($n = 11$) (151–153), was enriched among DE genes in preeclampsia [odds ratio (OR) = 3.4, $p = 6.9 \times 10^{-9}$]. This suggests that genes predominantly expressed by the placenta have pathologic and diagnostic significance in preeclampsia, a phenomenon indicated earlier by our targeted studies (153–155).

Subsequently, we investigated the genomic links among DE genes by searching for genomic regions associated with the observed placental transcriptomic changes. We found that Chr6 (OR = 1.54, $q = 1.6 \times 10^{-3}$) and Chr7 (OR = 1.42, $q = 0.02$) were particularly affected by these gene expression changes (Data S3 in Supplementary Material), while Chr19 (OR = 2.6, $q = 0.02$) was enriched in dysregulated transcription regulatory genes (Data S4 in Supplementary Material). Of interest, predominantly placenta-expressed genes were also enriched on Chr19 (OR = 2.5, $q = 0.002$) (Data S5 in Supplementary Material). **Figure 3B** shows the non-random genomic localization of DE genes in preterm preeclampsia and the pronounced gene dysregulation associated with Chr19. These results are consistent with the fact that Chr19 harbors large transcription regulatory gene families (156), and probably reflect its regulatory role in placental/trophoblastic gene expression and their dysregulation in preeclampsia.

Alterations in Biological Processes and Regulatory Networks in Preterm Preeclampsia

Next, we aimed to identify functional links among DE genes by identifying gene co-expression network modules and hub transcription regulatory genes driving differential expression in the modules. Weighted co-expression network analysis (WGCNA) was conducted among DE genes resulting in the assignment of these into four major modules, labeled as M1 (green, $n = 506$), M2 (red, $n = 442$), M3 (blue, $n = 381$), and M4 (orange, $n = 74$) (**Figure 3C**; Data S1 in Supplementary Material). Most predominantly placenta-expressed genes belonged to modules M1 ($n = 22$ genes) and M2 ($n = 12$). Module M1 was enriched in down-regulated genes (OR = 1.88, $p = 2.59 \times 10^{-8}$), while module M2 was enriched in up-regulated genes (OR = 6.47, $p = 2.2 \times 10^{-16}$), suggesting the presence of distinct dysregulated gene-networks. Genes with predominant VT expression (14%) were mainly down-regulated, while genes with predominant EVT expression (9%) were mainly up-regulated even though both sets had the most members in module M1 (**Figure 3D**). These data suggested that the functions of both VT and EVT are strongly impacted in preterm preeclampsia, albeit in different ways.

Predominantly placenta-expressed genes, down-regulated in module M1, are regulators of fetal growth (*CSH1*, *HSD11B2*) (157, 158), metabolism (*ESRRG*) (159), estrogen synthesis (*HSD17B1*) (160), and immune functions (*LGALS14*) (153), some of which were reported to be down-regulated in preeclampsia (30, 91, 130, 155, 161, 162). Within this module, *ESRRG*, *POU5F1*, and *ZNF554* transcription regulatory genes had the highest number of significant correlations with predominantly placenta-expressed genes

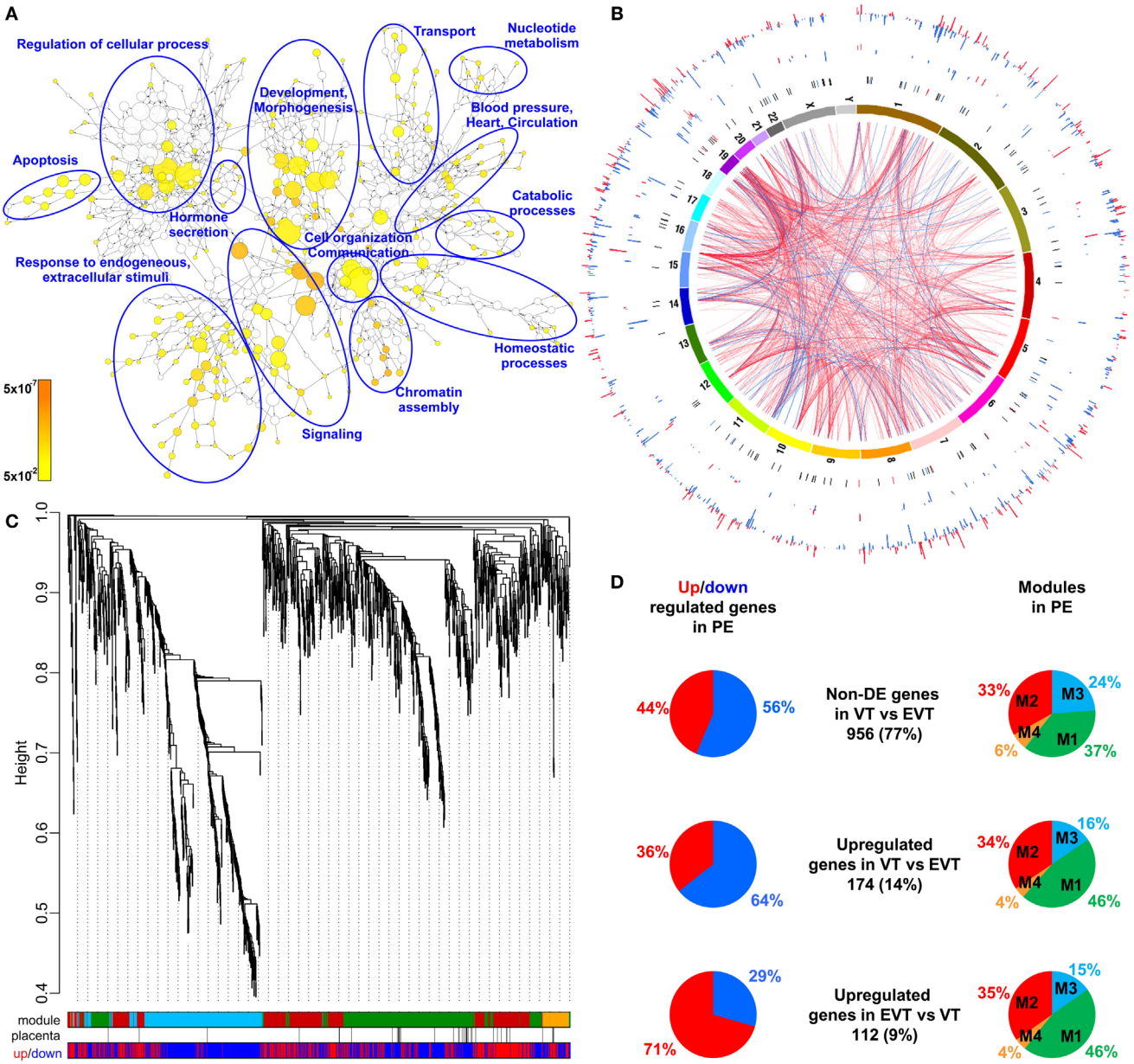


FIGURE 3 | Placental transcriptomic changes in preterm preeclampsia. **(A)** The network of biological processes enriched among differentially expressed (DE) genes. Circle sizes relate to the number of genes involved in the biological processes; colors refer to *p*-values according to the color code. Groups of the most enriched biological processes were circled and labeled. **(B)** Circos visualization of DE genes in preterm preeclampsia (PE). The inner circle shows chromosomes; curved lines represent correlation (red: positive; blue: negative) between transcription regulatory genes and their targets; the second circle shows genomic location of predominantly placenta-expressed genes (black lines: non-DE; red or blue lines: up- or down-regulated); third and fourth circles show the locations of DE transcription regulatory genes and their targets, respectively with blue (down-regulated) and red (up-regulated) bars. The height of the bars represents the magnitude of expression changes. **(C)** Dysregulated placental gene expression was characterized by four major disease gene modules within DE genes, marked with different colors. The height plotted on the *y*-axis represents distance metric. Of 38 predominantly placenta-expressed genes (black vertical lines), 33 belonged to the M1 (green, $n = 22$) and M2 (red, $n = 11$) modules. These modules were enriched in predominantly placenta-expressed genes and enriched in up-regulated (M2) or down-regulated (M1) genes marked under the modules with red or blue lines, respectively. **(D)** Pie charts depict the distribution of three gene sets among dysregulated genes or gene modules. Genes with similar expression in villous (VT) and extravillous trophoblast (EVT) had similar distribution among up- or down-regulated genes and in modules M1 and M2. Genes with up-regulation in VT compared to EVT were predominantly down-regulated in preeclampsia and positioned in module M1. Genes with up-regulation in EVT compared to VT were predominantly up-regulated in preeclampsia and positioned also in module M1.

and, hence, deemed as hub factors (Figure S1A in Supplementary Material). Of note, these transcription factors have been implicated in the regulation of stemness and differentiation (163, 164), pointing to the possible involvement of module M1 in the dysregulation of trophoblast differentiation in preterm preeclampsia. We selected *ZNF554* for functional studies, since it belongs to the

KRAB zinc finger family, crucial for early embryonic development and differentiation (165), and it may regulate genes in its co-expression network involved in biological processes affected by preeclampsia, such as development, chromatin assembly, signaling, adhesion, migration, and metabolism (Figure S1B in Supplementary Material).

In module M2, *FLT1,* which expresses sFlt-1, the main driver of BP elevation in the terminal pathway of preeclampsia (56, 90), was up-regulated. Moreover, module M2 genes were strongly overrepresented (OR = 29.9, $p = 6.54 \times 10^{-95}$) among genes that had correlated expression with mean arterial pressure (MAP) (Data S6 in Supplementary Material), suggesting a key role for this module in promoting hypertension. Within this module, *BCL6*, *BHLHE40*, and *ARNT2* had the highest correlation in gene expression with predominantly placenta-expressed genes, including *FLT1* (Figure S1A in Supplementary Material). Of note, these transcription regulatory hub genes are involved in hypoxia response. ARNT2 heterodimerizes with hypoxia-response regulator HIF-1α that is involved in trophoblast invasion and the pathogenesis of preeclampsia (166–169), and ARNT2 is a key regulator for adaption to hypoxic conditions at high altitudes, where the incidence of preeclampsia is much higher (16%) than at low altitude (170, 171). *BHLHE40* links immune and hypoxia-induced pathways (172). *BCL6,* a gene previously found to be up-regulated in the placenta in preterm preeclampsia, represses trophoblast differentiation and is regulated by stress-activated protein kinase signaling pathways (135, 173, 174). Our co-expression analysis revealed an enrichment of biological processes, such as differentiation, apoptosis, metabolism, signaling, and responses to stimuli including oxygen among genes co-expressed with *BCL6* (Figure S1C in Supplementary Material). Since *BCL6* is a key player in inflammation and oxygen-driven regulation of cell fate, we selected this gene for functional studies along with *ARNT2*.

The Association of Gene Modules With Clinical Parameters

Next, to validate the microarray results on a larger patient population ($n = 100$) comprised mostly of subjects of African-American origin presenting various phenotypes of preeclampsia (**Table 2**; Figure S2 in Supplementary Material), we selected 47 genes for qRT-PCR profiling of 100 placentas (**Table 3**), provided these genes were: (1) dysregulated genes with predominant placental and syncytiotrophoblastic expression, potentially encoding biomarkers; (2) dysregulated hub transcription regulatory genes that had a high co-expression with dysregulated, predominantly placenta-expressed genes in modules M1 and M2; and (3) non-dysregulated genes with roles in trophoblast differentiation, trophoblast-specific gene expression, or the pathogenesis of preeclampsia.

TABLE 2 | Demographics of American women included in the placental validation study.

Groups	Controls for preterm PE ($n = 20$)	Preterm PE ($n = 20$)	Preterm PE with SGA ($n = 20$)
Maternal age (years)[b]	22 (20–28.5)	23.5 (21–27)	22.5 (19.5–30)
Primiparity[a]	20	40	25
Gestational age (weeks)[b]	32.3 (28.2–34.9)	31.4 (29.6–33.6)	31.8 (29.7–34.4)
Race[a]			
Caucasian	5	10	10
African-American	95	90	90
Other	0	0	0
Systolic BP (mmHg)[b]	116 (110–125)	177 (166–187)[c]	171 (164–189)[c]
Diastolic BP (mmHg)[b]	65 (59–71)	105 (103–111)[c]	108 (94–118)[c]
Proteinuria[b]	0	3 (2–3)[c]	3 (3–3)[c]
Birthweight (g)[b]	1,635 (1,075–2,715)	1,488 (1,050–1,908)	1,173 (908-1,650)[d]
Birthweight percentile[b]	40.5 (31.9–53.4)	22.7 (18.3–32.9)[d]	6.7 (1–8.6)[c]
Cesarean delivery[a]	45	80[d]	75
Groups	**Controls for term PE ($n = 20$)**	**Term PE ($n = 10$)**	**Term PE with SGA ($n = 10$)**
Maternal age (years)[b]	22 (21–32)	19 (19–35)	26.5 (19–31)
Primiparity[a]	15	40	10
Gestational age (weeks)[b]	38.6 (38–39.1)	39.1 (38.6–39.6)	38.4 (37.3–38.9)
Race[a]			
Caucasian	15	0	0
African-American	80	100	100
Other	5	0	0
Systolic BP (mmHg)[b]	121 (111–134)	173 (165–178)[c]	169 (164–190)[c]
Diastolic BP (mmHg)[b]	70 (64–73)	106 (102–110)[c]	102 (97–104)[c]
Proteinuria[b]	0	3 (1–3)[c]	3 (1–3)[c]
Birthweight (g)[b]	3,215 (3,110–3,335)	3,123 (2,990–3,200)	2,405 (2,205–2,555)[c]
Birthweight percentile[b]	46 (37.2–63)	37.1 (28.5–48.8)	1.1 (1–3.5)[c]
Cesarean delivery[a]	55	40	20

BP, blood pressure; PE, preeclampsia; SGA, small-for-gestational age.

[a]*Percentage.*

[b]*Median (interquartile range).*

[c]$p < 0.001$.

[d]$p < 0.05$.

TABLE 3 | Genes included in the placental qRT-PCR validation study.

Gene symbol	DE genes in preterm PE (microarray)	Gene modules (microarray)	Hub TR genes in modules (microarray)	TR genes	PPE genes	DE genes in preterm PE (qRT-PCR)	DE genes in term PE (qRT-PCR)
ARNT2	Up	M2	M2	Yes	–	**Up**	–
BCL3	Up	M1	–	Yes	–	–	**Up**
BCL6	Up	M2	M2	Yes	–	**Up**	–
BTG2	Down	M2	–	Yes	–	**Down**	**Down**
CDKN1A	Down	M1	–	–	–	–	Up
CGB	Up	M4	–	–	Yes	–	**Up**
CLC	Up	M2	–	–	–	**Up**	**Up**
CLDN1	Down	M1	–	–	–	**Down**	**Down**
CRH	Up	M2	–	–	Yes	**Up**	–
CSH1	–	M1	–	–	Yes	Down	–
CYP19A1	–	–	–	Yes	Yes	–	–
DUSP1	Up	M2	–	–	–	**Up**	–
ENG	–	–	–	–	–	Up	Up
ERVFRDE1	–	–	–	–	Yes	–	–
ERVWE1	–	–	–	–	Yes	–	–
ESRRG	Down	M1	M1	Yes	Yes	**Down**	–
FBLN1	Down	M1	–	–	Yes	–	–
FLT1	Up	M2	–	–	–	**Up**	**Up**
GATA2	–	–	–	Yes	–	–	–
GCM1	–	–	–	Yes	Yes	Down	–
GH2	–	–	–	–	Yes	–	–
HLF	Down	M1	–	Yes	–	**Down**	–
HSD11B2	Down	M1	–	–	Yes	–	–
HSD17B1	Down	M1	–	–	Yes	**Down**	**Down**
IKBKB	Up	M1	–	–	–	–	–
INSL4	Up	M4	–	–	Yes	–	–
JUNB	Up	M2	–	Yes	–	**Up**	–
KIT	Up	M2	–	–	–	**Up**	–
LEP	Up	M2	–	–	Yes	**Up**	**Up**
LGALS13	–	–	–	–	Yes	Down	Down
LGALS14	Down	M1	–	–	Yes	**Down**	**Down**
LGALS16	–	–	–	–	Yes	–	–
LGALS17A	–	–	–	–	–	–	–
MAPK13	–	–	–	–	–	–	Up
NANOG	–	–	–	Yes	–	–	–
PAPPA	–	–	–	–	Yes	–	–
PAPPA2	Up	M2	–	–	Yes	**Up**	**Up**
PGF	–	–	–	–	Yes	–	–
PLAC1	Down	M1	–	–	Yes	**Down**	–
POU5F1	Up	M1	M1	Yes	–	–	–
SIGLEC6	Up	M2	–	–	Yes	**Up**	**Up**
TEAD3	–	–	–	Yes	Yes	–	Up
TFAM	Down	M3	–	Yes	–	–	Up
TFAP2A	–	–	–	Yes	Yes	–	–
TPBG	Up	M2	–	–	–	**Up**	**Up**
VDR	Up	M2	–	Yes	–	–	–
ZNF554	Down	M1	M1	Yes	–	**Down**	**Down**

PE, preeclampsia; DE, differentially expressed; TR, transcription regulatory; PPE, predominantly placenta-expressed.
Identical dysregulation in the validation cohort as in the microarray cohort is depicted in bold font.

As depicted in the heatmap representing qRT-PCR data for the 100 placentas and 47 genes (**Figure 4A**), validation data supported microarray experiments in terms of (1) differential expression of selected genes, (2) high correlation of genes within modules M1 and M2, and (3) separate dysregulation of modules M1 and M2 from each other. Comparison between preterm preeclampsia and gestational age-matched controls revealed that qRT-PCR data validated microarray results (differential or non-differential gene expression) for 33 of 47 genes (70%), and further confirmed the differential expression of four genes that were non-dysregulated in the microarray cohort (**Table 3**). As expected, the extent of changes in gene expression was less pronounced in term preeclampsia (**Table 3**; **Figure 4C**; Figure S3 in Supplementary Material). As a further confirmation, there was a strong correlation between qRT-PCR data as well as tissue microarray (TMA) and immunostaining results for selected proteins in module M2 (**Figures 4C–E**). The extent of dysregulation was larger in preterm phenotypes of preeclampsia than in term phenotypes, in agreement with the more severe placental pathology (44). Among transcription regulatory genes of module M1, *HLF* had strong down-regulation only in preterm preeclampsia while *ZNF554* was expressed at a lower

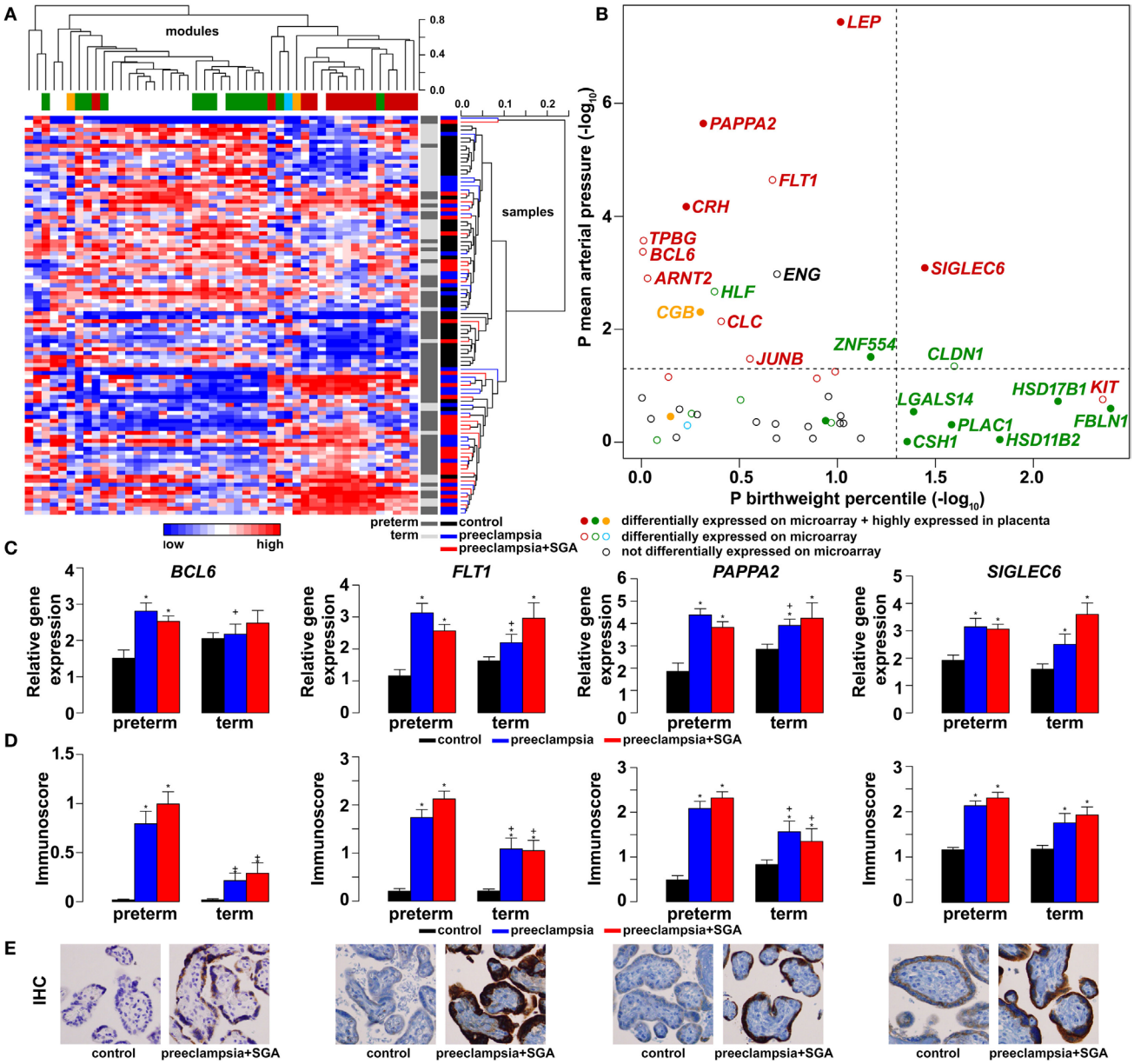


FIGURE 4 | Gene modules associated with blood pressure (BP) and birthweight (BW). **(A)** Hierarchical clustering of qRT-PCR data obtained with 100 samples and 47 genes. Pearson correlation was used for similarity analysis and average method for linkage calculation. Samples were colored according to patient groups and maturity status. M1 (green) and M2 (red) module genes and 34 of 60 samples from women with preeclampsia clustered together. **(B)** Association of gene expression with BP and BW. The significance *p*-values for these coefficients were plotted for all genes, colored according to module classification (black: not changed on the microarray). Filled circles represent predominantly placenta-expressed genes and dashed lines the significance threshold at $p = 0.05$. Seven of 9 genes related to BW belong to module M1, while 10 of 15 genes related to BP are from module M2. Gene expression **(C)** and protein immunostaining **(D)** of selected four genes in module M2 show similar patterns in the sub-groups of preeclampsia, and semi-quantitative immunoscorings validate gene expression data. Significant differences in preterm or term preeclampsia samples compared to gestational-age matched controls are shown with "*". When the change with preeclampsia in preterm samples was different from that in term samples, a "+" marks such an interaction. Comparisons for all genes are available in Figure S3 in Supplementary Material. **(E)** Representative images of the same placenta from a preterm control (left, 29 weeks) and a patient with preterm preeclampsia associated with SGA (right, 31 weeks) are shown for the immunostainings (hematoxylin counterstaining, 40× magnification).

level in all preeclampsia phenotypes (Figure S3 in Supplementary Material). Among the transcription regulatory genes in module M2, *ARNT2*, *BCL6,* and *JUNB* were highly expressed in preterm but not in term preeclampsia, suggesting that these might play a role only in the pathology of preterm cases (**Figure 4C**; Figure S3 in Supplementary Material). Overall, these data reflect the heterogeneous placental pathology and the more severely affected pathways in preterm preeclampsia.

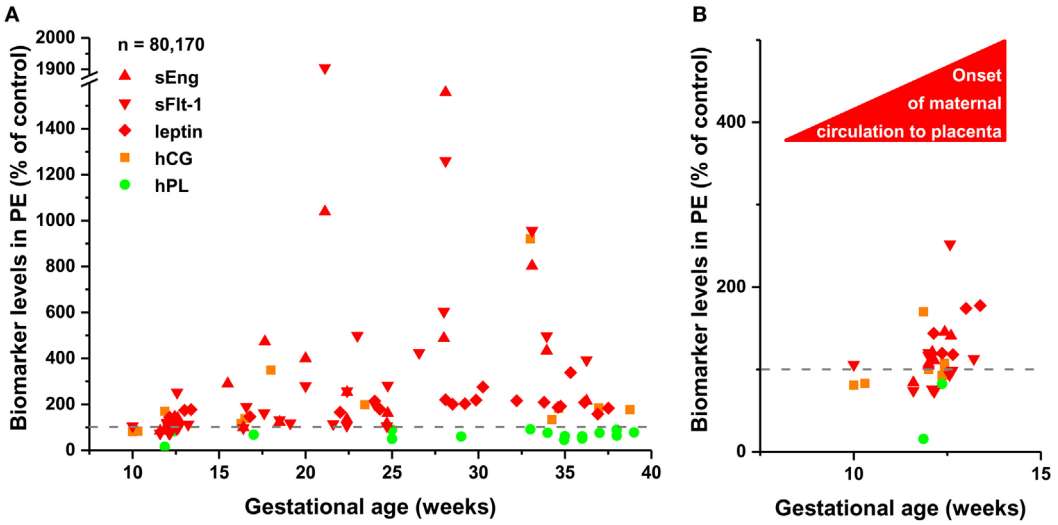


FIGURE 5 | The timing of gene module dysregulation in preterm preeclampsia. **(A)** A database of 80,170 measurements published in 61 reports (35, 61, 82, 88, 126, 178–233) was built for "virtual" liquid biopsy of the placenta in preterm preeclampsia with maternal blood levels of proteins with the highest expression in the placenta among all tissues [i.e. hCG, human placental lactogen (hPL), sEng, sFlt-1, and leptin]. Levels of these biomarkers in preterm preeclampsia were expressed as the percentage of control levels (dashed line) and were represented in scatter plots by different colors reflecting gene module classification. Based on qRT-PCR data, sEng belongs to module M2 (red). hPL (M1, green module) levels in preeclampsia were constantly below control levels during gestation, while the levels of module M2 biomarkers in preeclampsia had continuous elevation compared to control levels as a function of gestational age. **(B)** Analysis of first trimester data revealed the lower expression of all biomarkers in preeclampsia than in controls before the onset of maternal circulation, and the increasing expression of module M2 biomarkers in preeclampsia compared to controls after 12 weeks of gestation.

TABLE 4 | Demographics of Israeli women included in the maternal blood two-dimensional differential in-gel electrophoresis proteomics study.

Groups	Controls for preterm PE with SGA ($n = 5$)	Preterm PE with SGA ($n = 5$)	Controls for term PE ($n = 5$)	Term PE ($n = 5$)
Maternal age (years)[b]	30 (24–31)	29 (27–37)	29 (27–34)	26 (23–32)
Primiparity[a]	40	20	80	40
Gestational age at blood draw (weeks)[b]	10 (9–11)	8 (8–9)	8 (8–9)	9 (8–10)
Gestational age at delivery (weeks)[b]	39.7 (38.6–40.0)	34.9 (29.3–35.3)[d]	38.7 (38.6–41.0)	38.1 (38.0–38.1)
Race[a]				
Caucasian	100	100	100	100
African-American	0	0	0	0
Other	0	0	0	0
Systolic BP (mmHg)[b]	105 (104–110)	160 (150–165)[c]	110 (110–118)	150 (140–160)[c]
Diastolic BP (mmHg)[b]	60 (60–70)	100 (100–100)[c]	67 (63–68)	100 (90–100)[c]
Proteinuria[b]	0	4 (3–4)[c]	0	3 (3–4)[c]
Birthweight (g)[b]	2,955 (2,900–3,100)	1,720 (975–1,800)[c]	3,390 (3,270–3,600)	3,200 (3,150–3,210)
Cesarean delivery[a]	0	60[c]	60	40

BP, blood pressure; PE, preeclampsia; SGA, small-for-gestational age.
[a]*Percentage.*
[b]*Median (interquartile range).*
[c]*$p < 0.05$ when compared to corresponding controls.*
[d]*$p < 0.001$ when compared to corresponding controls.*

Because of the possible involvement of these two gene modules in distinct pathologic pathways, we correlated qRT-PCR data with maternal and fetal clinico-pathological indicators to further investigate their association (**Figure 4B**). This analysis showed that 7 of 9 genes related to the birthweight (BW) percentile were from module M1, while 10 of 15 genes related to BP were from module M2, confirming our observations with microarray data. Therefore, we decided to also refer to these as "M1-BW" and "M2-BP" modules. Of interest, the expression of most (9/14) M1 genes was negatively associated with the "maternal vascular malperfusion" score of the placenta, while the expression of most (10/14) M2 genes was positively correlated with this parameter (Data S7 in Supplementary Material). This observation fits well with the accepted concept that maternal vascular malperfusion of the placenta leads to oxidative stress (40, 41, 63, 175) and the increased placental expression of *FLT1* (63).

Maternal Study

Maternal Blood Reflects Gene Module Dysregulation in Preeclampsia

Next, we investigated whether placental gene module dysregulation can be detected in maternal blood and whether liquid biopsy

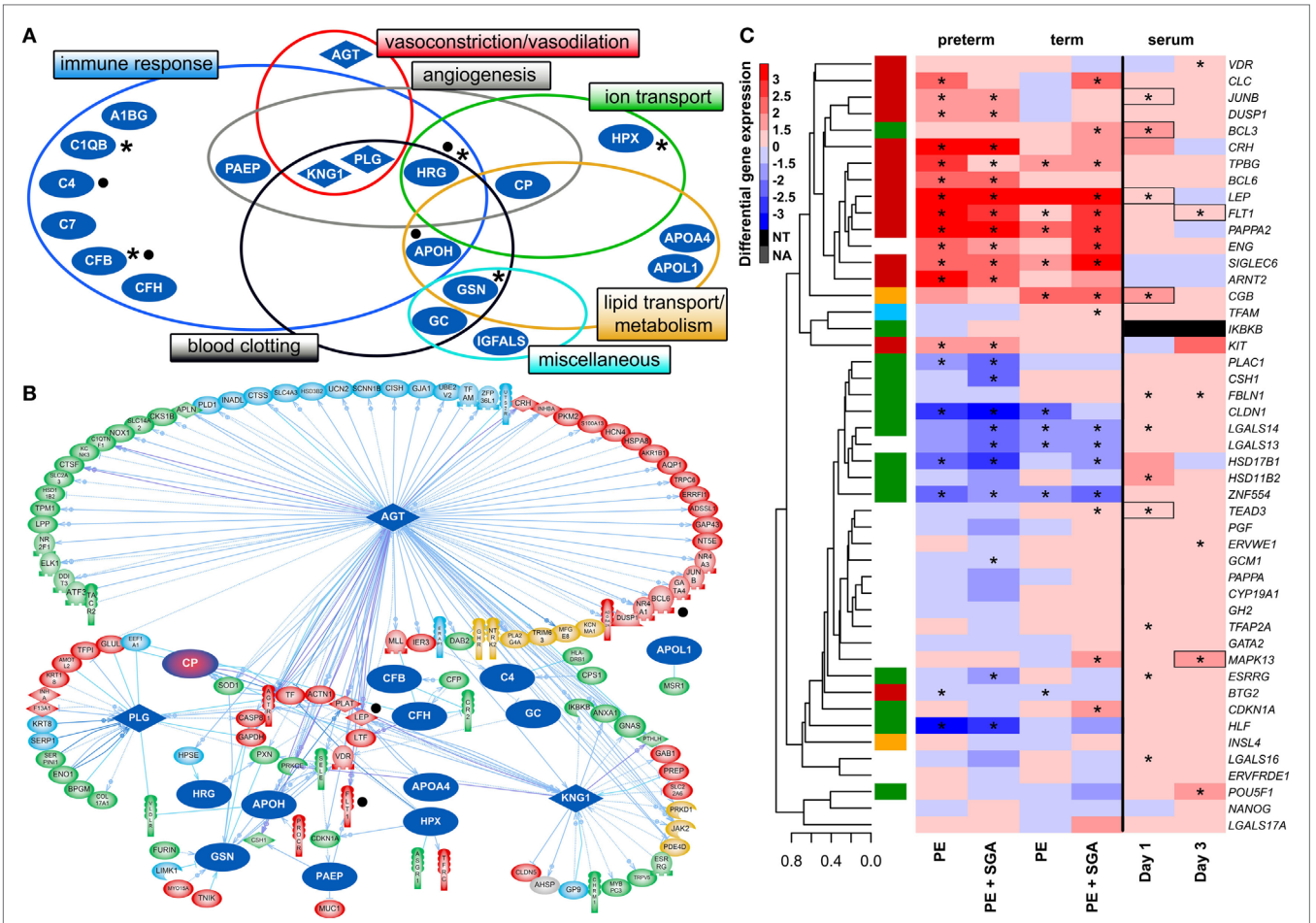


FIGURE 6 | Maternal blood proteomic changes and their effect on differentially expressed (DE) genes in preterm preeclampsia. **(A)** The 19 DE maternal serum proteins in preterm preeclampsia identified by two-dimensional differential in-gel electrophoresis could be classified into functional groups relevant for preeclampsia pathophysiology. Asterisks denote proteins reported by others to have the same direction differential abundance in the first trimester in patients developing preterm preeclampsia (126, 143, 239–241). Dots denote immune proteins that multiple reaction monitoring assays identified to have differential abundance in preterm preeclampsia in the same direction. **(B)** The 19 DE serum proteins have connections to 121 DE placental genes, among which 48 belong to module M2. Angiotensinogen has more connections than other proteins (OR = 1.9, $p = 4.9 \times 10^{-5}$), and the most connections to module M2 genes ($n = 35$); 77 of 86 connections of angiotensinogen have a directional effect toward the gene. Dots denote genes in connection to angiotensinogen found up-regulated in villous trophoblast (VT) upon treatment with first trimester serum from preeclamptic women. **(C)** Hierarchical clustering tree and heatmap representing differential placental gene expression in preeclampsia as in **Figure 4A**. The expression of the same genes (except *IKBKB*) was determined in primary VTs upon treatment with first trimester maternal serum. Serum from preeclamptic compared to control women induced the up-regulation of seven placental dysregulated genes (depicted with boxes) on the first and third days of VT differentiation, among which six were up-regulated in term and three in preterm preeclampsia. Stars depict significant changes, color bar encodes signed (up or down)-fold changes. Abbreviations: NA, not expressed; NT, not examined.

can be used to determine when the module dysregulation occurs. To study the relationship between placental gene expression and maternal biomarker concentrations, we selected predominantly placenta-expressed genes with the most differential expression in modules M1 (*CSH1*) or M2 (*LEP*), and correlated their placental expression levels with concentrations of their secreted protein products (hPL and leptin) in maternal blood collected in both first ($n = 12$) and third trimesters ($n = 19$) in a Hungarian patient population. Positive correlations were found between placental gene expression and maternal blood protein concentrations for both biomarkers in both trimesters (Figure S4 in Supplementary Material), suggesting that certain proteins in the maternal circulation reflect the placental expression of their encoding genes throughout pregnancy.

To detect disease-associated protein signatures of placental dysfunction in maternal blood, similar to plasma DNA tissue mapping for noninvasive prenatal assessments (176, 177), we performed "virtual" liquid biopsy of the placenta in preterm preeclampsia (Figure S5 in Supplementary Material; **Figure 5**). We identified five genes with predominant placental expression, which have products extensively investigated in maternal blood in preterm preeclampsia or in all cases of preeclampsia during all trimesters. From data of 61 reports (35, 61, 82, 88, 126, 178–233), we built a database of 80,170 measurements in which

TABLE 5 | Demographics of Hungarian women included in the maternal blood multiple reaction monitoring proteomics study.

Groups	Controls for preterm PE with SGA (n = 10)	Preterm PE with SGA (n = 5)
Maternal age (years)[b]	29.7 (28.9–31.5)	34.7 (28.6–35.1)
Primiparity[a]	20	80
Gestational age at blood draw (weeks)[b]	12.6 (12.6–12.8)	12.4 (12.4–13.0)
Gestational age at delivery (weeks)[b]	40.3 (39.9–40.8)	32.4 (26.4–34.0)[c]
Race[a]		
Caucasian	100	100
African-American	0	0
Other	0	0
Systolic BP (mmHg)[b]	124 (118–126)	170 (170–170)[c]
Diastolic BP (mmHg)[b]	75 (68–79)	100 (100–110)[c]
Proteinuria (g)[b]	0	3 (1–6)[c]
Birthweight (g)[b]	3,460 (3,308–3,623)	1,380 (790–1,450)[c]
Cesarean delivery[a]	30	100[c]

BP, blood pressure; PE, preeclampsia; SGA, small-for-gestational age.
[a]*Percentage.*
[b]*Median (interquartile range).*
[c]$p < 0.05$.

preeclampsia data were expressed as a percentage of average levels in controls. Regarding biomarkers in module M1, we found hPL levels in preterm preeclampsia to be consistently below control levels throughout pregnancy. This is substantiated by the down-regulation of another M1 biomarker, HSD17B1, in first trimester maternal blood (160), confirming a generalized down-regulation of M1 biomarkers in early pregnancy. By contrast, the levels of biomarkers in module M2 had constant elevation in preterm preeclampsia compared to controls during gestation. When analyzing only data collected in the first trimester, prior to the onset of maternal circulation to the placenta, levels of M1 and M2 biomarkers were lower in women with preeclampsia compared to controls, while after 12 weeks of gestation, following the opening of the intervillous spaces to maternal blood flow, patients had increasing levels of M2 biomarkers in maternal blood compared to controls. These findings offer support for the conclusion that placental transcriptomic changes typical for preeclampsia in the third trimester are rooted in the first trimester.

Altered Maternal Serum Proteome in Preeclampsia

Next, we investigated the maternal serum proteome in early pregnancy in distinct phenotypes of preeclampsia and the potential effects of proteomic changes on the placental transcriptome. Comparing samples from women with preterm preeclampsia associated with SGA and their respective controls from an Israeli patient population (n = 10, **Table 4**), 19 DE protein spots were identified and investigated by mass spectrometry. According to public data, many of these proteins have a role in immune response, complement and coagulation cascades, lipid transport and metabolism, angiogenesis, BP regulation, and ion transport (**Figure 6A**, Data S8 in Supplementary Material). Comparing samples from women with term preeclampsia and their respective controls from this Israeli patient population (n = 10, **Table 4**), 14 DE protein spots could be identified (Figure S6A; Data S8 in Supplementary Material). Many of the proteins found in these spots are the same as those found in preterm preeclampsia or function in the same pathways. Of note, these pathways and the 26 differentially abundant proteins identified in term and preterm preeclampsia had mostly been implicated in a later stage of preeclampsia (43, 101, 120, 234–238). We concluded that there is a common dysregulation of the maternal serum proteome in term and preterm preeclampsia; however, the extent of changes is larger in the latter, in agreement with the more fulminant and early pathogenesis.

Supporting our findings, five studies (126, 143, 239–241) found proteins with differential abundance in preterm preeclampsia in the same direction as our two-dimensional differential in-gel electrophoresis (2D-DIGE) study (**Figure 6A**). We also collected maternal blood specimens in the first trimester from a Hungarian patient population (n = 15, **Table 5**), and measured the concentrations of 10 immune proteins in patients with preterm preeclampsia associated with SGA and matched controls using liquid chromatography–mass spectrometry multiple reaction monitoring (MRM). In spite of the difference between the methods and ethnic background, MRM identified 4 of these 10 proteins as having differential abundance in the same direction as in the 2D-DIGE study, supporting the early pro-inflammatory changes in the maternal proteome in preterm preeclampsia (**Figure 6A**; Data S9 in Supplementary Material). MRM and proteomic evidence published to date supported 37% (7/19) of the proteomic changes detected by 2D-DIGE.

The *In Silico* Effects of Altered Maternal Serum Proteome on the Placenta

To reveal whether early proteomic changes in maternal blood may affect the placental transcriptome, placental DE genes with documented connections to DE proteins in maternal serum were identified by Pathway Studio. The 121 DE placental genes with connections to DE serum proteins in preterm preeclampsia were marginally over-represented by those from module M2 (48/121, OR = 1.4, $p = 0.057$). Angiotensinogen had the largest number of connections to DE genes including *FLT1* and *LEP* (n = 86, OR = 1.9, $p = 4.9 \times 10^{-5}$). This protein also had the largest number of connections to module M2 genes (n = 35), followed by plasminogen (n = 11) and kininogen-1 (n = 9), all involved in BP regulation (**Figure 6B**). These data were supported by the "renin-angiotensin signaling" as being a top pathway ($p = 1.28 \times 10^{-4}$) among the 35 angiotensinogen-connected DE genes (Data S10 in Supplementary Material). Similar results were obtained when analyzing connections between 116 DE placental genes and DE serum proteins in term preeclampsia (Figure S6B in Supplementary Material).

Trophoblast Study

The *In Vitro* Effects of an Altered Maternal Serum Proteome on the Trophoblast

Next, we aimed to study various factors implicated in the pathogenesis of preeclampsia in trophoblast models to determine whether these may drive the observed placental transcriptomic changes. Since the results noted above suggested that maternal serum proteins can influence the placental transcriptome, we

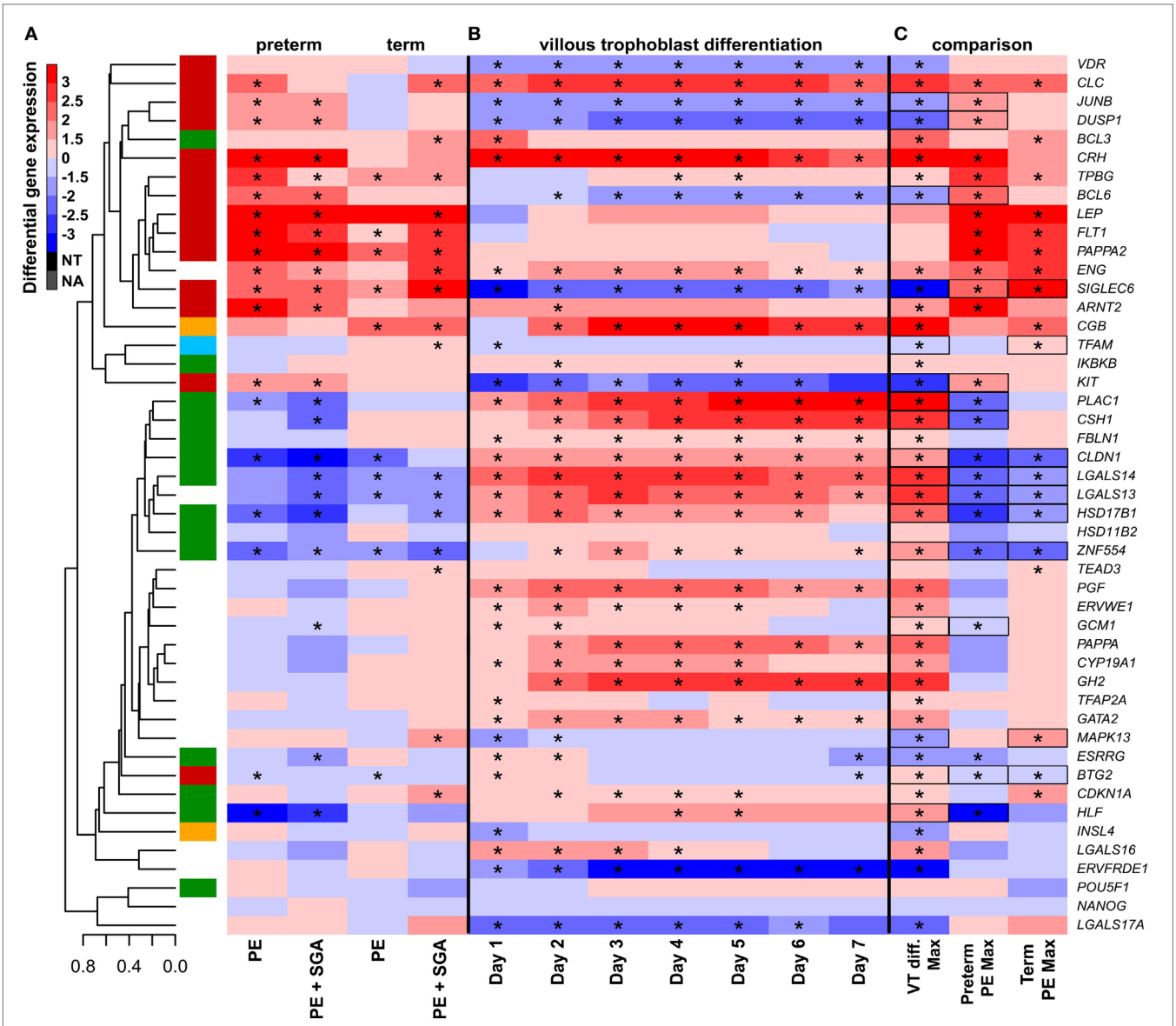


FIGURE 7 | The effect of trophoblast differentiation on differentially expressed genes in preeclampsia. **(A)** Hierarchical clustering tree of expression data for 47 genes in 100 placental specimens was augmented with a heatmap representing differential gene expression in term or preterm subgroups of preeclampsia. **(B)** Primary villous trophoblast (VT) differentiation time series expression data for 47 genes were depicted with a heatmap representing differential gene expression in each time point (days 1–7) compared to day 0. **(C)** Maximum expression values in the VT differentiation time series were presented alongside with maximum expression values in the placenta in preterm or term preeclampsia. Comparative visualization revealed the opposite-direction differential regulation of 17 genes in preeclampsia compared to VT differentiation as depicted with black boxes. Among these genes, 15 had this behavior in preterm preeclampsia and 9 in term preeclampsia ($p = 0.057$). In **(A–C)**, stars depict significant changes, color bar encodes signed (up or down)-fold changes. Abbreviations: NA, not expressed; NT, not examined.

first measured the effects of maternal blood from early pregnancy on trophoblastic gene expression. We treated primary VTs during differentiation with first trimester sera from women with preterm preeclampsia or normal pregnancy, and analyzed the expression of genes included in the placental validation study. Serum from preeclamptic women compared to controls induced up-regulation of seven placental DE genes on the first and third days of trophoblast differentiation, including *FLT1* and *LEP* (**Figure 6C**). Among these, six genes were up-regulated in the placenta in term and three in preterm preeclampsia. These results support *in silico* findings and suggest that maternal serum factors can up-regulate *FLT1* and may induce the terminal pathway.

The Effects of Altered VT Differentiation

Next, we examined whether disturbance in VT differentiation may be reflected in the placental gene expression signature in women with different preeclampsia phenotypes. Since there

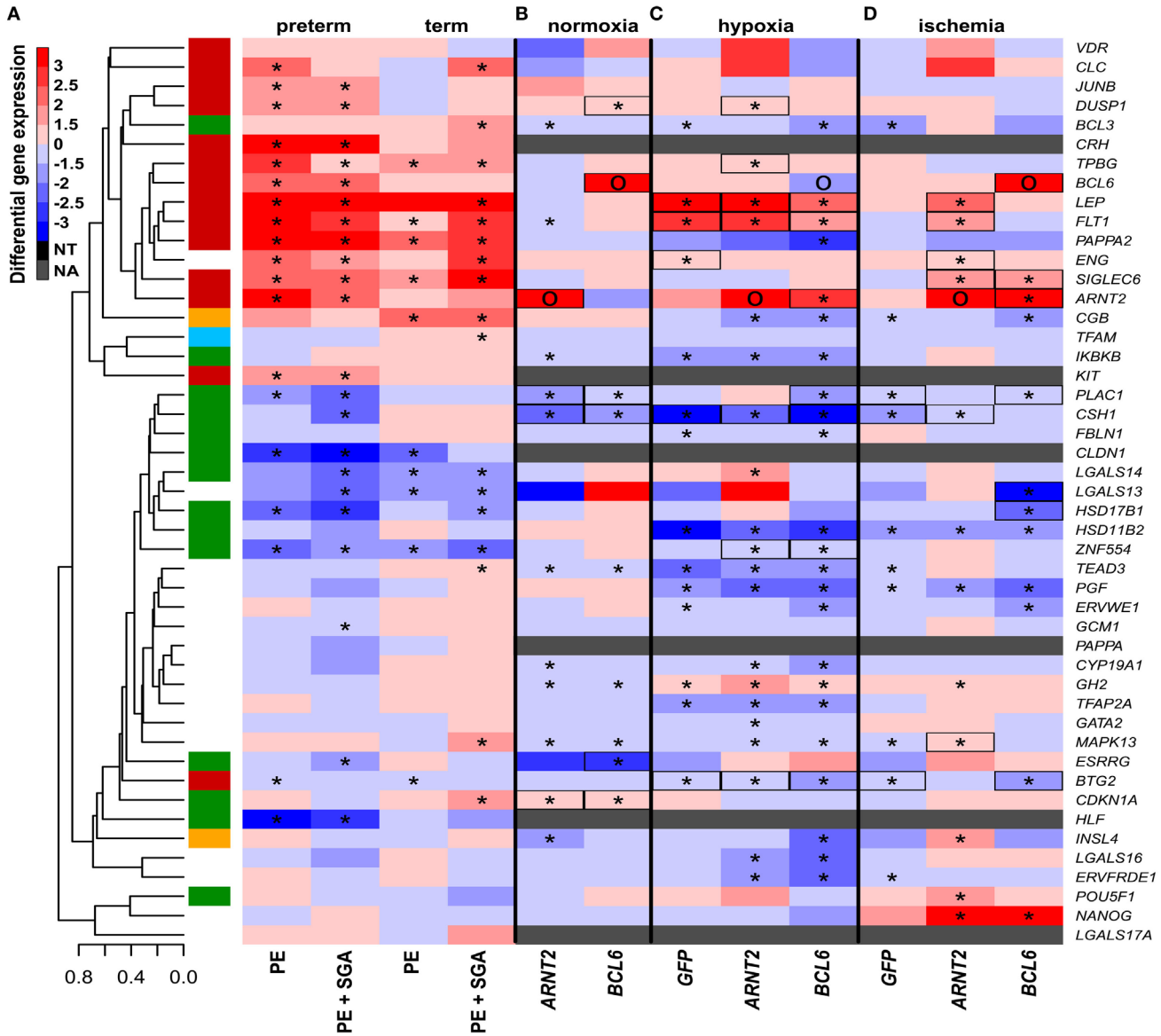


FIGURE 8 | The effect of O_2 levels, *BCL6* and *ARNT2* overexpression on differentially expressed genes in preeclampsia. **(A)** Hierarchical clustering tree and heatmap representing differential placental gene expression in preeclampsia. **(B)** The overexpression of *ARNT2* or *BCL6* in normoxic BeWo cells induced the dysregulation of three or five genes dysregulated in preeclampsia, respectively (boxed). **(C)** Hypoxia induced the dysregulation of five genes in BeWo cells also altered in preeclampsia. Hypoxia combined with *ARNT2* or *BCL6* overexpression led to the dysregulation of a large number of genes. **(D)** Ischemia induced the dysregulation of three genes in BeWo cells similar to preeclampsia. Ischemia combined with *ARNT2* or *BCL6* overexpression led to the dysregulation of 11 genes similar to preeclampsia. **(C,D)** Represents comparisons of gene expressions between hypoxia/ischemia vs. normoxia. In **(A–D)**, stars depict significant changes, "O" depicts "overexpressed," color bar encodes signed (up or down)-fold changes. Black boxes depict genes with similar expression changes *in vitro* as in the placenta in preeclampsia. Abbreviations: NA, not expressed; NT, not examined.

were no ($n = 46$) or subtle ($n = 2$) gestational age-dependent differences in the expression of selected target or housekeeping genes in third trimester control placentas in our microarray study (132), the comparison of placentas and trophoblasts from various gestational ages was deemed to be valid. Thus, we performed qRT-PCR profiling of primary VTs during 7 days of differentiation to reveal the dynamics in the expression of genes (**Figure 7B**), which we similarly profiled in the placenta (**Figure 7A**). We determined whether maximum expression change of selected genes during VT differentiation compared to day 0 inversely correlated with their maximum expression change in different preeclampsia phenotypes. We found that this was the case for 17 genes (**Figure 7C**), suggesting the delay or inhibition of VT differentiation-related expression change of these genes in

preeclampsia. Among these 17 genes, 15 showed this behavior in preterm and 9 in term preeclampsia, suggesting that VT differentiation problems are more pronounced in preterm than in term preeclampsia ($p = 0.057$).

The Impact of Hypoxia, Ischemia, and Overexpression of *BCL6* and *ARNT2* on the Trophoblast

Subsequently, we tested how other factors implicated in preeclampsia pathogenesis, namely physiologic hypoxia (2% O_2) (167) or alternating hypoxic (1% O_2) and normoxic (20% O_2) conditions (ischemia) (40, 41, 242), in combination with the overexpression of hub transcription regulatory factors, may affect trophoblastic gene expression in a widely used BeWo cell trophoblast model. Two percent O_2 induced the dysregulation of only five genes, including *LEP* and *FLT1*, from the set of genes investigated in the placenta in preterm preeclampsia (**Figure 8C**), while alternating O_2 concentrations induced the dysregulation of only three genes (**Figure 8D**). Since hypoxia or ischemia alone did not induce similar transcriptomic changes in BeWo cells as seen in the placenta in preeclampsia, we examined how the overexpression of hub transcription regulatory genes in module M2 may modify the effects of these two conditions. Of note, 2% O_2 combined with *ARNT2* or *BCL6* overexpression led to the dysregulation of a large number of genes (**Figure 8C**). There were 9 genes (6 in the M2 and 3 in the M1 modules) dysregulated in BeWo cells, including *FLT1*, *ARNT2*, and *ZNF554*, similar to preeclampsia. Alternating O_2 concentrations combined with *ARNT2* or *BCL6* overexpression led to the dysregulation of 11 genes (5 in the M2 and 3 in the M1 modules), including *LEP*, *FLT1*, and *ENG*, similar to preeclampsia (**Figure 8D**). However, *ARNT2* or *BCL6* overexpression at normoxic conditions did not lead to substantial gene expression changes (**Figure 8B**), underlining the importance of gene–environment interactions. A permutation test showed that *BCL6* overexpression in ischemia mimicked overall expression changes of module M1 and M2 genes in preterm preeclampsia, while *ARNT2* overexpression in ischemia (and also in hypoxia) mimicked the up-regulation of module M2 genes in term and preterm preeclampsia (Data S11 in Supplementary Material). Since *BCL6* overexpression up-regulated *ARNT2* both in ischemia and hypoxia but not *vice versa*, we propose that *BCL6* is upstream of *ARNT2*. The up-regulation of these two transcription regulatory genes sensitize the trophoblast to ischemia, leading to the early dysregulation of modules M1 and M2, thus promoting preterm preeclampsia (Figure S7 in Supplementary Material).

Since none of the investigated conditions could up-regulate *BCL6*, we wondered whether *BCL6* overexpression might have an epigenetic background. Treating BeWo cells with 5-azacitidine down-regulated *BCL6*, supporting that its expression is regulated by DNA methylation in the trophoblast (Figure S8 in Supplementary Material). Of note, a recent study described the first intron of *BCL6* to be key in its overexpression in Burkitt lymphoma *via* altered DNA methylation (243). The Human Reference Epigenome Mapping Project revealed a differentially methylated region (DMR) in this intron in H1 embryonic stem cells as well as trophoblastic and neuronal cells derived from H1 cells, suggesting that this DMR may be differentially methylated in the trophoblast compared to other cells. To address whether this intronic region may be affected in the trophoblast in preeclampsia, we investigated DNA methylation in this region in primary VTs compared to cord blood cells collected from the same normal pregnancies. Bisulfite sequencing showed that Chr3:187,458,083-187,458,651 and Chr3:187,460,304-187,460,374 regions contain 12 hypermethylated CpGs in the trophoblasts compared to cord blood cells (Figures S8 and S9 in Supplementary Material). Further, we tested DNA methylation in this region in 100 placentas with qRT-PCR data including patients with preeclampsia after laser capturing VTs. Three CpGs (Chr3:187,458,095, Chr3:187,458,163, and Chr3:187,458,327) were differentially methylated in preeclampsia (Figure S10 in Supplementary Material), of which CpG Chr3:187,458,163 was differentially methylated in preterm preeclampsia, suggesting that this CpG may have a role in *BCL6* dysregulation in preeclampsia.

The Effects of *ZNF554* Down-Regulation in VTs

Next, we were interested in how the dysregulation of module M1 genes may play a role in preeclampsia pathology. Among hub genes of this module, *ZNF554* was of most interest due to the biological processes enriched in its co-expression network (Figure S1B in Supplementary Material), and also to its potential placenta- and preeclampsia-related regulation by transposable elements. This hypothesis was based on the fact that insertion of transposable elements into regulatory regions can lead to transcriptional changes, especially in the placenta (155, 244–247), and that the 5′ flanking region of *ZNF554* contains many LTR10A copies which had top enrichment among module M1 genes (OR = 17.4, $p = 1.27 \times 10^{-7}$; Data S12 in Supplementary Material). Of note, LTR10A drives placenta-specific expression of *NOS3* (248), and it may also have a similar effect on *ZNF554*. Indeed, *ZNF554* had the highest expression in the placenta in comparison to 47 other human tissues, which mostly had negligible *ZNF554* expression (**Figure 9A**).

Subsequent *in situ* hybridization (**Figure 9B**) and immunostaining (**Figure 9C**) of first and third trimester placentas of women with a normal pregnancy showed dominant *ZNF554* expression in the syncytiotrophoblast but not in the villous cytotrophoblast. Thus, we investigated *ZNF554* expression during VT differentiation, in which it was up-regulated similar to *CSH1* (**Figure 9D**), supporting that *ZNF554* expression is developmentally regulated in VTs. Of interest, ZNF554 immunostaining was faint in the syncytiotrophoblast in preeclampsia compared to controls (**Figures 9E,F**). To characterize the loss of syncytiotrophoblastic ZNF554 function, we silenced *ZNF554* in BeWo cells. At 74% *ZNF554* knock-down ($p = 5.24 \times 10^{-6}$) (**Figure 9G**), decreased nuclear and cytoplasmic ZNF554 immunostaining was found (**Figure 9H**). Microarray analyses of *ZNF554*-silenced cells revealed 123 DE genes (Data S13 in Supplementary Material) including 9 DE placental genes in preeclampsia, and the dysregulation of the "glycolysis/gluconeogenesis" pathway (OR = 7.8, $q = 0.06$) as well as 18 molecular functions including "RNA binding" (down) and "activin binding" (up) (**Figure 9I**; Data S14 in Supplementary Material). The up-regulation of *FSTL3* was confirmed by qRT-PCR (2.7-fold, $p < 0.001$) (**Figure 9J**). *FSTL3* encodes a secreted glycoprotein that

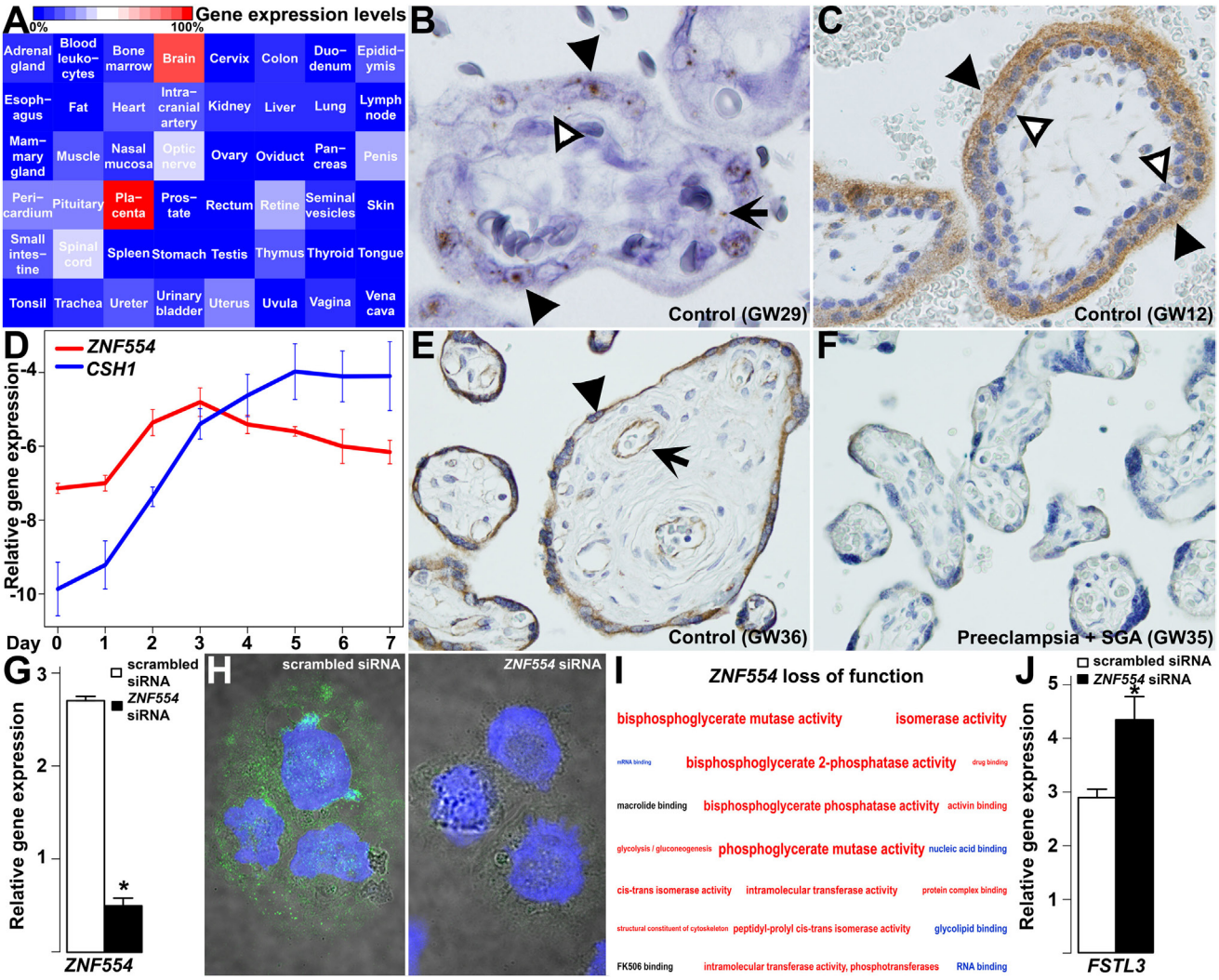


FIGURE 9 | *ZNF554* down-regulation in the villous trophoblast (VT) in preeclampsia. **(A)** Tissue qRT-PCR array revealed the highest *ZNF554* expression in the placenta among 48 human tissues. Color code depicts gene expression levels relative to that of the placenta (100%). **(B)** *In situ* hybridization of a third trimester control placenta (GW29) and **(C)** immunohistochemistry of a first trimester placenta (GW12) shows mainly syncytiotrophoblastic *ZNF554* expression (hematoxylin counterstaining, 1,400 and 400× magnifications, respectively). Black or white arrowheads depict syncytiotrophoblast or cytotrophoblast, while black arrow depicts fetal endothelium, respectively. **(D)** qRT-PCR revealed that *ZNF554* expression is up-regulated during VT differentiation in parallel with *CSH1*. **(E,F)** ZNF554 immunopositivity was faint in the syncytiotrophoblast in preeclampsia [**(F)**, GW35] compared to gestational-age matched controls [**(E)**, GW36] (hematoxylin counterstaining, 400× magnifications). Arrow and arrowhead depict syncytiotrophoblast and villous endothelium, respectively. **(G)** *ZNF554* mRNA expression was 74% lower in *ZNF554*-silenced BeWo cells compared to controls used for the microarrays ($p = 5.24 \times 10^{-6}$). **(H)** Nuclear and cytoplasmic ZNF554 immunofluorescence decreased in BeWo cells treated with *ZNF554* siRNA compared to control cells (3,500× magnifications). **(I)** Molecular functions and one Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway (glycolysis/gluconeogenesis) affected in *ZNF554*-silenced BeWo cells. Colors denote the proportions of up- or down-regulated genes (red: >0.5 up-regulated; blue:>0.5 down-regulated; black: 0.5–0.5 up- and down-regulated). Letter sizes represent the minus $\log_{10}$ of *p*-values of the given functions or pathway. **(J)** qRT-PCR validated *FSTL3* up-regulation (2.7-fold, $p < 0.001$) in BeWo cells upon *ZNF554* knock-down.

inactivates activin and other TGFβ ligands (249). It is involved in the regulation of EVT invasion (250, 251) and its placental up-regulation is associated with low BW in preeclampsia (252). This finding confirms that the dysregulation of *ZNF554* may have key downstream effects on the pathogenesis of preeclampsia.

The Effects of *ZNF554* Down-Regulation in EVTs

We supposed that *ZNF554* may also affect EVTs, since its expression in EVTs was detected in first and third trimester maternal decidua (**Figures 10A–D**), and ZNF554-positive intraluminal and endovascular trophoblasts were found in the wall of transformed spiral arteries (**Figure 10B**). In preterm preeclampsia, ZNF554 immunostaining of EVTs was weaker than in controls (**Figures 10C,D**). To characterize the loss of ZNF554 function in EVTs, we silenced *ZNF554* in trophoblastic HTR8/SVneo cells (253). At 87% knock-down ($p < 0.001$) (**Figure 10E**), we observed decreased nuclear and cytoplasmic ZNF554 immunostaining (**Figure 10F**). Microarray analysis

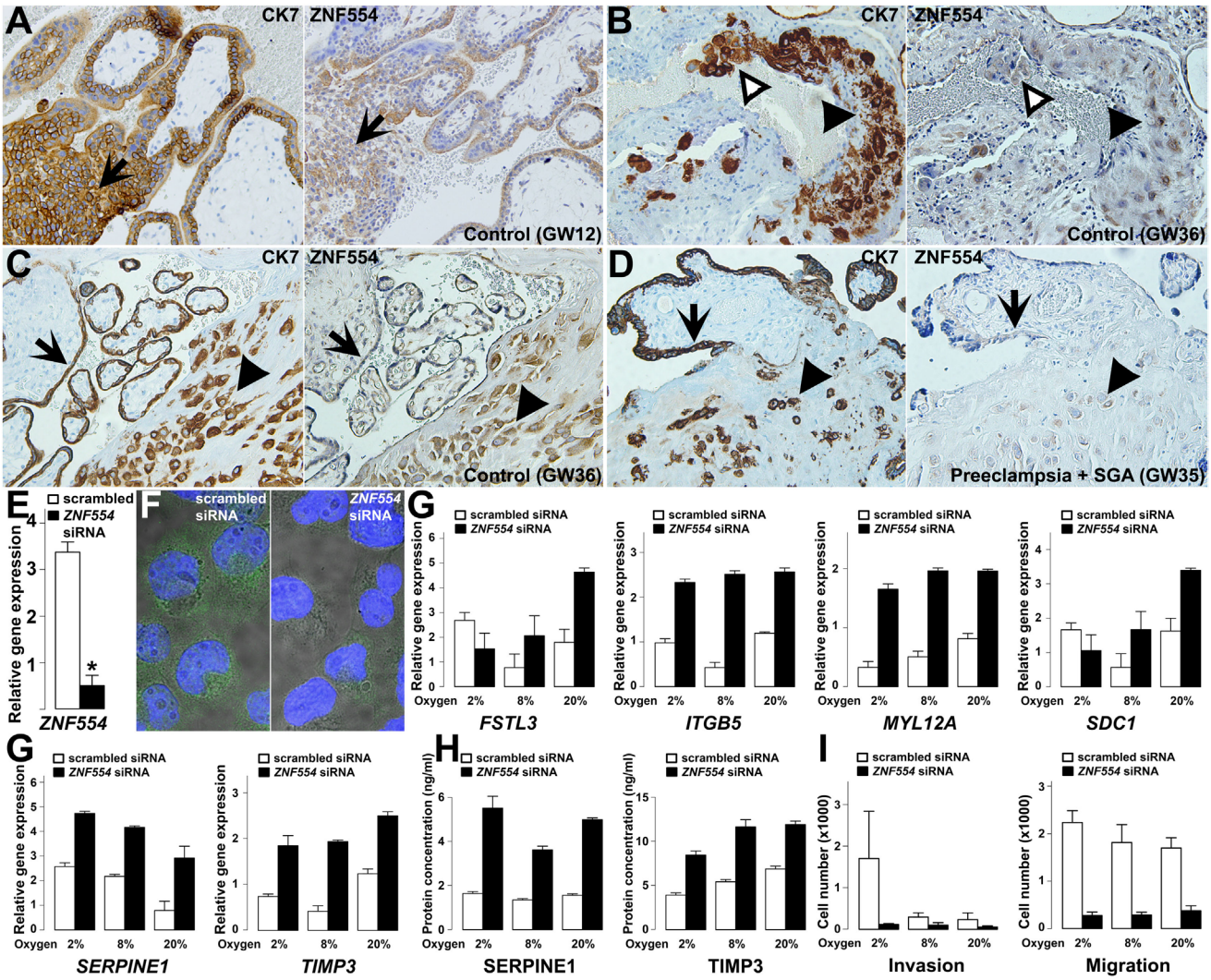


FIGURE 10 | *ZNF554* down-regulation in extravillous trophoblasts (EVTs) in preeclampsia. **(A)** EVTs in trophoblastic columns and **(B)** endovascular and intraluminal trophoblasts in the myometrium were immunostained for cytokeratin-7 (left) and ZNF554 (right). Arrows depict the direction of trophoblast invasion, black and white arrowheads point to EVTs in the wall and lumen of the spiral artery, respectively (serial sections, hematoxylin counterstaining, 200× magnifications). ZNF554 immunostaining in EVTs (arrowheads) and syncytiotrophoblast (arrows) was weaker in **(D)** preeclampsia than in **(C)** controls (serial sections, hematoxylin counterstaining, 200× magnifications). **(E)** *ZNF554* mRNA expression decreased to 13% upon knock-down ($p < 0.001$), **(F)** ZNF554 immunofluorescence was weaker in the nucleus and cytoplasm of *ZNF554*-silenced (left) than of control (right) HTR8/SVneo cells (3,500× magnifications). **(G)** The dysregulation of selected genes upon *ZNF554* knock-down was confirmed by qRT-PCR. **(H)** Plasminogen activator inhibitor-1 (PAI-1 / SERPINE1) and TIMP-3 proteins were increasingly secreted from *ZNF554*-silenced cells. **(I)** *ZNF554*-silenced cells had remarkably decreased invasive (left) and migratory (right) characteristics. O_2 concentrations are shown below the bars.

of *ZNF554*-silenced cells showed 185 DE genes (Data S15 in Supplementary Material) including 18 DE placental genes in preeclampsia. Gene ontology (GO) analysis revealed 16 molecular functions dysregulated, including "cyclin-dependent protein kinase regulator activity," "metalloendopeptidase inhibitor activity," and "insulin-like growth factor binding." The 67 enriched biological processes included "regulation of growth," "smooth muscle cell migration," "smooth muscle cell-matrix adhesion," and "response to oxygen levels," all relevant to trophoblast invasion and placental pathology of preeclampsia (Data S16 in Supplementary Material).

qRT-PCR confirmed the dysregulation of eight DE genes. Two genes (*CDKN1A*, *STK40*) are involved in the regulation of cell proliferation and differentiation (254, 255), and proliferation assays showed that *ZNF554* knock-down decreased cell proliferation in HTR8/SVneo cells slightly after 48 h (−14%, $p = 0.02$) (Figure S11 in Supplementary Material). Six genes (*FSTL3*, *ITGB5*, *MYL12A*, *SDC1*, *SERPINE1*, and *TIMP3*) encode proteins involved in cell adhesion, migration, invasion, and angiogenesis (**Figure 10G**). Since EVTs move through an environment with changing O_2 levels, we used O_2 concentrations for conditions relevant for endovascular (8%) and interstitial (2%) trophoblast invasion

besides standard cell cultures (20%). The effect of *ZNF554* knockdown was significant regardless of O_2 levels on four genes (*ITGB5*, *MYL12A*, *SERPINE1*, and *TIMP3*), while there was an interaction between O_2 levels and *ZNF554* silencing on two genes (*FSTL3* and *SDC1*) (**Figure 10G**).

The up-regulation of SERPINE1 (PAI-1) and tissue inhibitor of metalloproteinases-3 (TIMP-3) was also confirmed at the protein level in supernatants of *ZNF554*-silenced cells (**Figure 10H**). Both proteins have an inhibitory function on trophoblast migration and invasion (250, 256, 257), and TIMP-3 is the major tissue metalloproteinase inhibitor at the maternal–fetal interface, which is up-regulated in preeclampsia (106, 258, 259). These results suggested that *ZNF554*-silenced cells have reduced migratory and invasive functions. Indeed, functional assays revealed that *ZNF554* silencing had a strong inhibitory effect on trophoblast migration ($p = 1.9 \times 10^{-10}$) regardless of the O_2 concentration, and also on invasion ($p < 0.001$), especially at 2% O_2 concentration (**Figure 10I**). These data corroborated that *ZNF554* supports trophoblast invasion *via* modulating a set of key genes that are involved in this process.

DNA Methylation-Mediated Trophoblastic *ZNF554* Down-Regulation in Preeclampsia

We wondered whether placental *ZNF554* down-regulation might have an epigenetic background, given that the *ZNF554* flanking region contains several transposable elements, including several Alus, which are generally hypomethylated in germ cells and the placenta (260, 261), while their hypermethylation leading to altered gene expression may be detected in preeclampsia (262, 263). Thus, the down-regulation of *ZNF554* expression in the placenta of patients with preeclampsia may also be reflected in the DNA methylation of the transposable elements in its 5′ flanking region.

The treatment of BeWo cells with 5-azacitidine increased *ZNF554* expression, showing the role of DNA methylation in trophoblastic *ZNF554* regulation (**Figure 11A**). The subsequent search in the Human Reference Epigenome Mapping Project data revealed a DMR located in the AluY, which was hypomethylated in H1 embryonic stem cells and H1-derived trophoblasts compared to H1-derived neuronal cells (**Figure 11B**). This was of interest, since AluY is a retrotransposon evolved recently in primates, and its differential DNA methylation supports the expression of other gene transcripts in the placenta compared to somatic tissues (264). These data prompted us to investigate the DNA methylation in this genomic region in primary VTs and cord blood cells collected from the same normal pregnancies. In fact, bisulfite sequencing showed that the AluY, similarly to the AluSq2, is heavily methylated in cord blood cells compared to the hypomethylated trophoblast, suggesting its importance in the developmental regulation of *ZNF554* expression (**Figure 11B**; Figure S12 in Supplementary Material).

Further, we tested DNA methylation in this region in 100 placentas with qRT-PCR data, including patients with preeclampsia

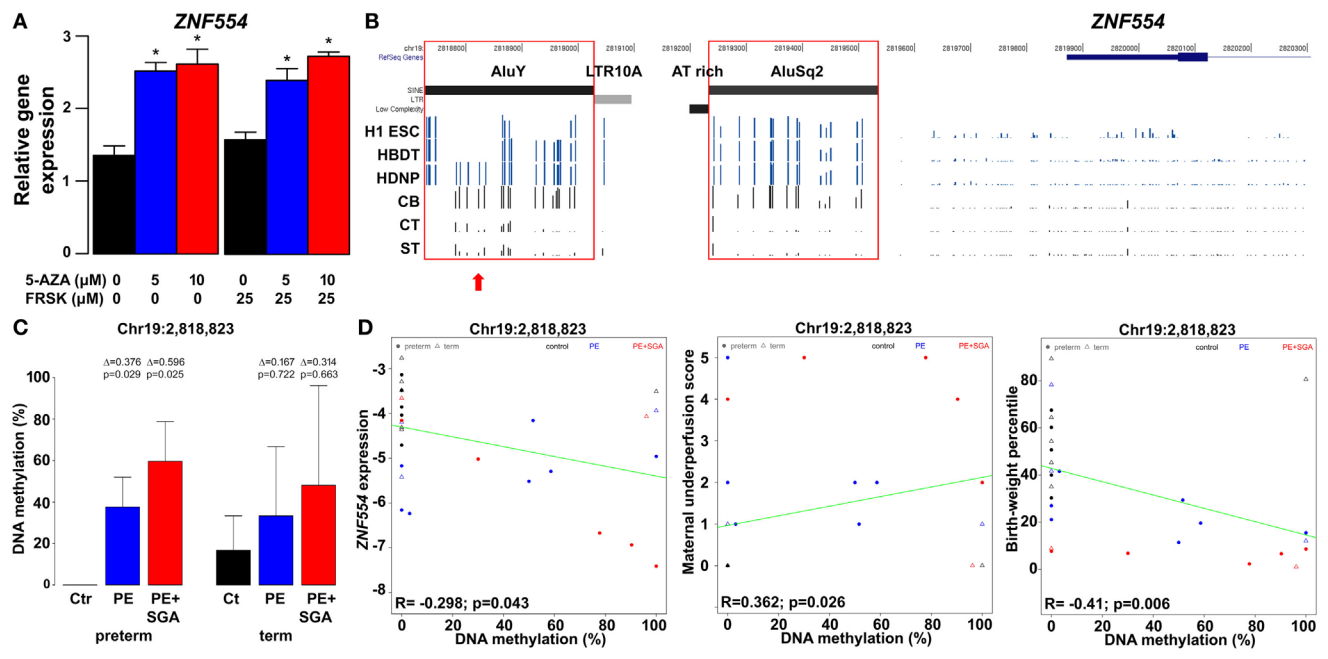


FIGURE 11 | The effect of trophoblastic DNA methylation on *ZNF554* expression and clinical parameters in preeclampsia. **(A)** Increased *ZNF554* expression in BeWo cells upon treatment with 5-azacitidine (5-AZA) irrespective of forskolin (FRSK) co-treatment. **(B)** *Upper three lanes*: whole genome bisulfite sequencing data of *ZNF554* 5′ flanking region from the Human Reference Epigenome Mapping Project. H1 ESC, H1 embryonic stem cell; HBDT, H1 BMP4-derived trophoblast; and HDNP, H1-derived neuronal progenitor. *Lower three lanes:* bisulfite sequencing data from this study. Abbreviations: CB, cord blood cell; CT, cytotrophoblast; ST, syncytiotrophoblast. *Red boxes:* AluY and AluSq2 are heavily methylated in cord blood cells and hypomethylated in trophoblasts. *Red arrow:* CpG Chr19:2,818,823. **(C)** CpG Chr19:2,818,823 was differentially methylated in trophoblasts in preterm preeclampsia ± SGA. **(D)** Correlation was observed between Chr19:2,818,823 CpG methylation and *ZNF554* expression, maternal vascular malperfusion score of the placenta, and birthweight percentile.

after laser capture of the VTs. Bisulfite sequencing of the trophoblastic DNA revealed four CpGs on AluY (Chr19:2,818,823, Chr19:2,818,864, Chr19:2,818,868, and Chr19:2,818,876) hypomethylated in controls but hypermethylated in preterm preeclampsia, with highest methylation in cases associated with SGA (**Figure 11C**; Figure S13 in Supplementary Material). Importantly, we found correlations between Chr19:2,818,823 CpG methylation and *ZNF554* expression ($R = -0.30$, $p = 0.04$), maternal vascular underperfusion score of the placenta ($R = 0.36$, $p = 0.03$), and BW percentile ($R = -0.41$, $p < 0.01$) (**Figure 11D**). These data collectively provide evidence that the hypermethylation of certain CpGs in AluY in the trophoblast may result in low *ZNF554* expression, impaired trophoblast invasion, preeclampsia, and fetal growth restriction.

DISCUSSION

The placenta has a key role in the pathogenesis of the terminal pathway of preeclampsia, which may be triggered by discrete disease pathways at early stages of pregnancy, leading to the development of different preeclampsia phenotypes. In this study, an integrated systems biology approach was employed to gain insights into these complex pathways, given that this strategy offered the ultimate analytic solution to investigate and understand the complex disease pathways of the syndrome of preeclampsia (265–268). We incorporated "omics," clinical, placental, and functional data from patients with distinct phenotypes of preeclampsia. We employed molecular network-based approaches to identify networks and modules of genes or proteins that are perturbed in the placenta and the maternal circulation of women with preeclampsia.

Our placental transcriptomics study identified 1,409 DE genes involved in many biological processes (e.g. BP regulation, apoptosis, development, hormone secretion, metabolism, and signaling) that were previously implicated in the pathogenesis of preterm preeclampsia by other placental transcriptomics studies (105, 110, 111, 113, 114, 116, 117, 122, 123, 125, 127, 128, 130, 131, 134, 137, 269–272). Despite the differences in patient populations, design, or methodologies between these studies, many DE genes on our list have also been found by other groups. Indeed, from the 40-gene meta-signature that characterized the significant intersection of DE genes from independent placental gene signatures in preeclampsia in the meta-analysis of Kleinrouweler et al. (135), our microarray and qRT-PCR studies found 26 (65%) to be DE in preterm preeclampsia. Of note, 16 of these 26 genes belong to module M2, while only six to the M1 module. This supports our observation that the dysregulation of module M2 is associated with BP elevation, the maternal disease condition required for patient inclusion into these studies. The weaker involvement of module M1 genes in the meta-signature may reflect the heterogeneity of preeclampsia transcriptomics studies regarding fetal (growth restriction) and placental disease conditions. Our microarray study was homogeneous for preterm preeclampsia cases with low BW and placental disease, while only a couple of other studies had this rigor. To overcome the inconsistency of smaller placental transcriptomics studies, Leavey et al. (140, 142) employed advanced bioinformatics methods to aggregate microarray datasets across multiple platforms to generate large datasets of patient samples. Unsupervised clustering of these datasets revealed three distinct molecular subclasses of preeclampsia. Among these, the "canonical" subclass, which is associated with the differential expression of our module M2 genes, was characteristic for preterm preeclampsia and consistent with stress response to poor oxygenation, further supporting our findings. However, our discovery on the two major dysregulated placental disease gene modules and their hub transcription regulatory genes, separately associated with maternal or fetal disease pathways, are novel (**Figure 12**).

Functional assays on hub transcription factors of these two disease gene modules demonstrated that *ZNF554* (M1) down-regulation leads to impaired trophoblast invasion, while *BCL6* and *ARNT2* (M2) overexpression sensitizes the trophoblast to ischemia, which are hallmarks in the pathogenesis of preterm preeclampsia. In the "ZNF554" pathway, hypermethylation of AluY in the *ZNF554 5′* flanking region inhibits gene expression, leading to impaired trophoblast invasion, placental vascular malperfusion, and low BW. In the "BCL6-ARNT2" pathway, which is activated only in preterm preeclampsia, ischemic stress of the trophoblast coupled with *BCL6* and *ARNT2* overexpression increases *FLT1* expression. This then eventually promotes the anti-angiogenic state, hypertension (37, 56, 57, 61, 62, 83), and the early onset of this syndrome (Figure S7 in Supplementary Material).

The perturbed placental disease gene modules in preterm preeclampsia can be detected by liquid biopsy in maternal blood in the early stages of pregnancy. Indeed, we detected the down-regulation of M1 and M2 disease gene module biomarkers in these patients during the first trimester. Of interest, the up-regulation of module M2 biomarkers can be detected after the establishment of maternal circulation in the intervillous space. These findings support the observation that placental transcriptomic changes, typical for preterm preeclampsia observed in the third trimester, are rooted in the first trimester. The positive correlation of gene expression in module M1 with BW and in module M2 with BP suggests that M1 genes may be biomarkers for placental and fetal growth and development while M2 genes can serve as biomarkers for placental stress.

First trimester maternal blood proteomics uncovered the altered abundance of proteins of the renin–angiotensin and immune systems as well as complement and coagulation cascades in patients who subsequently developed both preterm and term preeclampsia. The same proteins and pathways were found to be dysregulated in maternal blood in later stages of preeclampsia by other proteomics studies (108, 115, 118–120, 126, 129, 133, 136, 138, 139, 143); however, ours is the first revealing their dysregulation at an earlier stage of pregnancy when there is no or minimal direct connection between the placenta and the maternal circulation (273).

From these dysregulated maternal serum proteins, *in silico* analysis pointed to candidates, which may drive trophoblastic transcriptomic changes, and corroborated earlier findings on angiotensinogen/angiotensin II in driving hypertension indirectly through *FLT1* up-regulation in addition to its direct effects (91, 274). Moreover, *in vitro* functional assays revealed that altered maternal serum proteome in the first trimester can affect the trophoblastic transcriptome and up-regulate *FLT1*.

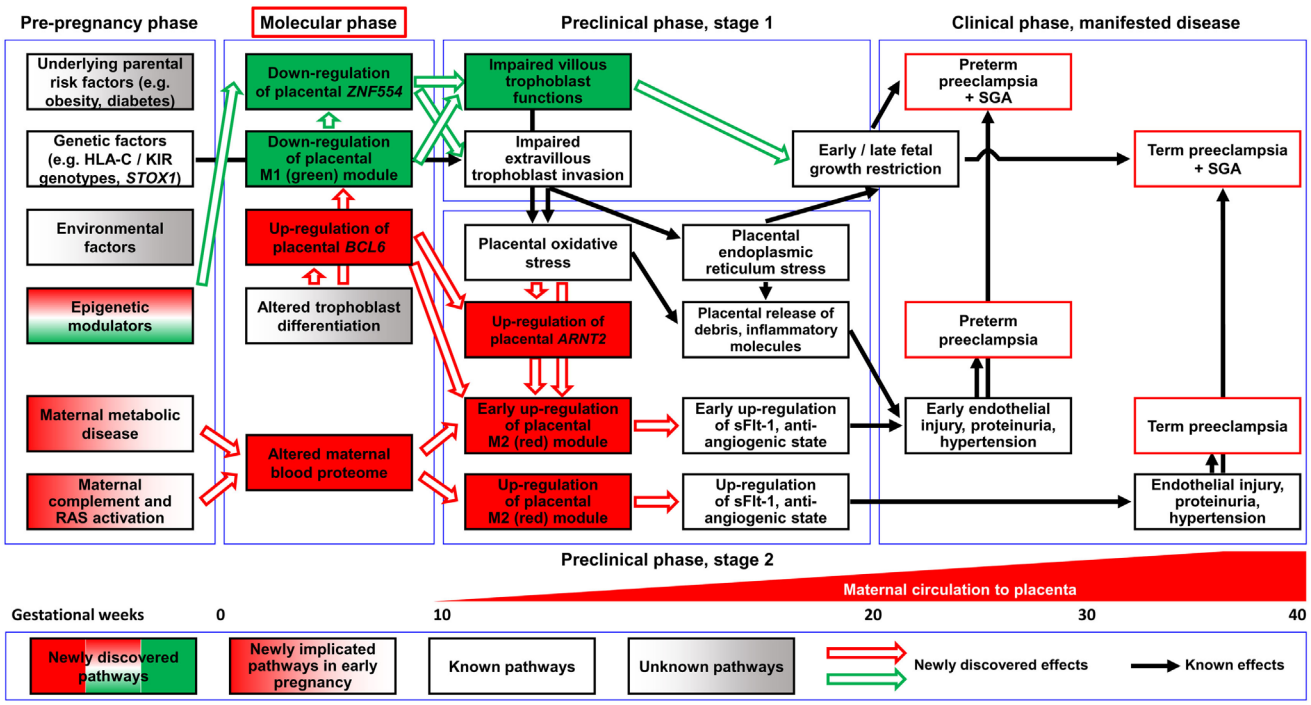


FIGURE 12 | Pathologic pathways in preeclampsia. *Maternal pathways:* alterations in the maternal blood proteome, including systemic inflammatory changes, can be observed in both preterm and term preeclampsia before the maternal circulation of the placenta has been established, supporting the observation that maternal factors have a key role in triggering early disease pathways. Later, these alterations can induce trophoblastic functional changes leading to the up-regulation of module M2 genes, the overproduction of sFlt-1, and an anti-angiogenic state through a trajectory that does not necessarily affect fetal growth. *Placental pathways:* altered differentiation of the trophoblast leads to the dysregulation of module M1 genes and hub factors in module M2. The down-regulation of *ZNF554* and module M1 genes involved in the regulation of fetal growth and metabolism imply impaired villous trophoblast (VT) functions besides abnormal extravillous trophoblast (EVT) invasion. The up-regulation of the *BCL6-ARNT2* pathway sensitizes the trophoblast to ischemia and increases *FLT1* expression after the maternal circulation to the placenta has been established. These changes are observed only in preterm preeclampsia, suggesting that this placental pathway promotes the early development of preeclampsia. The interplay of these molecular pathways leads to the complex pathogenesis of preeclampsia. Abbreviations: RAS, renin-angiotensin system; SGA, small-for-gestational age.

This is in agreement with reports indicating that maternal blood factors in preeclampsia can induce trophoblastic soluble endoglin overexpression and the development of preeclampsia-like symptoms in mice (85, 275).

Remarkably, most of the dysregulated maternal serum proteins in the first trimester in both preterm (11 of 19) and term (7 of 14) preeclampsia are implicated in immune functions (**Figure 6**; Figure S6 in Supplementary Material), suggesting a critical role for immune pathways and inflammation in the early pathogenesis of both phenotypes of preeclampsia. This is consistent with clinical, epidemiological, and immunological evidence showing that: (1) preeclampsia has multiple risk factors (e.g. dyslipidemia, hypertension, insulin resistance, and obesity) characterized by heightened inflammation (29, 276); (2) the combination of inhibitory decidual NK (dNK) cell killer immunoglobulin-like receptor and the fetal HLA-C2 genotype increases the susceptibility to preeclampsia due to the loss of activating interactions between trophoblasts and dNK cells at early stages of placentation (168, 277–283); (3) an altered local immune regulation and a shift toward the pro-inflammatory macrophage phenotype promotes a pro-inflammatory milieu in the maternal decidua in preeclampsia (284, 285); (4) an imbalance between Th1/Th2/Th17/Treg cells in preeclampsia leads to failure of maternal–fetal tolerance mechanisms (286–289); and (5) complement system activation in preeclampsia leads to the activation of innate immune cells and placental damage (101, 234, 236, 237, 290–292). The role of inflammation in early preeclampsia disease pathways is also supported by *in vivo* studies showing that bacterial endotoxin administration to pregnant rats induces placentation defects and symptoms consistent with preeclampsia (293–298).

Overall, our data show that there are distinct maternal and placental disease pathways, and their interaction influences the clinical presentation of preeclampsia. The activation of maternal disease pathways can be detected in both preterm and term preeclampsia earlier and upstream of placental dysfunction, not only downstream as described before (43), and distinct placental disease pathways are superimposed on these maternal pathways. This is a paradigm shift in our understanding of preeclampsia, which in agreement with epidemiological studies (25, 31) warrants for the central pathologic role of preexisting maternal diseases or perturbed maternal–fetal–placental immune interactions in preeclampsia.

The superimposed placental disease pathways differ between preterm and term preeclampsia. For preterm preeclampsia, our functional data suggest that placental disease pathways

are partly originated from altered trophoblast differentiation, which is followed by trophoblastic stress, induced by perturbed maternal blood proteome factors and/or ischemia after the onset of maternal circulation to the placenta. Our data are consistent with recent views indicating that defects in trophoblast proliferation, differentiation, invasion, and plugging are associated with defective decidualization (299), decidual inflammation (300), and the disturbance in endometrial-trophoblast dialog during the peri-conception (301) period. Abnormal trophoblast invasion and plugging will subsequently lead to the aberrant onset of maternal circulation (273) and malperfusion, causing placental oxidative stress in preterm preeclampsia (175, 273, 302). On the other hand, maternal disease pathways induce mainly placental dysfunction without maldevelopment in term preeclampsia. This is substantiated by the differences observed in the maternal proteome, placental transcriptome, and trophoblastic DNA methylation between term and preterm preeclampsia in our study. Moreover, this is also consistent with the major differences between these two preeclampsia phenotypes in etiology (31), placental histopathology (28, 33, 44–46), and stress levels (303) as well as clinical presentation (2, 25, 26, 29, 304).

Our findings are very timely in the light of recent clinical research showing that the administration of aspirin before 16 weeks of gestation to pregnant women at risk for preeclampsia prevents the preterm phenotype of this syndrome (24, 305–309). Thus, the anti-inflammatory and anti-platelet actions of aspirin (308–311) may ameliorate the early pro-inflammatory disease pathways leading to placental maldevelopment in preterm preeclampsia. Based on our discovery of these novel disease pathways and their hub molecules, we propose a "molecular phase" of preeclampsia (**Figure 12**), where early pathologic events can already be detected by maternal blood biomarkers, offering non-invasive diagnostics of maternal and placental disease pathways. Biomarkers of these disease pathways may open new venues for the molecular characterization of patients destined to develop preeclampsia, using multi-biomarker profiles that support preventive approaches for patients with distinct preeclampsia phenotypes.

MATERIALS AND METHODS

Placental Study

Placental Microarray Study

Study Groups and Clinical Definitions

Placental tissue and maternal blood samples were collected from Caucasian women at the First Department of Obstetrics and Gynaecology, Semmelweis University in Budapest, Hungary as described previously (132). Pregnancies were dated according to ultrasound scans between 8 and 12 weeks of gestation. Patients with multiple pregnancies or fetuses having congenital or chromosomal abnormalities were excluded. The collection and investigation of human clinical samples were approved by the Health Science Board of Hungary. Written informed consent was obtained from women prior to sample collection, and the experiments conformed to the principles set out in the World Medical Association Declaration of Helsinki and the Department of Health and Human Services Belmont Report. Specimens and data were stored anonymously.

Women were enrolled in the following groups: (1) preterm severe preeclampsia, with or without HELLP (hemolysis, elevated liver enzymes, low platelets) syndrome ($n = 12$) and (2) preterm controls (n = 5) (**Table 1**). Preeclampsia was defined according to the criteria set by the American College of Obstetricians and Gynecologists (1), and subdivided into preterm (<37 weeks) or term (≥37 weeks) groups. Severe preeclampsia was defined according to Sibai et al. (2). Preterm controls had no medical complications, clinical or histological signs of chorioamnionitis, and delivered neonates with a BW appropriate-for-gestational age (AGA) (312). SGA was defined as neonatal BW below the 10th percentile for gestational age. Cesarean delivery was performed in all cases due to severe symptoms as well as in all controls due to previous Cesarean delivery or malpresentation.

Placental Tissue and Maternal Blood Collection

Placental tissue specimens were processed immediately after delivery as described previously (132). For the microarray study, 1 × 1 cm villous tissue samples were excised from central cotyledons close to the umbilical cord to reduce the possible bias due to regional differences in gene expression (313, 314). These tissue blocks were then dissected from the choriodecidua on dry ice, snap-frozen, and stored at −80°C. For histopathologic evaluations, five representative tissue blocks were taken from each placenta to include central and peripheral cotyledons and the maternal side of the placenta with the fetal membranes. These blocks were embedded in paraffin after fixation in 10% neutral-buffered formalin (FFPE). Maternal blood samples were obtained at the time of admission into the delivery room; aliquots of maternal sera and plasma were stored at −80°C.

Histopathologic Evaluation of the Placentas

Placental specimens were examined according to a standard protocol, describing the topography and size of macroscopic lesions. Four micrometer sections were cut from the five FFPE blocks and mounted on SuperFrost/Plus slides (Fisherbrand, UK). After deparaffinization, slides were rehydrated, stained with hematoxylin and eosin, and examined in 10 randomly chosen microscopic fields using bright-field light microscopy by a pathologist blinded to the clinical information. Macroscopic and microscopic lesions were defined according to published criteria (315–317).

Placental Total RNA Isolation and Microarray Experiments

Tissues were homogenized using a ThermoSavant FastPrep FP120 Homogenizer (Thermo Scientific, Wilmington, DE, USA) with Lysing MatrixD (MP Biomedicals, Illkirch, France). Total RNA was isolated using RNeasy Fibrous Tissue Mini Kit (QIAGEN GmbH, Hilden, Germany), quantified with NanoDrop 1000 (Thermo Scientific), and assessed by Agilent 2100 Bioanalyzer (Matriks AS, Oslo, Norway). Total RNAs were labeled, and Cy3-RNAs were fragmented and hybridized to the Whole Human Genome Oligo Microarray G4112A (Agilent Technologies, Santa Clara, CA, USA) on an Agilent scanner, and processed with Agilent Feature Extraction software v9.5 according to the manufacturer's guidelines.

Data Analysis

Demographics data were compared by the Fisher's exact test and Mann–Whitney test using SPSS version 12.0 (SPSS Inc., Chicago, IL, USA). Microarray data analysis was performed using the R statistical language and environment[1] following the MIAME guidelines and methodologies described previously (318). Microarray expression intensities were background corrected using the "minimum" method in the "backgroundCorrect" function of the "limma" package (319). After $\log_2$ transformation, data were quantile-normalized. From the 41,093 probesets on the array, 93 were removed before differential expression analysis, lacking annotation in the array definition file (Agilent Technologies). Subsequently, an expression filter was applied to retain probesets with intensity greater than $\log_2(50)$ in at least two samples, yielding a final matrix of 30,027 probesets (15,939 unique genes). Differential gene expression was assessed using linear models including adjustment for batch effects, with coefficient evaluation *via* moderated *t*-tests. *P*-values were adjusted using the false discovery rate (FDR) method and Benjamini–Hochberg correction to compute *q*-values. Since there were no differences among patient groups in maternal age or ethnic background, we did not adjust for these parameters. Target gene Entrez IDs for the probesets were determined using the R package "hgu4112a.db." For probesets without annotation in the package, Entrez IDs were taken from the array definition file (Agilent Technologies). Probesets remaining un-annotated (without Entrez ID and/or gene symbol) were removed from further analysis. Probesets were defined as DE ($n = 1{,}409$) if they had a $q \leq 0.2$ and a fold-change $\geq$1.5 (Data S1 in Supplementary Material).

From the DE genes in preeclampsia, those encoding for proteins with functions in transcription regulation ($n = 137$) were identified using the Metacore (GeneGo Inc., Saint Joseph, MI, USA) and GeneCards v3[2] databases.

We downloaded the human U133A/GNF1H microarray data on 79 human tissues, cells, and cell lines from the Symatlas/BioGPS database (151) to identify human genes with predominant placental expression. A probeset was defined as having predominant placental expression if its placental expression was (1) $\geq$1,000 fluorescence units; (2) six times higher than the median value in 78 other tissue and cell sources; and (3) two times higher than its expression in the tissue with the second highest expression. The resulting 215 probesets corresponded to 153 unique genes. Eleven additional genes that were not present on the microarray platform used by Symatlas/BioGPS (Affymetrix, Santa Clara, CA, USA) were added to this list based on previously published evidence of placenta-specific expression (151–153) (Data S2 in Supplementary Material). Of the 164 predominantly placenta-expressed genes, 157 were present in our placental microarray data. These genes were tested for enrichment in DE genes compared to all genes on the array (1,409 of 15,939) using the Fisher's exact test.

The expression levels of DE genes in EVT compared to VT lineages were analyzed by retrieving published microarray datasets (320–322) and reanalyzing expression data. Raw Affymetrix GeneChip Human Genome U133A 2.0 Array data from Bilban et al. (320) and Tilburgs et al. (322) was downloaded from GEO (GSE9773) and ArrayExpress (E-MATB-3217) respectively, and was processed with the *"affy"* (323) and *"limma"* (324) packages of Bioconductor.[3] After RMA normalization, log-fold changes were calculated. Processed Illumina Human HT-12 V3 BeadArray data from Apps et al. (321) was downloaded from ArrayExpress (E-MATB-429), and then log-fold changes were calculated.

Chromosomal locations for all genes tested on the Agilent array were obtained from the R package "org.Hs.eg.db." Of the 15,939 unique and 1,409 DE genes on the array, 15,935 and 1,408 could be assigned to chromosomes, respectively. Mapping the microarray probesets on the Affymetrix human U133A/GNF1H chips to ENTREZ identifiers was performed using the Bioconductor "hgu133a.db" and "hgfocus.db" packages (325, 326). Chromosomal locations of the resulting list of genes were obtained from the package "org.Hs.eg.db" and from the National Center for Biotechnology Information for the 11 additional genes (327). Enrichment analyses for chromosomes among predominantly placenta-expressed genes, DE genes and DE genes encoding for transcriptional regulators (Data S3–S5 in Supplementary Material) were tested by the Fisher's exact test. Chromosomal locations of these genes were visualized by Circos (328).

Weighted gene co-expression network analysis (329, 330) was applied on the 1,409 DE genes across 17 samples to identify distinct regulatory modules and prioritize candidate genes for qRT-PCR validation. A gene pair-wise similarity (absolute Pearson correlation) matrix was first computed, then soft-thresholded by raising it to the power of 10 (chosen based on the scale-free topology criterion) to obtain an adjacency matrix. The topology overlap matrix (TOM) was then derived from the adjacency matrix. The topology overlap (331) measures the node interconnectedness within a network and was generalized to WGCNA (329). This measure defines the similarity between the two genes based on both correlations within themselves and outside other genes. Gene distance matrix was defined as 1-TOM and used for average linkage hierarchical clustering. A hybrid dynamic tree-cutting method (332) was applied to obtain modules (tree clusters).

Gene modules identified with this approach were further tested for enrichment in DE genes using the Fisher's exact test. Transcription regulatory genes expressed at high levels (average $\log_2$ intensity >9) and co-expressed (absolute Pearson coefficient >0.7) with the most genes among DE genes were treated as candidates for hub genes in the module. Hub genes then were selected based on the number and strength of their Pearson co-expression partners as well as their and their networks' biological activities. The networks of biological processes enriched among genes co-expressed with hub factors modules were created by BINGO and visualized with Cytoscape.

Enrichment analysis of transposable elements present in the 10,000 bp upstream region of DE genes was performed separately for the M1 (green) and the M2 (red) modules in preeclampsia versus all genes present on the microarray using the Fisher's exact

[1] www.r-project.org.

[2] www.genecards.org.

[3] www.bioconductor.org.

test. The locations of transposable elements and their families and classes were obtained from the "RepeatMasker" table of the UCSC Table Browser.[4] *P*-values <0.05 were considered significant.

Placental Validation Study

Study Groups and Clinical Definitions

Third trimester placentas ($n = 100$) collected predominantly from African-American women were retrieved from the Bank of Biological Specimens of the Perinatology Research Branch (Detroit, MI, USA). Pregnancies were dated according to ultrasound scans between 8 and 12 weeks. Patients with multiple pregnancies or fetuses having congenital or chromosomal abnormalities were excluded. The use of biological specimens and clinical data for research purposes was approved by the Wayne State University Human Investigation Committee and the Institutional Review Board of the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development (NICHD, NIH, DHHS). Written informed consent was obtained from women prior to sample collection, and the experiments conformed to the principles set out in the World Medical Association Declaration of Helsinki and the Department of Health and Human Services Belmont Report. Specimens and data were stored anonymously.

The following homogenous patient groups were selected from a cohort: (1) preterm severe preeclampsia ($n = 20$); (2) preterm severe preeclampsia associated with SGA ($n = 20$); (3) preterm controls ($n = 20$); (4) term severe preeclampsia ($n = 10$); (5) term severe preeclampsia associated with SGA ($n = 10$); and (6) term controls ($n = 20$). Term controls, consisting of normal pregnant women with ($n = 10$) or without ($n = 10$) labor, and preterm controls with preterm labor and delivery had no medical complications or clinical or histological signs of chorioamnionitis, and delivered an AGA neonate (333) (**Table 2**; Figure S2 in Supplementary Material).

Labor was defined by the presence of regular uterine contractions at a frequency of at least two contractions every 10 min with cervical changes resulting in delivery (334). Preeclampsia was defined according to the criteria set by the American College of Obstetricians and Gynecologists (1) and was subdivided into preterm (<37 weeks) or term (≥37 weeks) groups. Severe preeclampsia was defined according to Sibai et al. (2). SGA was defined as neonatal BW below the 10th percentile for gestational age (333). Cesarean delivery was performed in cases due to severe symptoms and in controls due to previous Cesarean delivery or malpresentation.

Placental Tissue Collection

Third trimester placental tissue specimens ($n = 100$) were processed immediately after delivery. For histopathologic evaluations, representative tissue blocks were taken from each placenta to include central and peripheral cotyledons, maternal side of the placenta, fetal membranes, and the umbilical cord. These blocks were embedded in paraffin after fixation in 10% neutral-buffered formalin (FFPE). Villous tissue blocks from central cotyledons were selected for TMA. For gene expression studies, systematic random sampling (335) was used to obtain villous tissues to reduce the possible bias due to regional gene expression differences (313, 314). Excised tissue blocks were homogenized and mixed in TRIzol reagent (Life Technologies), snap-frozen with liquid N_2, and stored at −80°C.

Histopathologic Evaluation of the Placentas

Placental specimens ($n = 100$) were examined according to a standard protocol, describing the topography and size of macroscopic lesions. Five μm sections were cut from the representative FFPE tissue blocks, stained with hematoxylin and eosin, and examined using bright-field light microscopy by two anatomic pathologists blinded to the clinical information. Histopathologic changes of the placenta were defined according to published criteria proposed by the Perinatal Section of the Society for Pediatric Pathology (336). "Maternal vascular malperfusion score" was calculated by summing the number of pathologic lesions consistent with this lesion category (46, 336) present in a given placenta.

Placental Total RNA Isolation and qRT-PCR

Total RNA was isolated from snap-frozen third trimester placental villous tissues ($n = 100$) with TRIzol reagent (Life Technologies) and RNeasy kit (QIAGEN, Valencia, CA, USA) according to the manufacturers' recommendations. The 28S/18S ratios and the RNA integrity numbers were assessed using an Agilent Bioanalyzer 2100, and RNA concentrations were measured with NanoDrop 1000. Total RNA (500 ng) was reverse transcribed with the High Capacity cDNA Reverse Transcription Kit using random hexamers (Applied Biosystems, Foster City, CA, USA). TaqMan Assays (Applied Biosystems; **Table 3**, Data S17 in Supplementary Material) were used for high-throughput gene expression profiling on the Biomark qRT-PCR system (Fluidigm, San Francisco, CA, USA) according to the manufacturers' instructions.

Placental TMA Construction, Immunohistochemistry, and Immunoscoring

Tissue microarrays were constructed from central tissue blocks of third trimester FFPE placentas ($n = 100$) as described earlier (337). Briefly, three 20 × 35 mm recipient blocks were made of Paraplast X-Tra tissue embedding media (Fisher Scientific, Pittsburgh, PA, USA). One mm diameter cores from tissue blocks were transferred in triplicate into recipient paraffin blocks using an automated tissue arrayer (Beecher Instruments, Inc., Silver Spring, MD, USA). Five μm sections cut from TMAs were placed on silanized slides and stained with antibodies and reagents (Data S18 in Supplementary Material) either on Ventana Discovery or Leica BOND-MAX autostainers.

Tissue microarray immunostainings were semi-quantitatively scored by three examiners blinded to the clinical information with an immunoreactive score modified from a previously published one (154). Immunostaining intensity was graded as follows: 0 = negative, 1 = weak, 2 = intermediate, and 3 = strong. All villi in a random field of each of the three cores were evaluated by all examiners, and scores within each core were averaged to represent the target protein quantity of that core. Thus, each

[4]http://genome.ucsc.edu/.

placenta had three scores corresponding to the three cores examined.

Data Analysis

Demographics data were compared by the Fisher's exact test and Mann–Whitney test using SPSS version 12.0 (SPSS). All other data were analyzed in the R statistical environment (see text footnote 1).

Placental qRT-PCR. Data were analyzed using the ΔΔCt method. Data were first normalized to the reference gene (*RPLP0*), and the batch effect was adjusted through calibrator samples. Log_2 mRNA relative concentrations were obtained for each sample as $-\Delta Ct_{(gene)} = Ct_{(RPLPO)} - Ct_{(gene)}$. The surrogate gene expression values ($-\Delta Ct_{gene}$) were used to perform a hierarchical clustering with 1-Pearson correlation distance and average linkage. Between-group comparisons (in which groups were predefined based on the clinical characteristics of the patients) were performed by fitting a linear model on −ΔCt values, using the group variable indicator and the maturity status of the fetus (term vs. preterm) as covariates while allowing for an interaction effect (**Figure 4**; Figure S3 in Supplementary Material).

Histopathology. The association between qRT-PCR gene expression and the "maternal vascular malperfusion" score was tested using a linear model. *P*-values of <0.05 were considered significant.

TMA Immunoscoring. Group comparisons using immunoscores were conducted in the same way as for the qRT-PCR data (**Figure 4**).

Correlation of Clinical Parameters and Placental Gene Expression

Data Analysis

To reveal whether the expression of any gene on the microarray was correlated with MAP while controlling for BW, a linear model (y~MAP + BW + Batch) was fit for every gene on the array, in which y represents gene expression, and the dependent variables represent MAP, BW, and batch, respectively. A moderated *t*-test was used to obtain *p*-values, which then were adjusted using the FDR method and Benjamini–Hochberg correction for multiple testing. Significance was determined using a $q \leq 0.2$. Gene modules were also tested for the enrichment in genes with their expression correlated with MAP using the Fisher's exact test (Data S6 in Supplementary Material).

In the qRT-PCR validation study, we extended our analysis to include all 100 patients to test for the association between gene expression and mean arterial BP as well as BW percentile while adjusting for gestational age. All variables were treated as continuous. *P*-values of <0.05 were considered significant (**Figure 4**).

Genomic DNA Methylation Analysis of the Trophoblast

Laser Capture Microdissection

Fifteen µm sections were cut from snap-frozen placentas that were also used for qRT-PCR expression profiling ($n = 100$) on 2 µm Glass Foiled PEN slides (Leica Microsystems). The trophoblast layer of 300–350 villi in each specimen was laser captured by a Leica DM6000B microscope (Leica Microsystems) into 0.5 ml microcentrifuge tubes. The captured material was digested with Proteinase K (PicoPure DNA Extraction Kit, Applied Biosystems) at 56°C by overnight incubation. Digestions were stopped at 95°C, and samples were stored at −70°C until DNA isolation.

Genomic DNA Isolation

Genomic DNA was isolated from laser captured VTs ($n = 100$), from primary VTs ($n = 3$) collected for functional studies described below, and from respective umbilical cord blood leukocytes ($n = 3$) taken from the same pregnancies. The EZ1 Advanced Nucleic Acid Isolation System using EZ1 DNA Tissue and EZ1 DNA Blood Kits (QIAGEN) were utilized for DNA isolation, and DNA samples were quantified with Quantifiler Human DNA Quantification Kit (Applied Biosystems) according to the manufacturers' instructions.

Primer Design and Validation

The methylation status of CpGs in human H1 embryonic stem cells, H1-derived trophoblast cultured cells, and H1-derived neuronal progenitor cultured cells obtained by whole-genome shotgun bisulfite sequencing (University of California, San Diego, USA; UCSD Human Reference Epigenome Mapping Project) were visualized by the Epigenome Browser[5] and used for the selection of regions of interest. Primer design, targeted amplification, and sequencing were conducted using the targeted sequencing service protocol of Zymo Research Corporation. For targeted bisulfite sequencing, 30 primer pairs were designed and validated. Primers were synthesized by Integrated DNA Technologies, Inc., (Coralville, IA, USA) and underwent quality control, which included duplicate testing for specific amplification of 1 ng bisulfite DNA using bisulfite converted human DNA. Quality control criteria included robust and specific amplification (Cp values <40 cycles and CV <10% for duplicates) of the bisulfite primers on a LightCycler 480 real-time qRT-PCR instrument (Roche Diagnostics Corp. Indianapolis, IN, USA).

Bisulfite Conversion, Multiplex Amplification, Bar-Coding and Adapterization PCR, and Next-Generation Sequencing

Genomic DNA samples from laser captured VTs, primary VTs, and umbilical cord blood cells as well as control samples were subjected to sodium bisulfite treatment using the EZ DNA Methylation-Direct Kit (Zymo Research Corporation). For non-methylated control, human DNA was extracted and purified with Quick-gDNA Miniprep Kit (Zymo Research Corporation) from the HCT116 cell line (American Type Culture Collection, Manassas, VA, USA), which is double knock-out for both DNA methyltransferases DNMT1 (−/−) and DNMT3b (−/−), and thus contains a low level (<5%) of DNA methylation. For methylated control, human DNA was purified similarly from the HCT116 cell line and was enzymatically methylated at all cytosine positions comprising CG dinucleotides by CpG Methylase (Zymo Research Corporation).

[5] www.epigenomebrowser.org.

Bisulfite-treated samples and 30 validated primer pairs were subjected to targeted amplification on the 48.48 Access Array System (Fluidigm), using the targeted sequencing service protocol of Zymo Research Corporation. Fluidigm's protocols were used for sample loading, harvesting, and pooling, 1:100 dilution of amplicon pools for each sample, and for amplification using barcoded adapter-linkers (Fluidigm). Reactions were cleaned up using the DNA Clean and Concentrator-5 (Zymo Research Corporation), and products were normalized by concentration and pooled. The sequencing library was denatured, diluted, and sequenced with a 150-base paired-end run on the MiSeq Benchtop Sequencer (Illumina) according to Illumina's protocols.

Sequence Alignment and Data Analysis

Sequence reads from bisulfite-treated libraries were identified using standard Illumina base-calling software, and then analyzed using a Zymo Research Corporation proprietary analysis pipeline. Residual cytosines (Cs) in each read were first converted to thymines (Ts), with each such conversion noted for subsequent analysis. Reads were aligned by Bismark, a Bowtie-based alignment tool for bisulfite converted reads.[6] The number of mismatches in the induced alignment was then counted between the unconverted read and reference, ignoring cases in which a T in the unconverted read was matched to a C in the unconverted reference. For a given read, only the best scored alignment was kept. If there were more than one best read, then only one was kept arbitrarily. The methylation level of each sampled cytosine was estimated as the number of reads reporting a C, divided by the total number of reads reporting a C or T. CpGs with coverage of less than four reads were removed from the analysis. The developed sensitive and robust bisulfite sequencing assays yielded a median total sequencing read of 533 (range: 30–1,725) per CpG in the trophoblast-fetal blood cell comparison and a median total sequencing read of 136 (range: 4–2,609) per CpG in the clinical sample comparison.

Multiple sequencing counts (total and methylated) were summed for each sample at each CpG site, and samples with a total count <4 were dropped from the analysis. The mean methylation ratio in each group was computed for genomic visualization. In the comparison of methylation levels between trophoblasts and cord blood cells, a group sample size of two was considered as a minimum. In order to fit the count data, we used a generalized linear model of Poisson distribution with log link. When all samples in any of the two groups being compared had zero methylation counts, the maximum likelihood estimation of the Poisson model went to infinity. In such cases, the Student's *t*-test was used alternatively. *P*-values and the group difference in methylation ratios were included above the bar plots in Figures S9 and S12 in the Supplementary Material. For comparisons of methylation levels between the clinical groups, only comparisons with a minimum group sample size of four were considered, and the Wilcoxon rank-sum test was used. *P*-values and the group difference in methylation ratios were included above the bar plots in **Figure 11** as well as Figures S10 and S13 in the Supplementary Material. Differential methylation was considered to be mild, moderate, or strong when the *p*-value was <0.05 and the difference in methylation ratios was ≥0.125, ≥0.25, or ≥0.5, respectively. The correlation between methylation levels on each CpG in clinical samples and various demographical, clinical, or histopathological variables were evaluated by Kendall's tau statistics. Correlation coefficients and *p*-values were plotted in the scatter plots. A Kendall's tau $p < 0.05$ was considered significant.

[6]http://www.bioinformatics.babraham.ac.uk/projects/download.html#bismark.

Maternal Study

"Virtual" Liquid Biopsy of the Placenta in Preterm Preeclampsia

Sample Collection

First trimester placentas and maternal blood samples were collected from healthy Caucasian women undergoing termination of pregnancy ($n = 12$), and processed at the Maternity Clinic and Semmelweis University in Budapest, Hungary. Villous tissues were dissected from the choriodecidua on dry ice and stored at −80°C. Aliquots of maternal sera and plasma were stored at −80°C.

Pregnancies were dated according to ultrasound scans between 5 and 13 weeks of gestation. Patients with multiple pregnancies were excluded. The collection and investigation of human clinical samples were approved by the Health Science Board of Hungary. Written informed consent was obtained from women prior to sample collection, and the experiments conformed to the principles set out in the World Medical Association Declaration of Helsinki and the Department of Health and Human Services Belmont Report. Specimens and data were stored anonymously.

Placental Total RNA Isolation and qRT-PCR

Total RNA was isolated from snap-frozen first trimester placental villous tissues ($n = 12$) with Direct-zol RNA MiniPrep Kit (Zymo Research Corporation) according to the manufacturer's recommendations. The 28S/18S ratios and the RNA integrity numbers were assessed using an Agilent Bioanalyzer 2100 (Agilent Technologies), and RNA concentrations were measured with NanoDrop 1000. Total RNA (500 ng) was reverse transcribed with the qScript cDNA Synthesis Kit (Quanta Biosciences, Gaithersburg, MD, USA). TaqMan Assays (Applied Biosystems; Data S17 in Supplementary Material) were used for expression profiling on the StepOnePlus Real-Time PCR System (Applied Biosystems).

Enzyme-Linked Immunosorbent Assays

Concentrations of leptin and human placental lactogen (hPL) in first ($n = 12$) and third ($n = 19$) trimester maternal blood samples were measured with sensitive and specific immunoassays (Leptin ELISA Kit, Abnova, Taipei City, Taiwan; Human Placental Lactogen ELISA Kit, Alpco, Salem, NH, USA) according to the manufacturers' instructions. Standard curves were generated, and sample assay values were extrapolated. The sensitivities of the assays were <4.4 ng/ml (leptin) and <550 ng/ml (hPL).

Correlation Analysis of Placental Gene Expressions and Maternal Plasma Protein Concentrations

Placental gene expression was measured with either microarray on third trimester samples or qRT-PCR on first trimester samples as described above. Maternal plasma protein concentrations were measured with the above-described immunoassays on respective blood samples taken from the same patients on the day of either delivery or termination of pregnancy. Correlations between placental gene expression and maternal plasma concentrations of gene product proteins were calculated with the Pearson method and visualized on scatter plots (Figure S4 in Supplementary Material).

Publication Search, Database Build, and "Virtual" Liquid Biopsy of the Placenta

To detect disease-associated protein signatures of placental dysfunction in maternal blood, similar to plasma DNA tissue mapping for noninvasive prenatal, cancer, and transplantation assessments (176), we performed "virtual" liquid biopsy of the placenta. Briefly, human microarray data on 79 human tissues and cells were downloaded from the BioGPS database, which was used for the generation of placenta enrichment scores (placental expression/mean expression in 78 other tissues and cells). Five genes (*CGB3*, *CSH1*, *ENG*, *FLT1*, and *LEP*) with enrichment scores between 1.4 and 1,490 were selected based on a literature search due to the extensive investigations of their gene products in maternal blood in preeclampsia (Figure S5 in Supplementary Material). Next, an extensive PubMed search was conducted for first trimester maternal blood protein measurements of these five gene products in patients who developed preeclampsia later in pregnancy. Altogether, 61 scientific reports were identified that met the inclusion criteria, which contained data for 80,170 measurements (35, 61, 82, 88, 126, 178–233). These reports were used to build a database for the "virtual" liquid biopsy. In this database, biomarker levels in preterm preeclampsia were expressed as the percentage of control levels as a function of gestational age. Then, the correlations of control percentage values with gestational age were evaluated using the Pearson method. Scatterplots were used to visualize data (Figure S5 in Supplementary Material; **Figure 5**).

Maternal Serum Proteomics Discovery Study

Study Groups, Clinical Definitions, and Sample Collection

Women were enrolled in a prospective, longitudinal, and multicenter study (196) in prenatal community clinics of the Maccabi Healthcare Services, Israel. Pregnancies were dated according to the last menstrual period and verified by first trimester ultrasound. Patients with multiple pregnancies or fetuses having congenital or chromosomal abnormalities were excluded. The collection and investigation of human clinical samples were approved by the Maccabi Institutional Review Board. Written informed consent was obtained from women prior to sample collection, and the experiments conformed to the principles set out in the World Medical Association Declaration of Helsinki and the Department of Health and Human Services Belmont Report. Specimens and data were stored anonymously.

Preeclampsia was defined according to the criteria set by the International Society for the Study of Hypertension in Pregnancy (338) and was subdivided into preterm (<37 weeks) or term (≥37 weeks) groups. Severe preeclampsia was defined by Sibai et al. (2). SGA was defined as neonatal BW below the 10th percentile for gestational age. Healthy controls had no medical or obstetric complications and delivered a neonate with a BW appropriate for gestational age.

Peripheral blood samples were obtained by venipuncture in the first trimester from women who subsequently developed preterm severe preeclampsia ($n = 5$) and term severe preeclampsia ($n = 5$) as well as healthy controls ($n = 10$) matched for gestational age at blood draw (**Table 4**). Blood samples were allowed to clot and were then centrifuged at 10,000 × *g* for 10 min to separate and collect sera. Serum samples were stored in aliquots at −20°C in the Maccabi Central Laboratory until shipped on dry ice to Hungary.

Sample Preparations, Immunodepletion of High-Abundance Serum Proteins

Sera were immunodepleted at Biosystems International Ltd., (Debrecen, Hungary) for 14 highly abundant serum proteins on an Agilent 1100 HPLC system using Multiple Affinity Removal LC Column-Human 14 (Agilent Technologies) according to the manufacturer's protocol. To improve the resolution of 2D gels, immunodepleted serum samples were lyophilized, delipidated, and salt-depleted at the Proteomics Laboratory of the Eotvos Lorand University (Budapest, Hungary) (339). The delipidated and salt-depleted plasma protein samples were dissolved in lysis buffer, and their pH was adjusted to 8.0.

Fluorescent Labeling and 2D-DIGE

Protein concentrations of the immunodepleted, desalted, and delipidated serum samples were between 2 and 4 μg/μl as determined with PlusOne 2D Quant Kit (GE Healthcare, Little Chalfont, United Kingdom). Samples were equalized for protein content, and then 5 μg of each protein sample was labeled with a CyDye DIGE Fluor Labeling kit for Scarce Samples (saturation dye) (GE Healthcare) according to the manufacturer's instructions. Individual samples from cases ($n = 2 \times 5$) and controls ($n = 2 \times 5$) were labeled with Cy5. An internal standard reference sample was pooled from equal amounts (2.5 μg) of all individual samples in this experimental set and was labeled with Cy3. Then, 5 μg of each Cy5-labeled individual sample was merged with 5 μg of the Cy3-labeled reference sample, and these 20 mixtures were run in 2 × 10 gels simultaneously. Briefly, labeled proteins were dissolved in isoelectric focusing (IEF) buffer and were rehydrated passively onto 24 cm immobilized non-linear pH gradient (IPG) strips (pH 3–10, GE Healthcare) for at least 14 h at room temperature. After rehydration, the IPG strips were subjected to the first dimension of IEF for 24 h to attain a total of 80 kVh. Focused proteins were reduced by equilibrating with a buffer containing 1% mercaptoethanol for 20 min. After reduction, IPG strips were loaded onto 10% polyacrylamide gels (24 × 20 cm) and SDS-PAGE was conducted at 12W/gel in the second dimension. Then, gels were scanned in a Typhoon TRIO + scanner (GE Healthcare) using appropriate lasers and filters with the photomultiplier tube biased at 600V. Images in different channels were overlaid using selected colors, and the differences were visualized using Image Quant software (GE Healthcare). Differential protein expression analysis was performed using the differential in-gel analysis and

biological variance analysis (BVA) modules of the DeCyder 6.0 software package (GE Healthcare).

Identification of DE Protein Spots

The internal standard reference sample representative of every protein present in all experiments was loaded equally in all gels and thus provided an average image for the normalization of individual samples. The determination of the relative abundance of the fluorescent signal between internal standards across all gels provided standardization between the gels, removing experimental variations and reducing gel-to-gel variations. According to the standard proteomic protocol, the threshold for differential expression was set at 1.05-fold minimum fold-change (340). A *p*-value was determined for each protein spot using the Student's *t*-test by the BVA module of the DeCyder software. A *p*-value of <0.05 was considered statistically significant.

Sample Preparation, Fluorescent Labeling, and Preparative 2D-DIGE

The density of spots in the case of Colloidal Coomassie Blue labeling depends only on the concentration of protein in the sample; however, the density of the spots in the case of saturation dye labeling also depends on the number of cysteines of the labeled proteins because the saturation dye labeling method labels all available cysteines on each protein. This results in the same pattern with different density among samples on the analytical and the preparative gels, rendering identification more difficult. To eliminate this problem for the exact identification of proteins in spots of interest, preparative 2D gel electrophoresis was performed using CyDye saturation fluorescent labeling and Colloidal Coomassie Blue labeling in the same gel. A total of 800 μg of proteins per each of the two gels was run. Following electrophoresis, gels were scanned in a Typhoon TRIO + scanner as described above, the significantly altered spots were matched among the "master" analytical and the fluorescent preparative gel images using the BVA module of the DeCyder 6.0 software package. The resolved protein spots were visualized by the Colloidal Coomassie Blue G-250 staining protocol. Individual spots of interest were excised from the gels based on the comparison of the matched images.

In-Gel Digestion, Liquid Chromatography–Tandem Mass Spectrometry (LC–MS/MS)

The excised protein spots were analyzed at the Proteomics Research Group of the Biological Research Center of the Hungarian Academy of Sciences (Szeged, Hungary); the detailed protocol is described in http://ms-facility.ucsf.edu/ingel.html. Briefly, salts, SDS, and Coomassie brilliant blue dye were washed out, disulfide bridges were reduced with dithiothreitol, and then free sulfhydryls were alkylated with iodoacetamide. Digestion with side-chain protected porcine trypsin (Promega, Madison, WI, USA) proceeded at 37°C for 4 h. The resulting peptides were extracted from the gel using 1% formic acid in 50% acetonitrile; then the samples were dried down and dissolved in 0.1% formic acid.

Samples were analyzed on an Eldex nanoHPLC system online coupled to a 3D ion trap tandem mass spectrometer (LCQ Fleet, Thermo Scientific) in "triple play" data-dependent acquisition mode, where MS acquisitions were followed by CID analyses on computer-selected multiply charged ions. HPLC conditions included in-line trapping onto a nanoACQUITY UPLC trapping column (Symmetry, C18 5 μm, 180 μm × 20 mm; and 15 μl/min with 3% solvent B) followed by a linear gradient of solvent B (5–60% in 35 min, flow rate: 300 nl/min, where solvent A was 0.1% formic acid in water and solvent B was 0.1% formic acid in acetonitrile) on a Waters Atlantis C18 Column (3 μm, 75 μm × 100 mm).

Database Search and Data Interpretation

Raw data files were converted into Mascot generic files (*.mgf) with the Mascot Distiller software v2.1.1.0 (Matrix Science Inc, London, UK). The resulting peak lists were searched against a human subdatabase of the non-redundant protein database of the NCBI (NCBInr, Bethesda, MD, USA) in MS/MS ion search mode on an in-house Mascot server v2.2.04 using Mascot Daemon software v2.2.2 (Matrix Science Inc). Monoisotopic masses with peptide mass tolerance of ±0.6 Da and fragment mass tolerance of ±1 Da were submitted. Trypsin with up to two missed cleavages was specified as an enzyme. Carbamidomethylation of cysteines was set as fixed modification, and acetylation of protein N-termini, methionine oxidation, and pyroglutamic acid formation from peptide N-terminal glutamine residues were permitted as variable modifications. Acceptance criterion was set to at least two significant (peptide score >40, $p < 0.05$) individual peptides per protein. Localization of identified peptides in the core protein sequences was visually analyzed in order to identify potential protein split products.

Biological functions of the altered serum proteins were retrieved from Pathway Studio 9.0 software (Ariadne Genomics Inc., Rockville, MD, USA), and from the open-access GO database[7] (**Figure 6**; Figure S6 and Data S8 in Supplementary Material). To elucidate possible interactions between the altered serum proteins and placental genes in the microarray data, bioinformatics analysis was performed using the same software. Molecular networks between the changed serum proteins in preterm ($n = 19$) or term preeclampsia ($n = 14$) and DE placental genes annotated in the GO database ($n = 1{,}142$) were built separately with a non-linear literature processing search engine, and the resulting connections were manually validated by reading full-text publications (**Figure 6**; Figure S6 in Supplementary Material). The Fisher's exact test was used to test for the enrichment of the connections between the altered serum proteins and (1) DE genes in individual modules, taking the connections between the proteins and DE genes in all modules as a background; and (2) DE placental genes, taking the connections between the proteins and all genes tested on the array as a background. To reveal the pathways enriched among the DE genes connected to angiotensinogen, the Ingenuity Pathway Analysis software (QIAGEN, Redwood City, Redwood City, CA, USA) was used, which utilizes Fisher's exact test and Benjamini–Hochberg correction for multiple testing for the analyses. Statistical significance was set at $q < 0.01$ (Data S10 in Supplementary Material).

[7] http://www.geneontology.org/.

Maternal Serum Proteomics Validation Pilot Study

Study Groups, Clinical Definitions, and Sample Collection

Caucasian women were enrolled in a prospective study at the Department of Obstetrics and Gynaecology of the University of Debrecen and at the Andras Josa Teaching Hospital in Nyiregyhaza, Hungary. Pregnancies were dated according to ultrasound scans between 8 and 14 weeks of gestation. Patients with multiple pregnancies or fetuses having congenital or chromosomal abnormalities were excluded. The collection and investigation of human clinical samples were approved by the Regional Ethics Committee of the University of Debrecen. Written informed consent was obtained from women prior to sample collection, and the experiments conformed to the principles set out in the World Medical Association Declaration of Helsinki and the Department of Health and Human Services Belmont Report. Specimens and data were stored anonymously.

Women were included in the following groups: (1) preterm severe preeclampsia with SGA ($n = 5$) and (2) controls ($n = 10$). Women were matched for gestational age at blood draw (**Table 5**). Preeclampsia was defined according to the criteria set by the American College of Obstetricians and Gynecologists (1) and was subdivided into preterm (<37 weeks) or term (≥37 weeks) groups. Severe preeclampsia was defined according to Sibai et al. (2). SGA was defined as neonatal BW below the 10th percentile for gestational age. Healthy controls had no medical or obstetric complications and delivered a neonate with a BW appropriate for gestational age.

Peripheral blood samples were obtained by venipuncture in the first trimester. Plasma samples were separated by double centrifugation for 10 min. Samples were stored in aliquots at −80°C.

Sample Preparations

All solvents were HPLC-grade from Sigma-Aldrich (St. Louis, MO, USA) and all chemicals, where not stated otherwise, were obtained from Sigma-Aldrich. Frozen plasma samples were thawed and denatured with denaturing buffer (Biognosys AG; Schlieren, Switzerland). Samples were alkylated using alkylation solution (Biognosys). Subsequently, samples were digested overnight with sequencing grade modified trypsin (Promega; Madison, WI, USA) at a protein:protease ratio of 50:1. C18 cleanup for mass spectrometry was carried out according to the manufacturer's instructions using C18 Micro Spin columns (Nest Group Inc.; Southborough, MA, USA). Peptides were dried down to complete dryness using a SpeedVac system. Dried peptides were redissolved with LC solvent A (1% acetonitrile in water with 0.1% formic acid). Final peptide concentrations were determined for all samples by 280 nm measurement (Spectrostar^{NANO}, BMG Labtech, Offenburg, Germany). Samples were spiked with PlasmaDive™ (Biognosys) reference peptides mix at known concentrations.

LC–MRM Measurements and Data Analysis

Peptides (1 µg per sample, corresponding to injection of 0.0259 µl of initial plasma sample) were injected to a self-packed C18column [75 µm inner diameter and 10 cm column length, New Objective (Woburn, MA, USA); column material was Magic AQ, 3 µm particle size, and 200Å pore size from Michrom] on a ThermoScientific EASY-nLC1000 nano-liquid chromatography system. LC–MRM assays were measured on a ThermoScientific TSQ Vantage triple quadrupole mass spectrometer equipped with a standard nano-electrospray source. The LC gradient for LC–MRM was 6–40% solvent B (85% acetonitrile in water with 0.1% formic acid) for 30 min followed by 40–94% solvent B for 2 min and 94% solvent B for 8 min (total gradient length was 40 min). A subset of Biognosys' PlasmaDive™ MRM Panel of 10 peptides representing 10 proteins (Data S9 in Supplementary Material) was used for the measurements of 10 altered proteins involved in immune responses. For the quantification of the peptides across samples, the TSQ Vantage was operated in scheduled MRM mode with an acquisition window length of 5 min. The LC eluent was electrosprayed at 1.9 kV and Q1/Q3 were operated at unit resolution (0.7 Da). Signal processing and data analysis were carried out using SpectroDive™ 8.0—Biognosys' software for multiplexed MRM/PRM data analysis based on mProphet (341). A q-value filter of 1% was applied.

Because of the small sample size of this pilot study, statistical simulations were carried out to predict power and significance in the extended validation study by multiple permutation testing. The probability of significant differences between the two groups was estimated by a paired t-test and the Mann–Whitney test. For both, $p < 0.05$ was taken as criteria to count successful simulations. The number of successful simulations was calculated for different sample sizes ($n = 10$ or 100) and repeats of simulations ($n = 10$, 100, or 1,000). The simulated significance level at $p < 0.05$ was accepted if the number of successful simulations was >25% (**Figure 6**; Data S9 in Supplementary Material).

Publication Search

An extensive PubMed search was conducted for first trimester maternal blood protein measurements of the DE proteins found by 2D-DIGE. Altogether five scientific reports were identified that met the inclusion criteria (126, 143, 239–241). **Figure 6** depicts biomarkers with the same direction differential abundance in preterm preeclampsia in published data as in 2D-DIGE assays.

Trophoblast Study

In Vitro Modeling of Placental Disease Pathways

Database Search and Data Analysis

To build optimal *in vitro* cellular models of trophoblastic disease, an extensive PubMed search was first conducted for similar assays. Since no human trophoblastic stems cells were yet available that would enable a natural proliferative trophoblastic pool with differentiation potential into villous or extravillous lineages, choriocarcinoma-derived trophoblastic cell lines or immortalized EVTs were mostly used for such purposes. Our search revealed BeWo cells (342) and HTR8/SVneo cells (253) as the increasingly most accepted cell model systems based on 1,500 published articles.

Primary VT Differentiation

For *in vitro* trophoblast experiments, placentas ($n = 6$) were collected prospectively at the Perinatology Research Branch (NICHD, NIH, DHHS) from normal pregnant women at term who delivered an AGA neonate with Cesarean section.

Cytotrophoblasts were isolated from these placentas by the modified method of Kliman et al. (343). Briefly, 100 g villous tissues were cut, rinsed in PBS, and sequentially digested with Trypsin (0.25%; Life Technologies, Grand Island, NY, USA) and DNAse I (60 U/ml; Sigma-Aldrich) for 90 min at 37°C. Dispersed cells were filtered through 100 μm Falcon nylon mesh cell strainers (BD Biosciences, San Jose, CA, USA), and then erythrocytes were lysed with 5 ml NH_4Cl solution (Stemcell Technologies, Vancouver, BC, Canada). Washed and resuspended cells were layered over 20–50% Percoll gradients and centrifuged for 20 min at 1,200 × *g*. Trophoblast containing bands were collected and non-trophoblastic cells were excluded by negative selection using anti-CD9 (20 μg/ml) and anti-CD14 (20 μg/ml) mouse monoclonal antibodies (R&D Systems, Minneapolis, MN, USA), MACS anti-mouse IgG microbeads, and MS columns (Miltenyi Biotec, Auburn, CA, USA). Then, primary VTs were plated on a collagen-coated 12-well plate (BD Biosciences; 3×10^6 cells/well) in Iscove's modified Dulbecco's medium (IMDM; Life Technologies) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). To test the effect of trophoblast differentiation on selected genes' expression, primary trophoblasts were kept in IMDM containing 5% non-pregnant human serum (SeraCare, Milford, MA, USA) and 1% P/S. The medium was replenished every 24 h, and cells were harvested for total RNA every 24 h between days 1 and 7.

The Effect of Maternal Serum on Trophoblastic Gene Expression

Villous trophoblasts were isolated from normal term placentas and plated as described above. To test the effect of preeclampsia serum on VTs at two time points of differentiation, VTs were kept in IMDM containing 1% P/S and 10% first trimester maternal sera from control or preeclamptic women. Medium was replenished after 48 h, and cells were harvested for total RNA either 24 or 72 h after the start of the human serum treatment. All experiments were run in triplicate.

The Effect of Oxygen Levels, BCL6, and ARNT2 Overexpression on Trophoblastic Gene Expression

BeWo cells (American Type Culture Collection) were incubated in a T-25 flask or 6-well plate with Ham's F12-K medium (Life Technologies) supplemented with 10% FBS and 1% P/S in a humidified incubator (5% CO_2, 20% O_2) at 37°C until reaching 50–80% confluence. To test the effect of *ARNT2* or *BCL6* overexpression on gene expression, cells were transiently transfected with *ARNT2*, *BCL6*, or control (GFP) vectors. Briefly, 4 μg expression plasmid (OriGene Technologies, Inc., Rockville, MD, USA) and 12 μl FuGENE HD transfection reagent (Promega) were mixed with 180 μl Ham's F12-K medium (10% FBS, 1% P/S), incubated at RT for 15 min and added to cell cultures with 1.8 ml medium in each well of 6-well plate. Twenty-four hours after transfection, cells were split into three treatment groups and kept under either normoxic (20% O_2), hypoxic (2% O_2), or ischemic (1% and 20% O_2 alternating for 6 h) conditions in an Oxycycler C42 (BioSpherix, Lacona, NY, USA) for 48 h before cell harvest. This study setup followed the generally accepted view in reproductive sciences that ~2% O_2 concentration represents physiologic hypoxia at the implantation site and that placental development occurs under physiologic hypoxia in the first trimester (242, 344).

Total RNA Isolation and qRT-PCR

Total RNA was isolated from primary VTs on days 0–7 of differentiation and from BeWo cell cultures with TRIzol reagent (Life Technologies) and RNeasy kit (QIAGEN) according to the manufacturers' recommendations. The 28S/18S ratios and the RNA integrity numbers were assessed using an Agilent Bioanalyzer 2100; RNA concentrations were measured with NanoDrop 1000. Total RNA (500 ng) was reverse transcribed with High Capacity cDNA Reverse Transcription Kit using random hexamers (Applied Biosystems). TaqMan Assays (Applied Biosystems; Data S17 in Supplementary Material) were used for high-throughput gene expression profiling on the Biomark qRT-PCR system (Fluidigm) according to the manufacturers' instructions.

Data Analysis

qRT-PCR. Data were analyzed using the ΔΔCt method. Data were first normalized to the reference gene (*RPLP0*), and $\log_2$ mRNA relative concentrations were obtained for each sample as $-\Delta Ct_{(gene)} = Ct_{(RPLPO)} - Ct_{(gene)}$.

Primary Trophoblast Differentiation. The overall changes in gene expression during the 7 days of differentiation were analyzed by comparing the mean expressions on a given day versus Day 0. The highest fold change for a given gene was defined as the maximum of the daily expression differences during the 7-day time-period. Significant differences were defined by a paired *t*-test ($p < 0.05$) (**Figure 7**).

Serum Treatment of Primary Trophoblast. Gene expression data were analyzed using the Student's *t*-test to compare the effect of preeclampsia serum with the effect of control serum on gene expression at Days 1 and 3 of trophoblast differentiation. *P*-values of <0.05 were considered significant (**Figure 6**).

BeWo Cell Transfections. Gene expression data were analyzed to compare the effect of *ARNT2* or *BCL6* overexpression with the effect of control vector overexpression on gene expression in normoxic conditions using a one-way ANOVA model. The same model was used to assess the differential effect of *ARNT2*, *BCL6*, or *GFP* overexpression on gene expression in hypoxic or ischemic conditions vs. normoxia. *P*-values of <0.05 were considered significant (**Figure 8**).

A *permutation test* was used to measure the statistical significance of the matching between differential gene expression patterns in *in vitro* and *in vivo* conditions. Genes were discretized into three states, i.e. up-regulated (UP), down-regulated (DN), or unchanged (NS). For each gene in the two conditions, a score of 1 was assigned for a perfect match of UP/UP or DN/DN, 0 for a neutral match of NS/NS, −1 for a perfect mismatch of UP/DN or DN/UP, and −0.5 for all other patterns. The matching score for any pair of conditions was computed as the sum of all scores for each individual gene. The significance of the scores was assessed *via* a permutation on the class labels. Permutations were

exhaustive when feasible, otherwise limited to a random sample of 5,000 (Data S11 in Supplementary Material).

Evaluation of Placental/Trophoblastic Expression and Function of ZNF554

Tissue qRT-PCR Array Expression Profiling

TaqMan assays for *ZNF554* and *RPLP0* (Data S17 in Supplementary Material) were run in triplicate for expression profiling of the Human Major Tissue qPCR Array (OriGene Technologies) that contains cDNAs from 48 different pooled tissues.

mRNA In Situ Hybridization

In situ hybridization on third trimester FFPE placental tissues ($n = 6$) retrieved from the Bank of Biological Specimens of the Perinatology Research Branch was carried out using the RNAscope 2.0 FFPE Assay–Brown (Advanced Cell Diagnostics, Hayward, CA, USA) on a HybEZ Hybridization System (**Figure 9**). Briefly, tissue sections were incubated with *ZNF554* target probe (Cat.No.: 423831, Advanced Cell Diagnostics) for 2 h at 40°C. After rinsing with 1× Wash Buffer, slides underwent a six-step amplification procedure at 40°C and were washed with 1× Wash Buffer between amplification steps. Chromogenic detection was performed using a 1:1 mixture of Brown-A and Brown-B solutions. Slides were counterstained with hematoxylin, dehydrated in graded ethanol, and mounted in xylene.

Immunohistochemistry

Third trimester placentas were retrieved from the Bank of Biological Specimens of the Perinatology Research Branch. First trimester placentas were collected from healthy Caucasian women undergoing termination of pregnancy and processed at the Maternity Clinic and Semmelweis University in Budapest, Hungary as described above. Five µm sections of first and third trimester FFPE placental tissues ($n = 15$) were placed on silanized slides and stained using anti-ZNF554 or anti-cytokeratin-7 antibodies as well as reagents listed in Data S18 (Supplementary Material) either on Ventana Discovery (Ventana Medical Systems, Inc, Tucson, AZ, USA) or Leica BOND-MAX (Leica Microsystems, Wetzlar, Germany) autostainers (**Figures 9** and **10**).

Primary VT Differentiation

The experimental procedures were embedded in the study on VT differentiation as described above (**Figure 9**).

BeWo Cell Cultures

BeWo cells were incubated in a T-25 flask or 6-well plate with Ham's F12-K medium (Life Technologies) supplemented with 10% FBS and 1% P/S in a humidified incubator (5% CO_2, 20% O_2) at 37°C until reaching 50–80% confluence.

To test the effect of *ZNF554* knockdown on gene expression, cells were treated either with 100 nM *ZNF554* siRNA (Ambion-Life Technologies, Foster City, CA, USA) or 100 nM scrambled (control) siRNA (Ambion) using X-tremeGENE siRNA transfection reagent (Roche, Mississauga, ON, Canada), and incubated at 37°C in 2 ml serum-free Opti-MEM (Gibco-Life Technologies) medium. After 6 h, the medium was replaced with 2 ml Ham's F12-K medium supplemented with 10% FBS. After 48 h, cells were collected for RNA isolation, microarray, and qRT-PCR as well as confocal microscopy (**Figure 9**).

To test the effect of DNA methylation on gene expression, BeWo cells were treated with 5 or 10 µM 5-azacitidine (Sigma-Aldrich), and control cells with DMSO. This experiment was also performed when both 5-azacitidine-treated and control cells received 25 µM forskolin (Sigma-Aldrich) to induce syncytialization. After 24 h incubation, cells were harvested for RNA isolation and qRT-PCR. The experiment was performed in six replicates (**Figure 11**; Figure S8 in Supplementary Material).

HTR8/SVneo Cell Cultures

HTR8/SVneo EVT cells (kindly provided by Dr. Charles H. Graham, Queen's University, Kingston, Ontario, Canada) were incubated in a 6-well plate with RPMI-1640 medium (Gibco-Life Technologies) supplemented with 10% FBS and 1% P/S in a humidified incubator (5% CO_2, 20% O_2) at 37°C until reaching 50% confluence.

To test the effect of *ZNF554* knockdown on gene expression and functions, cells were treated either with 100 nM *ZNF554* siRNA or 100 nM scrambled (control) siRNA as described for BeWo cells. After 6 h, the medium was replaced with 2 ml RPMI-1640 medium (Gibco-Life Technologies) supplemented with 10% FBS. On the following day, cells were kept in various O_2 concentrations (2, 8, or 20%) in an Oxycycler C42. Cells were collected for functional assays after 24 h, while cells were collected for RNA isolation, microarray, qRT-PCR, or confocal microscopy and their supernatants for ELISA after 48 h. Cell cultures were used for cell proliferation assays after 0, 24, and 48 h.

Total RNA Isolation, Microarray, and qRT-PCR

Total RNA was isolated from BeWo and HTR8/SVneo cell cultures with TRIzol reagent (Life Technologies) and RNeasy kit (QIAGEN, Valencia, CA, USA) according to the manufacturers' recommendations. The 28S/18S ratios and the RNA integrity numbers were assessed using an Agilent Bioanalyzer 2100; RNA concentrations were measured with NanoDrop 1000. DNase-treated RNA from BeWo and HTR8/SVneo cells (500 ng) was amplified and biotin-labeled with the Illumina TotalPrep RNA Amplification Kit (Ambion-Life Technologies). Labeled cRNAs were hybridized to a HumanHT-12v4 Expression BeadChip (Illumina, Inc., San Diego, CA). BeadChips were imaged using a BeadArray Reader (Illumina, Inc.), and raw data were obtained with BeadStudio Software V.3.4.0 (Illumina, Inc.). Total RNA (500 ng) was also reverse transcribed with High Capacity cDNA Reverse Transcription Kit using random hexamers (Applied Biosystems, Foster City, CA, USA). TaqMan Assays (Applied Biosystems; Data S17 in Supplementary Material) were used for high-throughput gene expression profiling on the Biomark qRT-PCR system (Fluidigm, San Francisco, CA, USA) according to the manufacturers' instructions.

Confocal Microscopy

ZNF554 knock-down and control BeWo and HTR8/SVneo cells cultured in 6-well plate were detached with 0.05% Trypsin-EDTA (Life Technologies), washed, and resuspended in PBS, and then 4×10^4 cells were cytospined to Superfrost Plus slides (Fisher

Scientific). Then, cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA), blocked with Protein Block (Dako North America, Inc., Carpinteria, CA, USA), and immunostained with anti-ZNF554 mouse polyclonal antibody (1:100 dilution, overnight; Abnova, Taipei City, Taiwan) and an AlexaFluor-488 goat anti-mouse antibody (1:1,000 dilution; Life Technologies). Cells were mounted with ProLong Gold antifade reagent with 4',6-diamidino-2-phenylindole (DAPI; Life Technologies), followed by confocal microscopy using a Leica TCS SP5 MP spectral confocal system (Leica Microsystems) (**Figures 9** and **10**).

Enzyme-Linked Immunosorbent Assays

Concentrations of human plasminogen activator inhibitor-1 (PAI-1) and TIMP-3 in HTR8/SVneo cell culture supernatants were measured with sensitive and specific immunoassays (Human PAI-1 ELISA Kit, Invitrogen; TIMP-3 Human ELISA Kit, Abcam Inc., Cambridge, MA, USA) according to the manufacturers' instructions. Standard curves were generated, and sample assay values were extrapolated. The sensitivities of the assays were <30 pg/mL (PAI-1) and <2 pg/ml (TIMP-3) (**Figure 10**).

Cell Proliferation Assays

Cell cultures of control and *ZNF554* siRNA treated HTR8/SVneo cells were assayed using the CellTiter 96 Aqueous Non-radioactive Cell Proliferation Assay (Promega) according to the manufacturer's instructions (Figure S11 in Supplementary Material).

Migration Assay

The migratory capacity of HTR8/SVneo cells was examined with 8μm-pore transwell inserts (Corning, NY, USA) inserted in a 12-well plate described previously (345). After transfection with *ZNF554* or scrambled siRNAs for 24 h, 5×10^5 HTR8/SVneo cells were plated in the upper chambers in a serum-free RPMI-1640 medium, whereas the lower chambers contained an RPMI-1640 medium supplemented with 10% FBS. After incubation for 36 h in 2, 8, or 20% O_2 concentrations, cells on the upper side of the membranes were removed by cotton swab, and the inserts were fixed in methanol for 10 min at RT and washed once with PBS. Then, the membranes were cut out and mounted on Superfrost Plus slides (Fisher Scientific) with ProLong Gold antifade reagent with DAPI. Comprehensive images of each membrane were taken using a Leica TCS SP5 MP spectral confocal system. The number of invaded cells was quantified using Image-Pro Premier v9.0.2 (Media Cybernetics, Inc., Rockville, MD, USA). The experiment was performed in six replicates (**Figure 10**).

Matrigel Invasion Assay

The invasiveness of HTR8/SVneo cells was examined with a Matrigel invasion assay using 8 μm-pore cell culture inserts (BD Biosciences) pre-coated with Matrigel (125 ng/ml; BD Biosciences) and inserted in a 24-well plate described previously (345). After transfection with *ZNF554* or scrambled siRNAs for 24 h, 2×10^5 cells were plated in the upper chambers in a serum-free RPMI-1640 medium, whereas an RPMI-1640 medium supplemented with 10% FBS was added to the lower chambers. After incubation for 48 h in 2, 8, or 20% O_2 concentrations, cells on the Matrigel side of the membranes were removed by cotton swab, and the membranes were processed as in the migration assay. Comprehensive images taken using the Leica TC5 SP5 spectral confocal system were quantified using Image-Pro Plus 6.2 (Media Cybernetics, Inc.). The experiment was performed in triplicate (**Figure 10**).

Data Analysis

Tissue qRT-PCR Array. The expression of *ZNF554* relative to *RPLP0* in the placenta was compared to 47 other human tissues using the Student's *t*-test. *P*-values of <0.05 were considered significant (**Figure 9**).

The Student's *t*-test was used to evaluate *ZNF554* knock-down efficiency and the effect of *ZNF554* knock-down on gene expression in BeWo and HTR8/SVneo cells (**Figures 9** and **10**).

BeWo and HTR8/SVneo Cell Microarray. Data were analyzed using the Bioconductor packages in R (346) following the MIAME guidelines and methodologies described previously (318). Raw microarray gene expression data were normalized by a quantile normalization approach. A moderated *t*-test was used to select DE genes using a cutoff of >1.5 fold-change and $q < 0.1$. GO analysis and pathway analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database were also performed.

HTR8/SVneo Cell qRT-PCR, Immunoassay, Cell Proliferation, Migration, and Invasion. qRT-PCR data were analyzed using the ΔΔCt method relative to *RPLP0* expression. The Student's *t*-test was used to evaluate *ZNF554* knock-down efficiency in HTR8/SVneo cells and the effect of *ZNF554* knock-down on gene expression and cell proliferation. A linear model was built to quantify the effects of *ZNF554* knock-down and various O_2 concentrations on the gene expression and protein secretion of HTR8/SVneo cells as well as on their migratory and invasive capacity. O_2 concentration was treated as a continuous variable, and the interaction between *ZNF554* knock-down and O_2 concentrations was retained in the model when the coefficient was significant. *P*-values of <0.05 were considered significant (**Figure 10**).

DATA AVAILABILITY

All relevant data are within the paper and its Supplementary Material files. MIAME compliant microarray data are available from the Gene Expression Omnibus (GEO) under accession numbers GSE65866, GSE65940, and GSE66273.

ETHICS STATEMENT

The collection and use of human biological specimens and clinical data for research purposes were approved by the Health Science Board of Hungary, the Institutional Review Board of the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development (NICHD, NIH, DHHS), the Wayne State University Human Investigation Committee, the Maccabi Institutional Review Board, and the Regional Ethics Committee of the University of Debrecen. Written informed consent was

obtained from women prior to sample collection, and the experiments conformed to the principles set out in the World Medical Association Declaration of Helsinki and the Department of Health and Human Services Belmont Report. Specimens and data were stored anonymously.

AUTHOR CONTRIBUTIONS

NGT conceptualized study and designed research. NGT, KAK, YX, KJ, RJL, EH-G, ZsD, AS, KE, SzSz, VT, HE-A, CL, AB, GSz, SL, and ZD performed research. NGT, RR, ALT, ZX, LO, OT, HM, SD, SSH, THC, CJK, and ZP contributed new reagents/analytic tools/clinical specimens. NGT, RR, ALT, KAK, YX, ZX, KJ, GB, ZsG, JP, THC, BAGy, AD, ASz, ZsD, GSz, IK, AF, MKr, MKn, OE, GJB, CJK, GJ, and ZP analyzed and interpreted data. All authors contributed to manuscript writing and approved the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01661/full#supplementary-material.

FIGURE S1 | Hub transcription regulatory genes in the M1 and M2 modules and their interaction network. **(A)** Co-expression matrix of transcription regulatory genes and predominantly placenta-expressed genes in the M1 and M2 modules. Within the M1 (green) module, *ESRRG*, *POU5F1*, and *ZNF554* transcription regulatory genes correlated most strongly with predominantly placenta-expressed genes. *ESRRG* and *ZNF554* had the most correlation with *CSH1* and *HSD11B2*, genes strongly implicated in fetal growth. Within the M2 (red) module, *BCL6*, *BHLHE40*, and *ARNT2* transcription regulatory genes were correlated most strongly with predominantly placenta-expressed genes. *BCL6* and *ARNT2* had the most correlation with *FLT1*. Heatmap represents Pearson coefficients. **(B,C)** The networks of biological processes enriched among genes dysregulated in preeclampsia and co-expressed with hub factors in M1 (*ZNF554*) and M2 (*BCL6*) modules were visualized with the BINGO module of Cytoscape. Sizes of the circles relate to the number of genes involved in the biological processes and colors refer to *p*-values. The groups of most enriched biological processes were manually circled and labeled. The color code depicts *p*-values.

FIGURE S2 | Clinical characteristics in the transcriptomic validation study groups. Blood pressure, birth weight, and gestational age data from the 100 pregnant women included in the validation study show that preeclampsia phenotypes are heterogeneous, underlining the complex pathways of disease in preeclampsia.

FIGURE S3 | Placental gene expression changes in various phenotypes of preeclampsia detected by qRT-PCR. Data represent gene expressions relative to *RPLP0* measured across 100 placentas. In each bar plot (mean ± SE), the left and right panels show significant differences ("*") in preterm and term preeclampsia associated with or without SGA samples compared to gestational age-matched controls, respectively. Changes in preterm preeclampsia samples significantly different to changes in term preeclampsia samples are indicated by "+".

FIGURE S4 | The correlation between placental gene expression and maternal plasma protein concentration. Placental *LEP* and *CSH1* gene expression was either measured with microarrays in the third trimester or with qRT-PCR in the first trimester. Maternal plasma leptin and serum human placental lactogen protein concentrations were measured with ELISA. Third trimester placental microarray data were correlated with ELISA data from maternal blood samples collected at the time of delivery from the same patients. qRT-PCR data from placentas taken from first trimester terminations were correlated with ELISA data from blood samples collected at the time of the procedure from the same patients. Correlations were investigated with the Pearson method and visualized on scatter plots. The two investigated genes' expression and their protein products' concentrations correlated both in the first and third trimesters.

FIGURE S5 | The timing of gene module dysregulation in preterm preeclampsia. **(A)** Human microarray data on 79 human tissues and cells downloaded from the BioGPS database was used for the generation of placenta enrichment scores (placental expression/mean expression in 78 other tissues and cells). Five genes with scores between 1.4 and 1,490 were selected based on literature search due to the extensive investigations of their gene products in maternal blood in preeclampsia. Colors depict gene module involvement. **(B–F)** The 80,170 measurements for five gene products published in 61 scientific reports (35, 61, 82, 88, 126, 178–233) were used for the virtual liquid biopsy of the placenta in preterm preeclampsia. Biomarker levels in preterm preeclampsia were expressed as the percentage of control levels (dotted lines) throughout pregnancy. Percentage values were represented in the scatter plots by different colors reflecting gene module classification. Based on qRT-PCR data, sEng belongs to M2 (red) module. The number of measurements, the Pearson correlation values for biomarker levels, and gestational age as well as corresponding *p*-values are depicted for each biomarker.

FIGURE S6 | Maternal blood proteomic changes in term preeclampsia and their effect on differentially expressed (DE) genes in the placenta. **(A)** The 14 DE maternal serum proteins in term preeclampsia belong to six functional groups. **(B)** These 14 proteins have connections with 116 DE placental genes, among which 46 belong to the M2 (red) module. Angiotensinogen has more connections than other proteins (OR = 2.5, $p = 1.6 \times 10^{-8}$) and the most with M2 (red) module genes ($n = 35$). Seventy seven of 86 connections of angiotensinogen have a directional effect toward the gene.

FIGURE S7 | Summary of functional experiments on module M2. Epigenetic changes to the trophoblast and abnormal trophoblast differentiation lead to a general down-regulation of gene expression and the up-regulation of hub factors in module M2 (e.g. *BCL6*). After placental circulation has been established and placental ischemic stress occurs, the up-regulation of *BCL6* sensitizes the trophoblast to ischemia by inducing *ARNT2* up-regulation and downstream increase of expression of *FLT1*, *ENG*, *LEP*, leading to the placental release of pro-inflammatory and anti-angiogenic gene products. This pathway is only observed in preterm preeclampsia, suggesting that the dysregulation of this placental pathway promotes the early development of preeclampsia. The alterations in maternal blood proteome can induce trophoblastic functional changes leading to the up-regulation of module M2 genes, the overproduction of sFlt-1 and an anti-angiogenic state through a trajectory that does not necessarily affect fetal growth.

FIGURE S8 | DNA methylation regulates *BCL6* expression in the trophoblast. **(A)** Decreased *BCL6* expression was observed in BeWo cells upon treatment with 5-azacitidine (5-AZA) irrespective of Forskolin (FRSK) co-treatment. **(B)** Upper three lanes: whole genome bisulfite sequencing data of *BCL6* first intron from the Human Reference Epigenome Mapping Project. H1 ESC; H1 embryonic stem cell; HBDT, H1 BMP4-derived trophoblast; and HDNP, H1-derived neuronal progenitor. Lower three lanes: bisulfite sequencing data in this study. Abbreviations: CB, cord blood cell; CT, cytotrophoblast; ST, syncytiotrophoblast. Red box: differentially methylated region; red arrow: CpG Chr3:187458163.

FIGURE S9 | DNA methylation levels at individual CpGs in *BCL6* in the trophoblast and umbilical cord blood cells. DNA methylation levels (0–100%) at individual CpGs in *BCL6* in umbilical cord blood cells (CB), cytotrophoblasts (CT), and differentiated syncytiotrophoblasts (ST) are depicted in the bar plots that represent means and SEs. Umbilical cord blood cells and cytotrophoblasts were obtained from the same fetuses. The genomic coordinates of the CpGs, the group differences (CB vs. CT; CT vs. ST) in mean DNA methylation levels and the *p*-values are shown above the bar plots. The number of samples analyzed with methylation reads above the threshold are shown below the bar plots (only comparisons with a group sample size of minimum two were considered). Differential methylation was claimed to be mild, moderate, or strong when the *p*-value was <0.05 and the difference in methylation level was ≥0.125, ≥0.25, or ≥0.5, respectively.

FIGURE S10 | DNA methylation levels at individual CpGs in *BCL6* in the trophoblast in controls and in cases of preeclampsia. DNA methylation levels (0–100%) at individual CpGs in *BCL6* in laser captured trophoblasts are depicted in the bar plots that represent means and SEs. The genomic coordinates of the CpGs, the group differences (compared preterm or term controls) in DNA methylation levels and the *p*-values are shown above the bar plots. The number of samples analyzed with methylation reads above the threshold are shown below the bar plots (only comparisons with a group sample size of minimum four were considered). Differential methylation was claimed to be mild, moderate, or strong when the *p*-value was <0.05 and the difference in methylation level was ≥0.125, ≥0.25, or ≥0.5, respectively. Preterm (left) and term (right) groups of patients were analyzed separately. Abbreviations: PE, preeclampsia; PE + SGA, preeclampsia associated with small-for-gestational age.

FIGURE S11 | The effect of *ZNF554* knock-down on cell proliferation in HTR8/SVneo extravillous trophoblastic cells. **(A)** Cell proliferation assays showed that *ZNF554* knock-down slightly but significantly decreased (−14%, $p = 0.02$) cell proliferation rate in HTR8/SVneo extravillous trophoblastic cells after 48 h. *Y*-axis depicts viable cell number, *X*-axis shows incubation time. **(B)** The differential expression of *CDKN1A* (cyclin-dependent kinase inhibitor 1A) and *STK40* (serine/threonine kinase 40), genes involved in the regulation of cell cycle, upon *ZNF554* knock-down was confirmed by qRT-PCR.

FIGURE S12 | DNA methylation levels at individual CpGs in *ZNF554* in the trophoblast and umbilical cord blood cells. DNA methylation levels (0–100%) at individual CpGs in *ZNF554* in umbilical cord blood cells (CB), cytotrophoblasts (CT), and differentiated syncytiotrophoblasts (ST) are depicted in the bar plots that represent means and SEs. Umbilical cord blood cells and CT were obtained from the same fetuses. The genomic coordinates of the CpGs, the group differences (CB vs. CT; CT vs. ST) in mean methylation levels and the *p*-values are shown above the bar plots. The number of samples analyzed with methylation reads above the threshold are shown below the bar plots (only comparisons with a group sample size of minimum two were considered). Differential methylation was claimed to be mild, moderate, or strong when the *p*-value was <0.05 and the difference in methylation level was ≥0.125, ≥0.25, or ≥0.5, respectively.

FIGURE S13 | DNA methylation levels at individual CpGs in *ZNF554* in the trophoblast in controls and in cases of preeclampsia. DNA methylation levels (0–100%) at individual CpGs in *ZNF554* in laser captured trophoblasts are depicted in the bar plots that represent means and SEs. The genomic coordinates of the CpGs, the group differences (compared preterm or term controls) in methylation levels, and the *p*-values are shown above the bar plots. The number of samples analyzed with methylation reads above the threshold are shown below the bar plots (only comparisons with a group sample size of minimum four were considered). Differential methylation was claimed to be mild, moderate, or strong when the *p*-value was <0.05 and the difference in methylation level was ≥0.125, ≥0.25, or ≥0.5, respectively. Preterm (left) and term (right) groups of patients were analyzed separately. Abbreviations: PE, preeclampsia; PE + SGA, preeclampsia associated with SGA.

DATA S1 | Genes differentially expressed in the placenta in preterm preeclampsia.

DATA S2 | Predominantly placenta-expressed genes.

DATA S3 | The enrichment of differentially expressed genes on chromosomes.

DATA S4 | The enrichment of differentially expressed transcription regulatory genes on chromosomes.

DATA S5 | The enrichment of predominantly placenta-expressed genes on chromosomes.

DATA S6 | Genes associated with blood pressure.

DATA S7 | The association of gene expression with placental pathology.

DATA S8 | Maternal blood proteomic changes in preeclampsia—two-dimensional differential in-gel electrophoresis.

DATA S9 | Maternal blood proteomic changes in preeclampsia—multiple reaction monitoring.

DATA S10 | Placental pathways enriched among the differentially expressed genes connected to angiotensinogen.

DATA S11 | Permutation test of functional experiments.

DATA S12 | Enrichment of transposable elements in genes within the M1 and M2 gene modules.

DATA S13 | Genes differentially expressed in ZNF554-silenced BeWo cells.

DATA S14 | Enrichment analysis of ZNF554-silenced BeWo cells.

DATA S15 | Genes differentially expressed in ZNF554-silenced HTR8/SVneo cells.

DATA S16 | Enrichment analysis of ZNF554-silenced HTR8/SVneo cells.

DATA S17 | TaqMan assays.

DATA S18 | Immunostaining conditions and antibodies.

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Human Innate Lymphoid Cells: Their Functional and Cellular Interactions in Decidua

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Innate lymphoid cells (ILC) are developmentally related cell subsets that play a major role in innate defenses against pathogens, in lymphoid organogenesis and in tissue remodeling. The best characterized ILC are natural killer (NK) cells. They are detectable in decidua in the early phases of pregnancy. During the first trimester, NK cells represent up to 50% of decidua lymphocytes. Differently from peripheral blood (PB) NK cells, decidual NK (dNK) cells are poorly cytolytic, and, instead of IFNγ, they release cytokines/chemokines that induce neo-angiogenesis, tissue remodeling, and placentation. dNK interact with resident myeloid cells and participate in the induction of regulatory T cells that play a pivotal role in maintaining an efficient fetal–maternal tolerance. dNK cells may originate from $CD34^+$ precursor cells present *in situ* and/or from immature NK cells already present in endometrial tissue and/or from PB NK cells migrated to decidua. In addition to NK cells, also ILC3 are present in human decidua during the first trimester. Decidual ILC3 include both natural cytotoxic receptor $(NCR)^+$ and NCR^- cells, producing respectively IL-8/IL-22/GM-CSF and TNF/IL-17. NCR^+ILC3 have been shown to establish physical and functional interactions with neutrophils that, in turn, produce factors that are crucial for pregnancy induction/maintenance and for promoting the early inflammatory phase, a fundamental process for a successful pregnancy. While NCR^+ILC3 display a stable phenotype, most of NCR^-ILC3 may acquire phenotypic and functional features of NCR^+ILC3. In conclusion, both NK cells and ILC3 are present in human decidua and may establish functional interactions with immune and myeloid cells playing an important role both in innate defenses and in tissue building/remodeling/placentation during the early pregnancy. It is conceivable that altered numbers or function of these cells may play a role in pregnancy failure.

Keywords: innate lymphoid cell, innate immunity, natural killer cells, human pregnancy, neutrophils, stromal cells, inflammation, tolerance

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INTRODUCTION

The fetus can be considered as a semi-allograft to the maternal host; therefore, pregnancy should include mechanisms to prevent allograft rejection (1–3). During the early phases of pregnancy, an appropriate balance between inflammation and tolerance is critical for a successful pregnancy (4, 5). Indeed, pro-inflammatory cytokines have been shown to contribute to tissue building/remodeling

and neo-angiogenesis, thus favoring embryo implantation (6–8). The inflammatory phase is followed by a regulatory phase characterized by an increase in regulatory T cells (Tregs) that prevent an excessive inflammation and avoid fetal immuno-mediated rejection (9). Thus, relevant interactions among cells involved in immune response may occur at the fetal–maternal interface and play a fundamental role for a successful pregnancy.

GENERAL CHARACTERISTICS OF INNATE LYMPHOID CELLS (ILC) SUBSETS

Innate lymphoid cells are immune effector cells involved in host defenses against pathogens and tumors, in lymphoid organogenesis and in secondary lymphoid organ remodeling after birth. ILCs are tissue-resident cells mainly found at the mucosal surfaces of intestine (10), lungs (11), decidua (12), and skin (13, 14). Thanks to their strategic location, ILC are among the first immune cells to respond to pathogens. Recently, on the basis of their cytokine profile and transcription factors (TF), ILC have been classified into two main groups: cytotoxic- and helper-ILC (15–18). Natural killer (NK) cells, representing cytotoxic-ILC, are the first innate lymphoid cell population described, featuring the capacity of killing virus-infected or tumor cells and to release pro-inflammatory cytokines and chemokines. Human NK cell function is regulated by an array of inhibitory receptors, such as the HLA-I-specific killer immunoglobulin-like receptors and CD94/NKG2A, and by activating receptors, including natural cytotoxicity receptors (NCR, i.e., NKp46, NKp30, and NKp44), NKG2D, DNAM-1, and CD16 (19, 20). The other ILC are represented by "helper"-ILC that are further classified into three main subsets (ILC1/ILC2/ILC3) (21). ILC1 mainly produce IFNγ and provide defenses against intracellular bacteria and protozoa (22). In humans, two different subsets of ILC1 have been described in the intestine (23, 24). ILC2 mainly release type-2 cytokines, such as IL-5, IL-13, and IL-4 and contribute to type-2 immune responses. Finally, ILC3 are a heterogeneous subset, their signature cytokines are represented by IL-17 and IL-22 (25). ILC3 were first identified in the fetus and were originally defined lymphoid tissue inducer (LTi) cells because of their key role in driving lymphoid organogenesis. In particular, during embryogenesis, LTi cells interact with stromal cells and induce upregulation of adhesion molecules thus promoting the development of lymph node structure. After birth, ILC3 are mainly located in secondary lymphoid organs (SLO), tonsils, decidua, and intestinal lamina *propria* where they contribute to host defenses against extracellular pathogens and are defined as LTi-like cells. In humans, LTi/LTi-like cells are lineage (CD3/CD19/CD14/CD56)-negative and express CD127, CD117, retinoic acid receptor-related orphan receptor (ROR)-γt TF, and secrete primarily IL-17 and TNFα. A population of cells referred to as NCR + ILC3, sharing common features with both LTi-like cells and NK cells (type of cytokines production and NCR expression, respectively), has recently been identified in mucosal tissues and prevalently releases IL-22. ILC3-derived IL-22 acts on intestinal epithelial cells and induces not only production of antimicrobial peptides but also epithelial cell migration and wound healing. Moreover, ILC3 promote tissue repair and remodeling of SLO damaged by inflammatory processes. Conversely, ILC3 may also exert a pro-inflammatory role in intestinal inflammatory diseases.

All ILC subsets are developmentally related. Evidence in mice and humans indicates that NK cells and helper-ILC derive from a common ILC progenitor (CILP). As B and T lymphoid progenitors, the CILP derive from the common lymphoid progenitor. The acquisition of mature stages is dependent by different TF. Thus, NK cell differentiation involves Eomes, which regulates the expression of IFNγ and of the cytolytic machinery, while terminal differentiation of helper-ILC is regulated by other TF. In particular, ILC1 requires Tbet, ILC2 GATA3, and RORα, and ILC3 RORγt and AhR (26–29). Although, specific ILC3-committed precursors have been defined, a precise identification of a common ILC precursor in humans is still lacking. Moreover, it is still only partially understood which signals from the microenvironment are driving their differentiation. The low numbers of ILC3 that can be generated *in vitro* has so far hampered studies aimed to answer these questions. Moreover, limitations in cell numbers may be an obstacle for clinical application of ILC. Thus, the development of protocols allowing the generation of suitable numbers of given subsets of ILC for their use in adoptive cell therapy is required.

Along this line, it has become more evident that the fate of ILC determination and their stability is not set in stone, but that there is some plasticity between different ILC subsets, depending on various signaling, including cytokines and exposure to different tissue-specific microenvironments. This would indicate that microenvironmental conditions might drive this plasticity from an ILC subset to another (29). Accordingly, it is conceivable that also putative differentiated ILC may display intermediate phenotypic/functional characteristics (30, 31).

ILC IN HUMAN DECIDUA AND THEIR INTERACTIONS WITH DECIDUA MICROENVIRONMENT

Innate immune cells are important components of the decidual microenvironment. In this tissue, the best characterized and most abundant ILCs are NK cells (1, 2, 32). Remarkably, while the function of peripheral blood (PB) NK cells is to defend the host against infections and tumors, thanks to their cytolytic activity and production of cytokines, such as IFN-γ and TNF, decidual NK (dNK) cells are characterized by a regulatory function (33). It has been shown that the NK cell function is greatly influenced by the microenvironment, including cytokines (34), chemokines, and cell-to-cell interactions. A paradigmatic example of how NK cell function may be regulated in tissues is provided by human dNK cells. They represent as much as 50–70% of decidual infiltrating lymphocytes during the first trimester of pregnancy, and are characterized by $CD56^{bright}CD16^{-}KIR^{+}CD9^{+}$ phenotype (2, 35). In spite of their high content of cytolytic granules, dNK cells are poorly cytotoxic and release very low amounts of IFNγ as compared to PB-NK cells (2, 36–39). On the other hand, dNK cells release peculiar cytokines and chemokines, such as vascular endothelial growth factor (VEGF), stromal derived

factor-1 (SDF-1 also identified as CXCL12), and IFN-γ-inducing protein 10 (IP10 also known as CXCL10), that mediate neo-angiogenesis, tissue remodeling, and placentation (3). Similarly, to humans, also murine dNK cells are abundant during the early phases of pregnancy and display unique phenotypic and functional features (40–44). Several reports revealed that NK cells are present also in non-pregnant endometrial tissue and that their proportions may vary during the menstrual cycle. It has been shown that, besides NK cells, other ILC populations are present in human decidua during the early phases of pregnancy (12, 41) This finding supports the notion that ILC participate to defensive/tissue building processes necessary for the maintenance/success of pregnancy (45, 46). Notably, decidual tissues contain different RORγt$^+$ ILC3 subsets displaying functional features similar to those previously described in other tissues. In particular, dILC3 not only express IL-22, but are also the main IL-8 producers, a functional activity previously assigned to dNK cells. Moreover, dILC3 have been shown to induce the expression of adhesion molecules (a functional activity referred to LTi-like cells) on decidual stromal cells (DSC), thus suggesting that, also dILC3, may play a role in tissue building/remodeling during the early phases of pregnancy.

ORIGIN OF DECIDUAL ILC

Given the relevant role of dNK cells in the maintenance of pregnancy, an important issue is to clarify their origin. NK cells are known to originate from CD34$^+$ hematopoietic stem cell, as revealed by a number of studies both *in vitro* and *in vivo*. These precursors are present mainly in the bone marrow, and also in PB and cord blood. Although, it is possible that dNK cells may derive from PB-NK cells migrated in decidua, where they acquire unique functional features upon exposure to the decidual microenvironment (47, 48), they may also derive from CD34$^+$ precursors detectable in decidua. In this context, previous studies described the presence of CD34$^+$VEGFR$^-$ precursors in decidual tissues and of immature NK cells in endometrial tissue (47–51). Both cell populations could undergo differentiation into dNK cells during pregnancy. Remarkably, dCD34$^+$ cells are committed toward the NK cell lineage as revealed by experiments showing that they undergo rapid *in vitro* differentiation toward mature NK cells both in the presence and in the absence of cytokines, provided they are co-cultured with DSC (3, 52).

The issue of the developmental relationship between different ILC is a matter of debate. In particular, the relationship between NK cells and ILC3 is unclear in humans, because the phenotypic features of immature NK cells and ILC3 are partially overlapping. It was originally proposed that NCR$^+$ILC3 represent an immature stage of NK cell development (53, 54). However, decidual ILC3, despite their extensive proliferative capacity, maintain their phenotypic and functional characteristic, while only a minor fraction could differentiate toward CD94$^+$ NK cells even in the presence of IL-15. Similarly, to NCR$^+$ILC3, also LTi-like cells virtually failed to generate NK cells. Thus, it is conceivable that the majority of NK cells present in decidual tissue may derive from PB-NK cells migrated to decidua, as well as from CD34$^+$ cell precursors detectable *in situ*. On the other hand, our recent studies support the notion that a developmental relationship exists between the two ILC3 subsets present in decidua. Indeed, NCR$^+$ILC3 (expressing CD56) could be derived from LTi-like CD56$^-$ cells. In these *in vitro* experiments, only a small percentage of LTi-like cells retained their phenotypic characteristics (Lin$^-$CD56$^-$CD117$^+$CD127$^+$), while they acquired the Lin$^-$CD56$^+$CD117$^+$CD127$^+$CD94$^-$NCR$^+$ phenotype (12). It cannot be excluded that, similarly to NK cells, also ILC3 may derive from precursors present in decidua. Indeed, ILC3 are not present in PB, implying that they should develop *in situ* from a precursor. Interestingly, dCD34$^+$ precursors are characterized by the expression of ID2 transcription factor, which is required for ILC development, suggesting that dCD34 cells may give rise not only to dNK cells but also to NCR$^+$ILC3 and LTi-like cells (12).

Although NK cells belong to the innate immune system, different reports suggested that they may display adaptive-like properties. These adaptive features include clonal expansion and the generation of long-lived memory cells (55–58). In humans, "trained/memory" NK cells are characterized by the expression of HLA-class I-specific activating receptor NKG2C (59). The NKG2C$^+$ NK cell subset undergo great expansion following human cytomegalovirus infection (55, 60). Several observations indicate that a major risk of a deficient placentation occurs in women that undergo first pregnancy (61, 62). These data suggest that, after the first pregnancy, the uterine microenvironment may acquire the ability to better sustain the early phase of placentation, including the inflammatory process. In this context, it has been shown recently that the dNK cell repertoire significantly differs in primigravidae as compared to multigravidae. Mulitgravid women display higher percentages of dNK cells expressing NKG2C and LILRB1, which also produce higher amounts of IFN-γ and VEGF as compared to dNK cells detectable in primigravid women. Precursors of these cells are present in the uterus between pregnancies and may become activated by the uterine microenvironment once a new pregnancy occurs. These cells could represent "trained" NK cells that would improve endometrial vascularization, angiogenesis, and maintenance of decidua through a more prompt and abundant secretion of functional VEGF and IFN-γ (63).

INVOLVEMENT OF ILC IN THE INFLAMMATORY AND TOLEROGENIC PHASES OF PREGNANCY

A successful pregnancy requires an early inflammatory phase that is necessary for successful implantation, while, subsequently, a regulatory/immunosuppressive phase should follow to prevent fetal rejection (5, 8, 64). Several studies highlighted the role of innate and adaptive immune cells in promoting either an inflammatory or an immunosuppressive environment. It is conceivable that functional activities thought to be exclusive of adaptive immunity, may actually be mediated also by ILC (65). Thus, not only uterine Th17 but also dILC releasing pro-angiogenetic factors are likely to play a role in the neo-angiogenesis and antimicrobial defenses during pregnancy.

The Inflammatory Phase

Different studies revealed the presence of neutrophils (N) with pro-angiogenetic capability in human decidua during the first and the second trimesters of pregnancy (66, 67). In particular, during the first trimester, N present in human decidual tissues are characterized by an "activated" phenotype, production of fibro/angiogenic factors and a prolonged survival. Previous studies reported that NCR$^+$ILC3 could mediate neutrophil activation in spleen *via* GM-CSF (68). It has been shown that dILC3 could functionally interact with N in decidual tissues (**Figure 1**). In particular, it is conceivable that dN recruitment to decidua, their activation, and expression of peculiar immunoregulatory factors (HB-EGF and IL1ra) may be related to interaction with dILC3 (69, 70). Indeed, thanks to their ability to release IL8 and GM-CSF, dILC3 could promote neutrophil recruitment and activation, respectively. These observations support the notion that both ILC3 and N may play an important role in early inflammatory phase and, subsequently, in the induction of tolerance.

Recent studies revealed that also cytokines released by NK cells might induce neutrophil activation, expression of activation markers, and production of cytokines and angiogenic factors (71–73). In addition, under suitable experimental conditions, NK cells have been shown to induce neutrophil apoptosis (74). Reciprocally, N may inhibit NK cell proliferation, cytolytic activity, cytokine production, and survival *via* contact-dependent or -independent mechanisms (73). In agreement with the concept of NK/N interactions in decidua, it has been observed that dN localize in close proximity of CD56$^+$ cells, suggesting a potential crosstalk with dNK cells. Thus, it is possible to speculate that also decidual N by interacting with dILC3 and/or dNK cells may contribute to the regulation of innate/adaptive immune responses occurring during pregnancy, by the release of soluble factors, or cell-to-cell contacts (67). These data shed new light on the cellular and molecular mechanisms involved in the initiation of an inflammatory response in the decidua.

The Tolerogenic Phase

Since fetus is a semi-allograft, successful pregnancy should also include mechanisms capable of preventing allograft rejection. Indeed, while effective immune responses must be maintained in order to protect the mother from harmful pathogens, immune reaction against fetal antigens should also be avoided. dNK cells express normal levels of HLA-class I-specific inhibitory receptors. Notably, certain HLA-class I molecules are expressed by trophoblast and are involved in the regulation of trophoblast growth, differentiation, and invasion (75). The fact that dNK cells are unable to kill different target cells has been tentatively explained with the poor ability to form appropriate immunological synapses and/or the expression of the inhibitory form of 2B4 co-receptor (39, 76). In addition, the fact that dNK cells do not kill trophoblastic

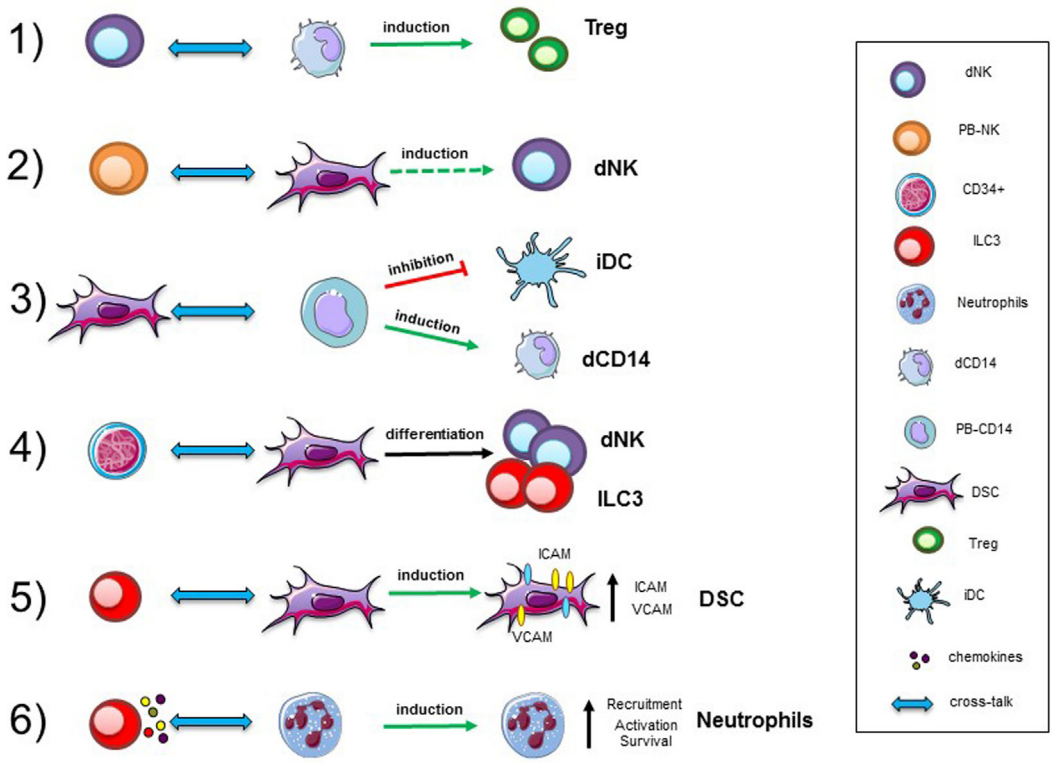


FIGURE 1 | Cellular interactions in human decidua. Representative interactions between immune cells, precursors, and stromal cells occurring in decidual tissues during the first trimester of pregnancy. Abbreviations: dNK, decidual natural killer cells; PB-NK, peripheral blood NK cells; ILC, innate lymphoid cells; dCD14, decidual myeloid cells; PB-CD14, peripheral blood myeloid cells; DSC, decidual stromal cells; Treg, regulatory T cells; iDC, immature dendritic cells.

cells has also been explained with the expression of inhibitory NK receptors, such as CD94/NKG2A specific for HLA-E, recognizing HLA-G KIR2DL4 and KIR2DL1/2/3 specific for HLA-C, i.e., the HLA-class I molecules expressed by human trophoblast. Therefore, both the poor ability to kill of dNK cells and a number of fail-safe mechanism may be responsible for the inability of dNK cells to kill the invading trophoblast (7). Notably, also Tregs have been detected during early pregnancy and are thought to exert an important protective role when the maternal immune cells come into contact with fetal antigens expressed by invading trophoblast cells. Accordingly, studies in humans revealed the presence of Tregs in the PB during the early phases of normal pregnancy, while a low Treg numbers have been reported in cases of recurrent pregnancy loss (77). In this context, it should be stressed that different Treg subpopulations occur, including naturally occurring Tregs, which derive from the thymus, and adaptive Tregs, which develop in the periphery, The development of Tregs requires the transcription factor Forkhead box P3 which currently represents their most specific marker. The secretion of inhibitory cytokines and contact-dependent inhibition are two identified mechanisms of Treg-mediated suppression. Tregs may contribute to prevent autoimmune diseases and play a role in transplantation tolerance. Previous studies indicated that the increase of Treg in decidual tissue may be due either to the local expansion or to their selective recruitment at the maternal–fetal interface (9, 78).

Another remarkable cell population present in the decidua is represented by $CD14^+$ cells, generally described as decidual myeloid cells (monocytes/macrophages) that did not express CD1a, a dendritic cell (DC) marker (79, 80). Histochemical analysis revealed that they may be in close association with dNK cells, and be involved in functional crosstalks with NK cells. *In vitro* experiments revealed that the interaction between dNK and $dCD14^+$ cells results in the production of IFN-γ which, in turn, induces indoleamine 2,3-dioxygenase (IDO) expression in $dCD14^+$ cells. Such "conditioned" $dCD14^+$ cells acquire the ability to induce Treg by a mechanism that involves TGF-β production. Our perception of the role of dNK cells has thus evolved indeed, not only they do not attack trophoblastic cells but also they can play a major role in immune regulation, by promoting the development of Tregs upon functional interaction with $dCD14^+$ cells (81) (**Figure 1**).

Decidual stromal cells represent other important component of decidual tissue. Indeed, decidual leukocytes are deeply influenced by these cells. On the other hand, also immune cells may modulate DSC function (82). Experimental evidences revealed that DSC, present in the early and late phases of pregnancy, may contribute to the induction of an anti-inflammatory and tolerogenic microenvironment crucial for the establishment/maintenance of successful pregnancy. In this context, DSC have been shown to induce downregulation of major activating NK receptors and to inhibit NK cell proliferation, cytotoxicity, and IFN-γ production. These inhibitory activities are related to the production of PGE2 and to the expression of IDO, resulting in kynurenine production. DSC display another important functional capability related to their ability to promote $CD34^+$ cell differentiation. In this context, it has been described that endometrium and decidua contain $CD34^+$ cell precursors ($dCD34^+$) capable of differentiating *in vitro* into NK cells. Interestingly, even in the absence of exogenous cytokines, $dCD34^+$ could give rise to dNK cells when co-cultured with DSC. Other leukocyte functional capabilities may be affected as a result of crosstalk with DSC (**Figure 1**). Thus, DSC, through IDO and PGE2, may affect the differentiation of PB-$CD14^+$ cells toward DCs (82). Moreover, previous studies showed that placenta-derived stromal cells can mediate T cell suppression and induce Treg expansion through a mechanism involving IDO, PGE2, and PD-L1 (83).

Interaction among different cell types may imply a bi-directional crosstalk. Accordingly, also immune cells may influence DSC properties. For example, decidual ILC3 (in particular LTi-like cells) can induce upregulation of ICAM1 and VCAM1 on DSC. This effect is one of the starting events necessary for the development of secondary and tertiary lymphoid organs. In decidua, this effect could be involved in tissue remodeling, placentation, and leukocyte recruitment. Moreover, engagement of RANK has been shown to promote production of CCL2, which, in turn, favors DSC survival and proliferation (84). Moreover, since decidual ILC3 express RANKL it would be of interest to explore whether they can interact with DSC *via* RANKL/RANK-mediated mechanisms.

CONCLUSION

As outlined in this contribution, during the first trimester of pregnancy, different cellular players deeply involved in the balance between inflammation and tolerance are present in decidual. In particular, decidual ILC, including NK cells, ILC3 and ILC1, upon interaction with stromal cells, neutrophils, myelomonocytic cells, and T lymphocytes may play a key role in the induction and maintenance of pregnancy. Notably, decidual ILC can originate from $CD34^+$ precursors or immature lymphoid cells present *in situ*. In view of the role of ILC in pregnancy, it is possible to speculate that defects in ILC generation or functional interactions in decidual tissues may be a possible cause of fetal losses. While a better knowledge on these cells is clearly required before planning any future clinical application, these concepts open new scenarios of investigation both in innate immunity and in reproductive immunology.

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All authors discussed together the general outline of the article. PV, CV, and LM wrote the first draft that was subsequently reviewed by MAC, EM, and MCM. Thereafter, all authors contributed to the elaboration of the final version of the manuscript.

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Clonally Expanded Decidual Effector Regulatory T Cells Increase in Late Gestation of Normal Pregnancy, but Not in Preeclampsia, in Humans

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Background: Regulatory T (Treg) cells are necessary for the maintenance of allogenic pregnancy. However, the repertoire of effector Treg cells at the feto-maternal interface in human pregnancy remains unknown. Our objective was to study T cell receptor (TCR) repertoires of Treg cells during pregnancy compared to normal and complicated pregnancies.

Methods: Paired samples of peripheral blood and decidua in induced abortion and miscarriage cases were obtained from consenting patients. $CD4^+CD25^+CD127^{low/-}CD45RA^-$ effector Treg cells were single-cell sorted from mononuclear cells. cDNAs of complementarity determining region 3 (CDR3) in TCRβ were amplified from the single cells by RT-PCR and the sequences were analyzed. The TCRβ repertoires were determined by amino acid and nucleotide sequences. Treg cells were classified into clonally expanded and non-expanded populations by CDR3 sequences.

Results: We enrolled nine induced abortion cases in the 1st trimester, 12 cases delivered without complications in the 3rd trimester, 11 miscarriages with abnormal chromosomal karyotyped embryo, seven miscarriages with normal chromosomal karyotyped embryo, and seven cases of preeclampsia [median gestational week (interquartile range): 7 (7–9), 39 (38–40), 9 (8–10), 8 (8–10), and 34 (32–37), respectively]. The frequency of clonally expanded populations of effector Treg cells increased in decidua of 3rd trimester cases compared to 1st trimester cases [4.5% (1.4–10.8%) vs. 20.9% (15.4–28.1%), $p < 0.001$]. Clonally expanded Treg cells were rarely seen in peripheral blood. The ratio of clonally expanded populations of decidual effector Treg cells in miscarriages with abnormal and normal embryos was not significantly different compared with that in 1st trimester normal pregnancy. Interestingly, clonally expanded populations of effector Treg cells decreased in

preeclampsia compared with that in 3rd trimester normal pregnancy [9.3% (4.4–14.5%) vs. 20.9% (15.4–28.1%), $p = 0.003$]. When repertoires in previous pregnancy and subsequent pregnancy were compared, some portions of the repertoire were shared.

Conclusion: TCR repertoires of decidual effector Treg cells are skewed in the 3rd trimester of normal pregnancy. Failure of clonal expansion of populations of decidual effector Treg cells might be related to the development of preeclampsia.

Keywords: effector regulatory T cell, human pregnancy, miscarriage, preeclampsia, regulatory T cell, T cell repertoire

INTRODUCTION

Regulatory T (Treg) cells are important in maintaining feto-maternal tolerance during pregnancy in humans and mice (1–6). Previous studies demonstrated the existence of fetal antigen specific Treg cells in a murine model of pregnancy (7–9). Rowe et al. (9) demonstrated that memory type-fetal antigen specific Treg cells induced fetal antigen specific tolerance in second pregnancy in mice. These results may explain why first pregnancy is a risk factor for preeclampsia. We have reported that fetal antigen specific Treg cells are recruited to uterine draining lymph nodes just before implantation in mice (10). Tilburgs et al. (11) suggested that human decidual Treg cells recognize self-fetal antigens by mixed lymphocyte reaction against umbilical cord blood. A suppressive reaction by decidual Treg cells, but not by systemic Treg cells, has been described (11). These findings suggest that fetal antigen specific memory type Treg cells induce feto-maternal tolerance at the feto-maternal interface in both mice and humans, although fetal antigen specific Treg cells have not yet been identified as a T cell receptor (TCR) repertoire in humans.

Human $CD4^+FoxP3^+$ cells contain a $CD4^+CD45RA^-FoxP3^{high}$ effector/activated Treg cell subset, $CD4^+CD45RA^+FoxP3^{low}$ naïve/resting Treg cell subset, and $CD45^+CD45RA^-FoxP3^{low}$ effector T cell subset (12, 13). $CD4^+CD45RA^-FoxP3^{high}$ effector Treg cells have the highest suppressive capability among these subsets. During human late gestation, $CD4^+CD45RA^-FoxP3^{high}$ effector Treg cells are the dominant Treg cell subset in peripheral blood and decidua (14). We have shown that $CD4^+CD45RA^-FoxP3^{high}$ effector Treg cells are significantly decreased in decidua of cases of miscarriage with normal chromosomal karyotyped embryo (15). Thus, the effector Treg cell subset might contain fetal antigen specific populations in human.

Previous reports suggested that systemic and local maldistribution and dysfunction of Treg cells could be one of the etiologies of miscarriage and preeclampsia (16–21). T cell receptor β variable (TRBV) repertoires of total Treg cells in peripheral blood and decidua were not significantly different between preeclampsia and normal pregnancy (22). Thus, how Treg cells relate to the development of preeclampsia remains unknown.

We hypothesized that decidual effector Treg cells that recognize fetal antigens are clonally expanded at the feto-maternal interface. To study the clonality of effector Treg cells, we used a single-cell based TCR repertoire analysis method that we previously described (23, 24). We also aimed to show whether altered TCR repertoires of effector Treg cells are present in miscarriage or preeclampsia.

MATERIALS AND METHODS

Subjects

The enrolled cases included nine cases of artificial abortion in the 1st trimester, 11 cases of 1st trimester miscarriage with abnormal embryo karyotype, seven cases of 1st trimester miscarriage with normal embryo karyotype, 12 cases delivered without pregnancy complications in the 3rd trimester, and seven cases delivered in the 3rd trimester with preeclampsia. Written informed consent was obtained from all the patients in accordance with a protocol approved by the Ethical Review Board of University of Toyama. Fetal heartbeat was confirmed before artificial abortion (induced abortion in the 1st trimester of normal pregnancy). Miscarriage was diagnosed when the fetal heart beat was lost, or when the fetal heartbeat was not detected inside the gestational sac for more than 2 weeks. All artificial abortion and miscarriage treatments were performed by dilation and curettage. Fetal chromosomal karyotype was determined by G-band staining in miscarriage cases. Preeclampsia was diagnosed when blood pressure exceeded 140/90 mmHg and urinary protein exceeded 0.3 g per day after the 20th week of gestation (25). Ten milliliters of venous blood and decidual sample were obtained simultaneously when induced abortion was performed or when subjects delivered a baby. The patients were recruited at Toyama University Hospital, Otogi no Mori Lady's Clinic and Yoshie Ladies Clinic.

Mononuclear Cell Isolation

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll Hypaque (Lymphoprep™; Alere Technologies, Norway) density gradient centrifugation. First trimester decidua was isolated from uterine content that was collected by induced abortion. Third trimester decidua was dissected from maternal surface of the delivered placenta. Decidua was rinsed with phosphate buffered saline (PBS) until the blood was removed, minced with a pair of scissors to produce 1–2 mm pieces, and filtered through 32-μm nylon mesh. All samples were cryopreserved.

Single-Cell Sorting

To sort the effector Treg cells, the following monoclonal antibodies were used: anti-CD3 (APC; BD Bioscience, USA), anti-CD4 (PerCP cy5.5; BD Bioscience), anti-CD45RA (APC

cy7; BioLegend, USA), anti-CD25 (PE cy7; BioLegend), and anti-CD127 (PE; BD Bioscience). PBMC and decidual mononuclear cells were stained by these antibodies for 20 min on ice. After staining, the cells were washed with PBS and analyzed using a FACSAria II flow cytometer (BD Biosciences). $CD3^+CD4^+CD45RA^-CD25^+CD127^{low/-}$ effector Treg cells were single-cell sorted into wells of a 96-well PCR plate. The gating strategy used to sort the effector Treg cells is presented in **Figure 1**.

TCR Repertoire Analysis of Effector Treg Cells by Single-Cell RT-PCR and Sequencing

TCRs and FoxP3 cDNAs were amplified from single cells using one-step multiplex RT-PCR as described previously (24). All PCR primers are listed in **Supplementary Table 1**. Contents of the PCR reaction mixture are listed in **Supplementary Table 2**. Five microliters of the RT-PCR mixture was added to each well containing a single effector Treg cell. One-step RT-PCR was performed with the following program: 40 min at 45°C for the RT reaction, 98°C for 1 min and 30 cycles of 98°C for 10 s, 52°C for 5 s, and 72°C for 1 min. The products were diluted 10-fold and 2 μL of each was added to 18 μL of the second PCR mixture. In the second cycle, TCRβ and FoxP3 cDNAs were amplified. The program for the second PCR was as follows: 98°C for 1 min and 35 cycles of 98°C for 10 s, 52°C for 5 s, and 72°C for 30 s. The second PCR products were used for direct sequencing to determine CDR3 of TCRβ. The TCR repertoire was analyzed with the IMGT/VQuest tool (http://www.imgt.org/). We classified effector Treg cells with identical CDR3 as the clonal population and those with unique CDR3 as the non-clonal population. To compare the clonality of the effector Treg cells, frequencies of clonal populations among the analyzed TCRs were calculated. For the assessment of TCR repertoire distribution, we calculated the Gini-coefficient as previously described (26). This coefficient was originally used in economic studies to describe income distribution. However, it is also useful to describe TCR repertoire distribution (26, 27). The Gini coefficient (G) is calculated as:

$$G = \sum_{i=1}^{n} (2i - n - 1)\, xi / n \sum_{i=1}^{n} xi$$

where "xi" indicates the abundance of the i^{th} sequence and "n" indicates the total number of TCR sequences. The score ranges from 0 to 1; $G = 1$ means that all the TCR clones are the same. When the variation of TCR repertoire is large, G approaches 0. The raw data supporting the conclusions of this manuscript, except the private information of the subjects, will be made available by the authors, without undue reservation, to any qualified researchers.

Statistical Analyses

Statistical analyses were performed with the JMP Pro 13.0.0 statistical analysis program (SAS Institute Inc., USA) and SPSS version 23 software (IBM, USA). The statistical tests used to determine statistical significance are indicated in the respective figure legends. Continuous variables are presented as median values with interquartile range, unless otherwise specified. A two-tailed $p < 0.05$ was considered significant.

RESULTS

Clinical Characteristics

Clinical characteristics of the subjects are shown in **Table 1**. Maternal ages of 1st trimester miscarriage and 3rd trimester normal pregnancy were higher than that of 1st trimester normal pregnancy. The frequency of cesarean section showed no significant difference between 3rd trimester normal pregnancy and preeclampsia.

TCR Repertoire Analysis of Effector Treg Cells in Normal Pregnancy

TCRβ and FoxP3 cDNAs were amplified from single cells and electrophoresed in agarose gel (**Figure 2A**). The number of analyzed TCRβ sequences of decidual effector Treg cells in 1st trimester normal pregnancy, 3rd trimester normal pregnancy, 1st trimester miscarriage with abnormal or normal embryo, and 3rd trimester pregnancy with preeclampsia for each subject was 49 (35.5–60), 46 (32.3–77.3), 36 (29–56), 43 (27–53), and 42 (31–45), respectively. The number of analyzed TCRβ sequences of effector Treg cells in PBMC in 1st trimester and 3rd trimester normal pregnancies was 70 (61.5–73.5) and 52 (43.5–60.8), respectively. Representative TCRβ repertoires in 1st trimester and 3rd trimester normal pregnancies are shown in **Figure 2B**. In the 3rd trimester, decidual effector Treg cells were clonally expanded and TCR repertoires were skewed (**Figure 2B**).

In normal pregnancy, the ratio of clonal populations of decidual effector Treg cells in the 3rd trimester was increased compared with the 1st trimester [4.5% (1.4–10.8%) vs. 20.9% (15.4–28.1%), $p < 0.001$]. In peripheral blood, the ratio for clonal populations of effector Treg cells was significantly smaller than that in paired decidual samples [0.0% (0.0–3.0%) vs. 4.5% (1.4–10.8%), $p = 0.039$ in the 1st trimester, 0.0% (0.0–3.3%) vs. 20.9% (15.4–28.1%), $p < 0.001$ in the 3rd trimester] and was not increased even in the 3rd trimester [0.0% (0.0–3.0%) vs. 0.0% (0.0–3.3%), $p = 0.935$; **Figure 3A**]. The Gini coefficient of the decidual TCRβ repertoire of effector Treg cells was higher in the 3rd trimester than in the 1st trimester [0.04 (0.02–0.09) vs. 0.22 (0.17–0.36), $p < 0.001$]. The Gini coefficient of the TCRβ repertoire of effector Treg cells in PBMC was lower than that in paired decidual samples in the 1st and 3rd trimesters [0.00 (0.00–0.03) vs. 0.04 (0.02–0.09), $p = 0.046$ in the 1st trimester, 0.00 (0.00–0.04) vs. 0.22 (0.17–0.36), $p < 0.001$ in the 3rd trimester; **Figure 3B**].

Frequencies of effector Treg cells among total Treg cells of 1st trimester decidua were significantly higher than that of peripheral blood [93.0% (82.4–94.6%) vs. 74.6% (58.0–91.4%), $p = 0.032$]. In the 3rd trimester, the p-value did not reach a significant level [87.0% (82.4–91.7%) vs. 79.6% (58.7–87.6%), $p = 0.094$; **Figure 3C**].

Next, we compared the TCR repertoires of peripheral blood and decidual effector Treg cells in each case. Common clonotype of effector Treg cells between PBMC and decidua appeared only

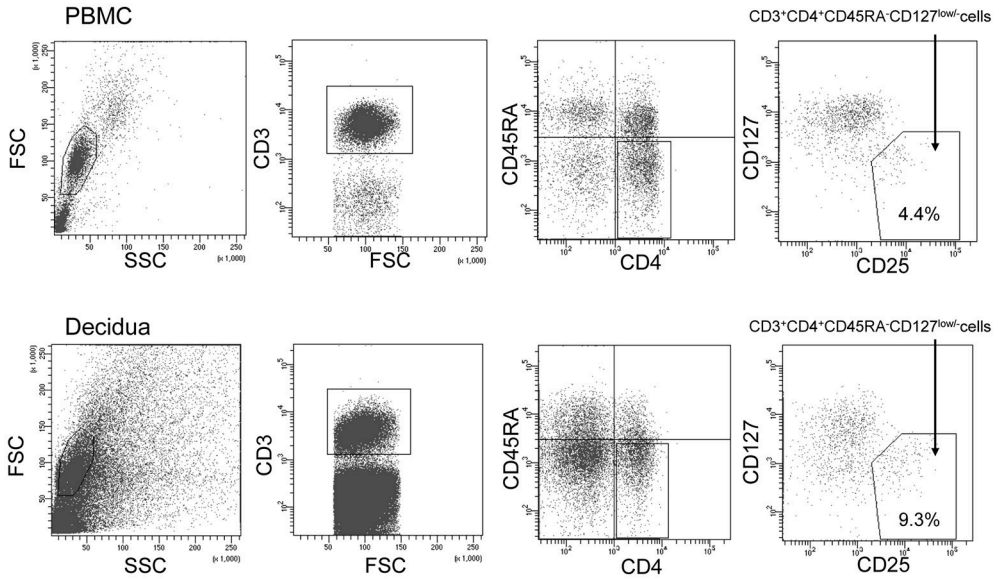


FIGURE 1 | Gating strategy to obtain $CD4^+CD45RA^-CD25^+CD127^{low/-}$ effector Treg cells. Lymphocyte in the peripheral blood (upper column) and decidua (lower column) were gated on forward and side scatter parameters. $CD3^+CD4^+$ T cells were classified into $CD45RA^+$ naïve T cells and $CD45RA^-$ effector T cells. Among $CD45RA^-$ effector T cells, $CD25^+CD127^{low/-}$ effector Treg cells were single-cell sorted.

TABLE 1 | Demographic and clinical characteristics.

	1st trimester normal pregnancy	3rd trimester normal pregnancy	1st trimester miscarriage with abnormal embryo	1st trimester miscarriage with normal embryo	3rd trimester preeclampsia
	(*n* = 9)	(*n* = 12)	(*n* = 11)	(*n* = 7)	(*n* = 7)
Maternal age (years), median (IQR)	28 (24–31)	36 (33–38)†	39 (38–41)†	38 (32–40)†	38 (31–41)
Gravidities, median (IQR)	3 (1–4)	5 (2–5)	3 (2–4)	3 (2–4)	2 (1–4)
No. of liveborn children, median (IQR)	0 (0–2)	0 (0–1)	0 (0–0)	0 (0–1)	0 (0–1)
No. of miscarriages, median (IQR)	0 (0–2)	2 (0–3)	1 (0–2)	1 (1–3)	(0–2)
Past history of stillbirth, *n* (%)	0 (0.0)	3 (25.0)	1 (9.1)	1 (14.3)	0 (0.0)
Nullipara, *n* (%)	5 (55.5)	5 (41.7)	8 (72.7)	3 (42.9)	4 (57.1)
Gestational weeks, median (IQR)	7 (7–9)	39 (38–40)	9 (8–10)	8 (8–10)	34 (32–37)
Cesarean section, *n* (%)		4 (33.3)			5 (71.4)

IQR, interquartile range. Steel–Dwass test for continuous variables and Fisher's exact test for categorical variables. $^{\dagger}p < 0.05$ *vs. 1st trimester normal pregnancy.*

in case #4 (two clones among 128 clones; 56 from decidua and 72 from PBMC) and case #16 (two clones among 82 clones; 40 from decidua and 42 from PBMC). The findings suggested marked differences in the characteristics of Treg cells between peripheral blood and decidua (**Figure 4**).

TCR Repertoire of Decidual Effector Treg Cells Between Previous and Subsequent Pregnancy

When repertoires of effector Treg cells were compared between past and subsequent pregnancies of the same subjects, sharing of some part of the repertoire of decidual effector Treg cells was evident. In case A, the TCR repertoires of cases #10 and #20, a pair of previous and subsequent 3rd trimester normal vaginal deliveries, shared three clones among 149 clones (**Figure 5A**; shared clones are underlined). In case B, the TCR repertoires of cases #13 and #17 revealed four shared clones among 129 clones (**Figure 5B**; underlined). In case C, case #25 was a previous pregnancy and resulted in miscarriage with normal embryo and case #47 was a subsequent pregnancy of the same subject and resulted in miscarriage with abnormal embryo. This pair shared one clone among 84 clones (**Figure 5C**; underlined). None of the TCR repertoires of effector Treg cells in PBMC were shared between previous and subsequent pregnancies (data not shown). Among the subjects who experienced pregnancies several times during the study period, no subject had their pregnancies ending up in preeclampsia.

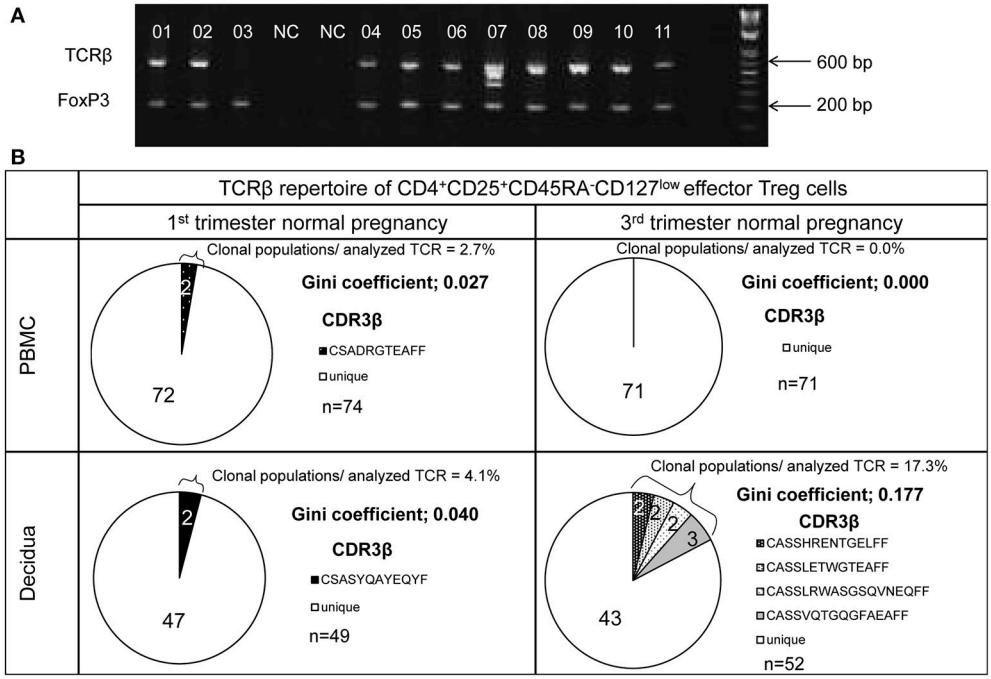


FIGURE 2 | TCRβ repertoire analysis of $CD4^+CD45RA^-CD25^+CD127^{low/-}$ effector Treg cells. **(A)** TCRβ and FoxP3 cDNAs amplified by multiplex one-step RT-PCR were resolved by agarose gel electrophoresis. Each single cell was numbered. NC denotes the cell-free negative control. FoxP3 and TCRβ mRNAs were expressed in 11/11 and 10/11, respectively. **(B)** Representative data of the TCRβ repertoire of $CD4^+CD45RA^-CD25^+CD127^{low/-}$ effector Treg cells in PBMC and decidua. The TCR repertoires were determined by amino acid and nucleotide sequences of complementarity determining region 3 (CDR3) of TCRβ chain. Each pie chart slice (shaded or closed) indicates clonal T cell population with the same clonotypic TCRβ. An open pie chart slice indicates T cells with unique TCRβ. Frequency of clonal populations among analyzed TCRβ and the Gini coefficients of the TCRβ repertoire were calculated.

TCR Repertoire of Decidual Effector Treg Cells of Normal Pregnancy and Miscarriage

In 1st trimester decidua, the frequencies of clonal populations of effector Treg cells and the Gini coefficients of TCR repertoires of miscarriage with abnormal embryo showed no significant differences between normal pregnancy, miscarriage with abnormal embryo, and miscarriage with normal embryo (**Figures 6A,B**). Proportions of $CD4^+CD45RA^-CD25^+CD127^{low/-}$ effector Treg cells among $CD4^+CD25^+CD127^{low/-}$ total Treg cells in decidua were significantly lower in miscarriage with normal embryo than normal pregnancy [80.0% (65.0–83.3%) vs. 90.6% (82.3–94.6%), $p = 0.049$; **Figure 6C**].

TCR Repertoire of Decidual Effector Treg Cells of Normal Pregnancy and Preeclampsia in 3rd Trimester

In 3rd trimester decidua, frequencies of clonal populations of effector Treg cells (**Figure 7A**) and Gini coefficients of TCR repertoires (**Figure 7B**) were significantly lower in preeclampsia than normal pregnancy [9.3% (4.4–14.5%) vs. 20.9% (15.4–28.1%), $p = 0.003$ and 0.09 (0.04–0.17) vs. 0.22 (0.17–0.36), $p = 0.005$, respectively]. The proportions of $CD4^+CD45RA^-CD25+CD127^{low/-}$ decidual effector Treg cells among $CD4^+CD25+CD127^{low/-}$ decidual total Treg cells did not differ significantly between the two groups [85.8% (71.1–88.4%) vs. 87.0% (82.4–91.7%), $p = 0.331$] (**Figure 7C**).

DISCUSSION

This study is the first report of the increase of clonally expanded decidual effector Treg cells in late gestation of normal pregnancy. In contrast, the repertoire of effector Treg cells of PBMC was not skewed in the 3rd trimester. A previous study showed that TRBV repertoires of total Treg cells differ in PBMC and decidua in the 3rd trimester (22). Our data support this finding. Neller et al. used 25 TRBV monoclonal antibodies in a TCR repertoire analysis. The analysis could not determine whether the skewed TCR repertoire occurred due to clonal expansion or not. We analyzed TCR repertoires more precisely based on CDR3 sequences that are a part of antigen binding site and provide high variety of TCR. Our results reveal that the skewed TCR repertoire in effector Treg cells in decidua, but not in peripheral blood during pregnancy, reflects clonal expansion of effector Treg cells in the decidua.

Concerning the relationship between the maldistribution or dysfunction of Treg cells and pregnancy complications, such as recurrent pregnancy loss or preeclampsia, the failure to maintain feto-maternal tolerance is thought to be one of the causes of these diseases (16–21). In the 1st trimester, miscarriage with normal embryo showed decreased population of decidual effector

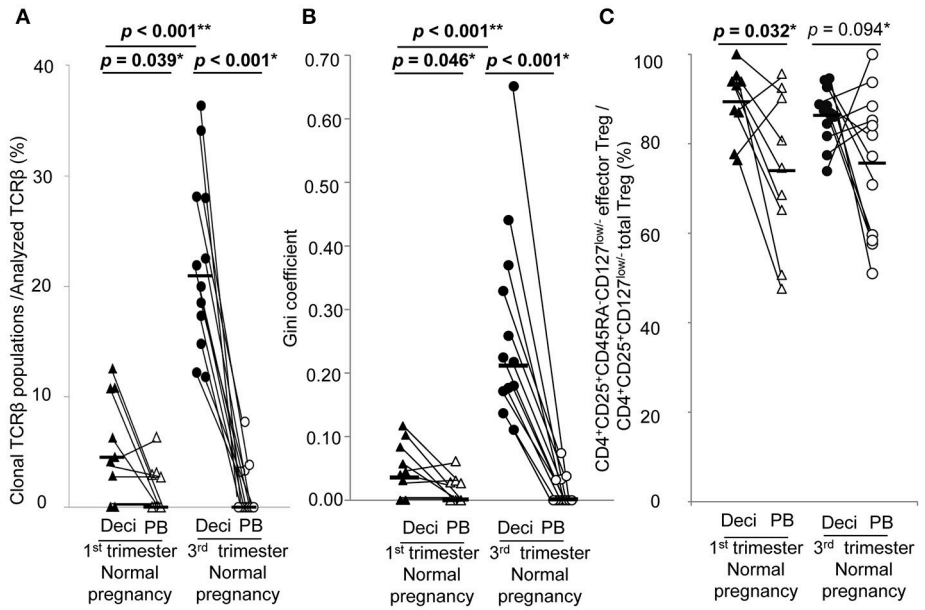


FIGURE 3 | TCRβ repertoire and flow cytometric analysis of effector Treg cells in 1st and 3rd trimester decidua and peripheral blood in normal pregnancy. **(A)** Frequencies of clonal populations among the analyzed TCRβ of effector Treg cells in 1st ($n = 9$) and 3rd trimester ($n = 12$) decidua and peripheral blood in normal pregnancies. **(B)** Gini coefficient of TCRβ repertoire of effector Treg cells. **(C)** Ratio of $CD4^+CD45RA^-CD25^+CD127^{low/-}$ effector Treg cells per $CD4^+CD25^+CD127^{low/-}$ total Treg cells. *p from Wilcoxon signed-rank test and **p from Mann–Whitney U-test. Each dot represents one donor, lines indicate median. Deci, decidua; PB, peripheral blood.

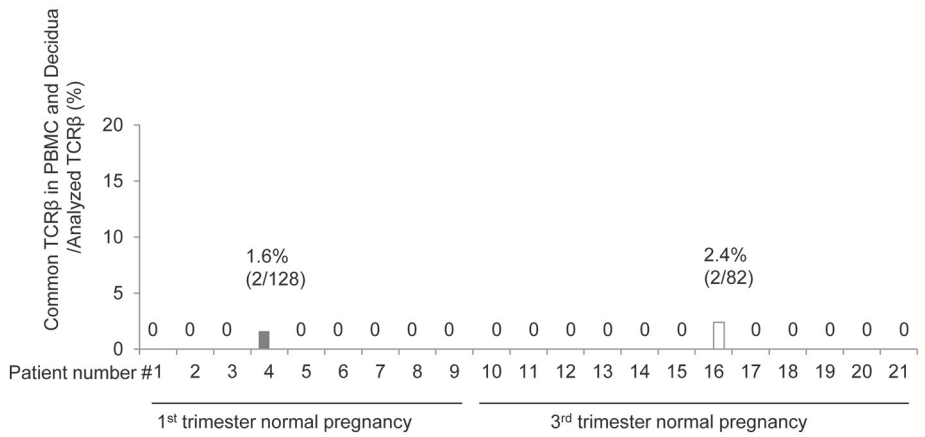


FIGURE 4 | Common clonotypic effector Treg cells between PBMC and decidua in 1st and 3rd trimester decidua and peripheral blood in normal pregnancy. Frequencies of common TCRβ in PBMC and decidua among analyzed TCRβ of effector Treg cells.

Treg cells compared with normal pregnancy. This result is consistent with our previous report of the decreased proportion of effector Treg cells in miscarriage with normal embryo than 1st trimester normal pregnancy (15). In contrast, the TCR repertoire in this study showed no significant skew between these populations (**Figure 6**). Taken together, our findings suggest that the decreased number of decidual effector Treg cells might be related to the pathogenesis of miscarriage with normal fetal karyotype, rather than an altered TCR repertoire. On the other hand, preeclampsia showed insufficient clonal expansion of decidual effector Treg cells compared with normal pregnancy in the 3rd trimester. In the 3rd trimester, effector Treg cells are the most dominant subset among Treg cells in PBMC and at the uteroplacental interface (14). The decrease in clonal populations of decidual effector Treg cells might be related to the pathogenesis of preeclampsia.

Concerning the TCR clonotypes of decidual effector Treg cells, we show for the first time that some TCR clonotypes in decidual effector Treg cells are shared between previous and subsequent pregnancies of the same subjects, but not those

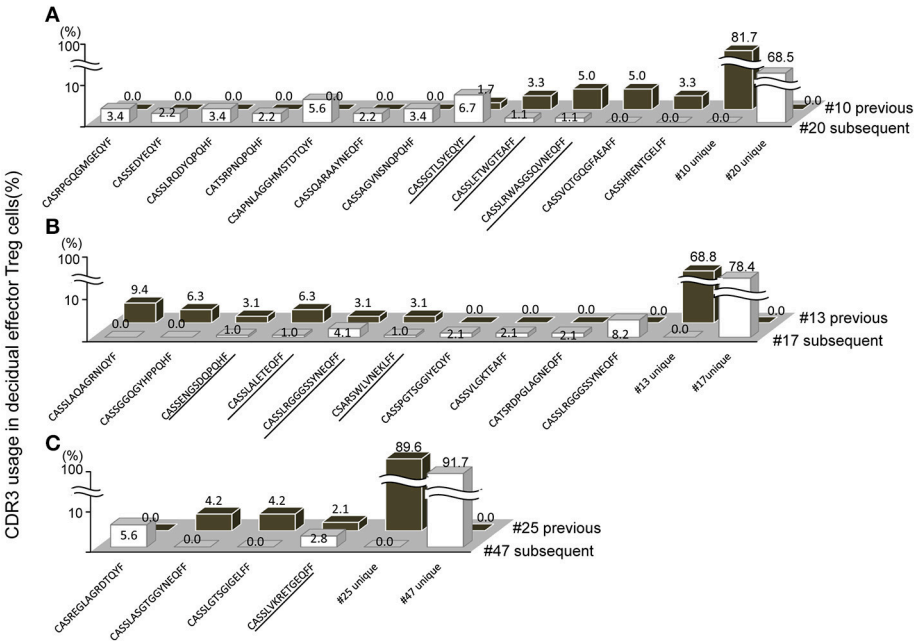


FIGURE 5 | Shared TCRβ repertoire of decidual effector Treg cells between previous and subsequent pregnancy. **(A–C)** Frequencies of decidual effector Treg cell clones expressing clonotypic TCRβ with the indicated CDR3 amino acid sequence. The underlined CDR3 sequences were shared in previous and subsequent pregnancies. **(A)** Case #10 previous pregnancy and case #20 subsequent pregnancy ending in the 3rd trimester with a normal vaginal delivery. **(B)** Case #13 previous pregnancy and case #17 subsequent pregnancy ending in the 3rd trimester with a normal vaginal delivery. **(C)** Case #25 previous pregnancy ending in miscarriage with a normal embryo and case #47 subsequent pregnancy ending in miscarriage with an abnormal embryo.

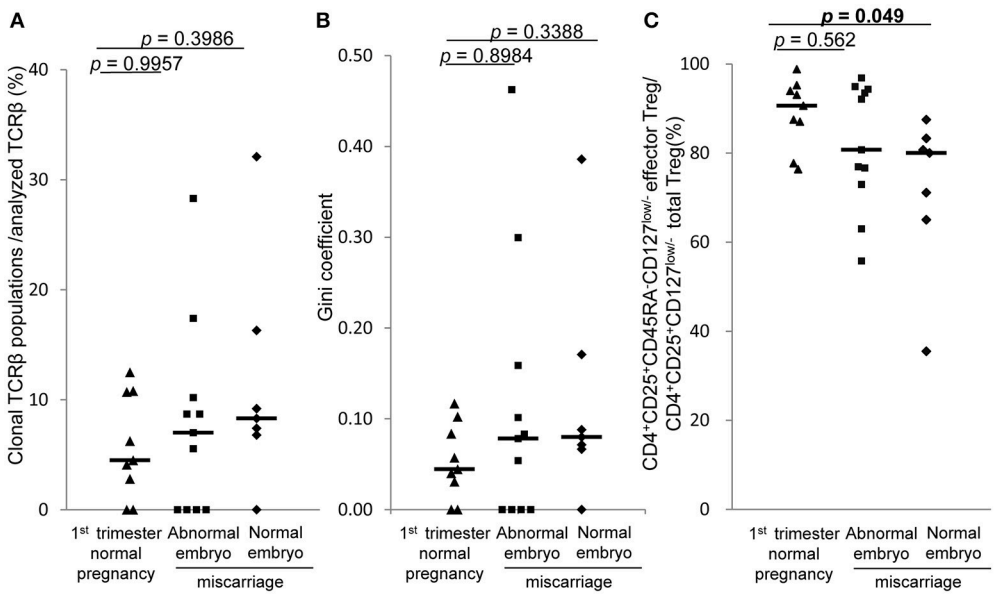


FIGURE 6 | TCRβ repertoire and flow cytometric analysis of decidual effector Treg cells in 1st trimester normal pregnancy and miscarriage. **(A)** Frequencies of clonal populations among analyzed TCRβ of effector Treg cells in 1st trimester normal pregnancy ($n = 9$) and miscarriage with abnormal embryo ($n = 11$) or normal embryo ($n = 7$). **(B)** Gini coefficient of TCRβ repertoire of effector Treg cells. **(C)** Ratio of $CD4^+CD45RA^-CD25^+CD127^{low/-}$ effector Treg cells per $CD4^+CD25^+CD127^{low/-}$ total Treg cells. *p from Steel's test. Each dot represents one donor; lines indicate median.

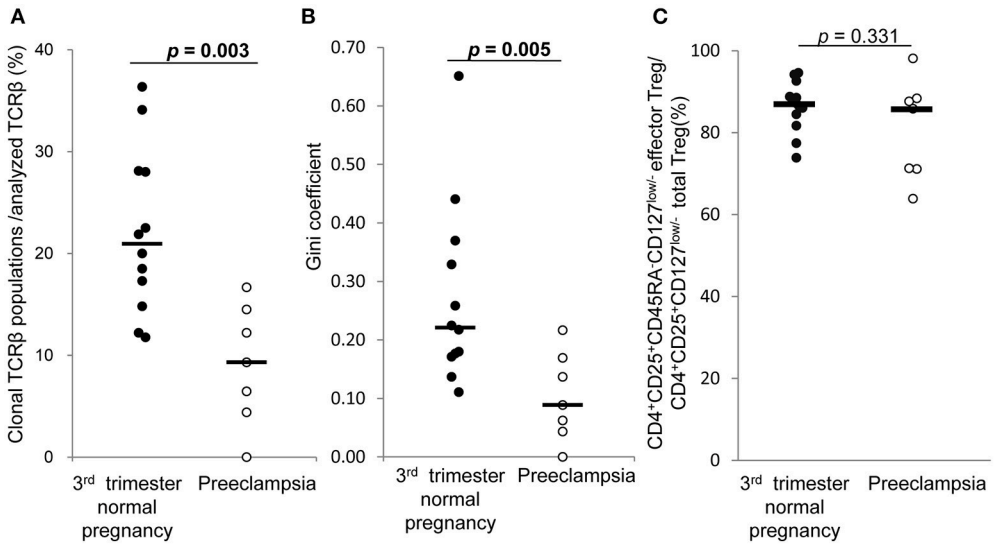


FIGURE 7 | TCRβ repertoire and flow cytometric analysis of decidual effector Treg cells in 3rd trimester normal pregnancy and preeclampsia. **(A)** Frequencies of clonal populations among analyzed TCRβ of effector Treg cells in 3rd trimester normal pregnancy ($n = 12$) and preeclampsia ($n = 7$). **(B)** Gini coefficient of TCRβ repertoire of effector Treg cells. **(C)** Ratio of $CD4^+CD45RA^-CD25^+CD127^{low/-}$ effector Treg cells per $CD4^+CD25^+CD127^{low/-}$ total Treg cells are shown. *p from Mann–Whitney U-test. Each dot represents one donor; lines indicate median.

in PBMC. The lower limit for the number of different CDR3 amino acid sequences in TCRβ is estimated to be $\sim 2 \times 10^7$ in young humans (28). This indicates that there is a very low probability of coincidence of the CDR3 amino acid sequence between two independent Treg cells if there is no force to skew the populations. Thus, effector Treg cell clones shared between previous and subsequent pregnancies might be recruited by reacting to the same antigens expressed in feto-maternal interface, suggesting that fetal antigen-specific Treg cells might accumulate at the feto-maternal interface.

The existence of fetal antigen-specific Treg cells and those recruited to the feto-maternal interface during pregnancy were reported in mice (8–10). An examination of functional differences of Treg cells from PBMC and decidua in humans led to the suggestion that decidual Treg cells contain fetal-antigen specific populations (11). However, fetal antigen-specific Treg cell clones have not been identified in humans. The present finding of common Treg cell clones in the decidua in previous and subsequent pregnancies raises the possibility that these clones might react to antigens expressed in feto-maternal interface and, thus, could be candidates of fetal antigen-specific Treg cells. Regarding the target antigen presenting cells for Treg cells in human decidua, it has been reported that HLA-C mismatched pregnancies feature an increased amount of Treg cells and higher activation of conventional T cells than non-mismatched pregnancies (29). HLA-C, E, F, and G are expressed in extravillous trophoblasts (30–33). These expressed HLAs might be potent antigens recognized by Treg cells.

Some limitations of our study have to be considered. Firstly, we could not amplify TCRα as efficiently as TCRβ. Thus, the TCR repertoire was analyzed based on TCRβ and not paired TCR. Secondly, our single cell TCR repertoire analysis covered only a limited number of Treg cell clones compared with the bulk TCR repertoire analysis by next-generation sequencing. Thirdly, the functionality of TCRs was not assessed. TCRs derived from antigen-specific Treg cells and their function were demonstrated by using TCR^{mini} mice, whose TCRβ is identical in all T cells (34, 35). In humans, a functional assay of target-specific polyclonal Treg cells has been developed (27). However, a direct functional assay of TCRs obtained from Treg cells is still not available, reflecting the limited knowledge about epitopes from physiological targets of Treg cells (27, 36). TCRs from tumor specific conventional $CD4^+$ T cells and $CD8^+$ T cells were identified using single-cell RT-PCR methods (23, 24, 37, 38). We also demonstrated a rapid and efficient functional assay for TCRs directly obtained from $CD8^+$ T cells using TCR-transduced lymphocytes in the absence of information on antigen or MHC haplotype (38). We tried to apply this technique to mixed lymphocyte reaction of paired αβ TCR-transduced maternal T cells or TCR-negative T cell lines against self-fetal umbilical cord blood but were unsuccessful. An assay method capable of identifying target-specific Treg or $CD4^+$ T cells using TCR-transduced responder cells in the absence of information concerning their antigen or MHC haplotype, to the best of our knowledge, has not been reported yet. It might be technically more difficult than analysis of TCRs obtained from $CD8^+$T cells, thus novel strategy might be needed to improve assay method of TCRs obtained from $CD4^+$ T cells. When the assay method is established, the function of obtained TCRs will be explored. Fourthly, we have not been able to demonstrate a relationship between the TCR clonotype and effector molecule expression. Zemmour et al. showed that Treg cells, which share the same

TCR clonotype, display similar transcriptional identities by single-cell analysis in mice (39). Combined single-cell TCR and gene expression analysis should be addressed, thereby providing further insight into features of heterogeneous decidual Treg cells.

In summary, we show for the first time that effector Treg cells are clonally expanded in 3rd trimester decidua, but not in peripheral blood in humans. In preeclampsia, the TCR repertoires of decidual effector Treg cells were not skewed. Clonally expanded effector Treg cell populations might be more important in the 3rd trimester than in the 1st trimester. Insufficient expansion of clonal effector Treg cells in decidua might be an etiological aspect in the development of preeclampsia. On the other hand, the frequency of effector Treg cells among the total Treg cells decreased in cases of miscarriage with normal fetal chromosomal content, but clonal effector Treg cells did not decrease. Our findings further the understanding of the mechanisms of feto-maternal tolerance and could provide clues for understanding the different aspects of the pathophysiology of preeclampsia and miscarriage.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Ethical Guidelines for Medical and Health Research Involving Human Subjects, the Ministry of Health, Labor and Welfare, Japan. The protocol was approved by the ethics review committee of University of Toyama (Rin 28-144). All subjects gave written informed consent in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

SS, HK, and AM: conception and design; ST, XZ, and AU: acquiring and processing samples; ST, XZ, HK, HH, AU, and KT: execution of experiment; ST and XZ: analysis of data; ST, XZ, HK, HH, TS, and SS: interpretation of data; ST, TS, and SS: drafting manuscript; XZ, HK, HH, TS, AU, KT, and AM: revision of the manuscript for important intellectual content.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.01934/full#supplementary-material

Supplementary Table 1 | Primers for single-cell RT-PCR.

Supplementary Table 2 | Contents of single-cell RT-PCR reaction mix.

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

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REVIEW
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Prenatal Immune and Endocrine Modulators of Offspring's Brain Development and Cognitive Functions Later in Life

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Milestones of brain development in mammals are completed before birth, which provide the prerequisite for cognitive and intellectual performances of the offspring. Prenatal challenges, such as maternal stress experience or infections, have been linked to impaired cognitive development, poor intellectual performances as well as neurodevelopmental and psychiatric disorders in the offspring later in life. Fetal microglial cells may be the target of such challenges and could be functionally modified by maternal markers. Maternal markers can cross the placenta and reach the fetus, a phenomenon commonly referred to as "vertical transfer." These maternal markers include hormones, such as glucocorticoids, and also maternal immune cells and cytokines, all of which can be altered in response to prenatal challenges. Whilst it is difficult to discriminate between the maternal or fetal origin of glucocorticoids and cytokines in the offspring, immune cells of maternal origin—although low in frequency—can be clearly set apart from offspring's cells in the fetal and adult brain. To date, insights into the functional role of these cells are limited, but it is emergingly recognized that these maternal microchimeric cells may affect fetal brain development, as well as post-natal cognitive performances and behavior. Moreover, the inheritance of vertically transferred cells across generations has been proposed, yielding to the presence of a microchiome in individuals. Hence, it will be one of the scientific challenges in the field of neuroimmunology to identify the functional role of maternal microchimeric cells as well as the brain microchiome. Maternal microchimeric cells, along with hormones and cytokines, may induce epigenetic changes in the fetal brain. Recent data underpin that brain development in response to prenatal stress challenges can be altered across several generations, independent of a genetic predisposition, supporting an epigenetic inheritance. We here discuss how fetal brain development and offspring's cognitive functions later in life is modulated in the turnstile of prenatal challenges by introducing novel and recently emerging pathway, involving maternal hormones and immune markers.

Keywords: pregnancy, fetal brain development, prenatal infection, maternal distress, maternal microchimeric cells, cytokines, glucocorticoids (GC), epigenetic aberrations

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INTRODUCTION

The vertical transmission of maternal immune and endocrine markers is increasingly recognized to modulate fetal neurodevelopment and future mental health of the offspring. Emerging evidence arising from observational studies in humans reveals that prenatal environmental challenges such as maternal distress perception and infections are associated with an impaired fetal neurodevelopment and increased risk for neurological or psychiatric disorders later in life (1–6). Insights into the underlying mechanisms and pathogenesis of prenatally programmed poor mental health are increasingly emerging. It is well known that neurodevelopment results from the interaction of genetic, epigenetic and environmental factors, through which proliferation, migration of neural progenitor cells and establishment of neuronal circuits are modulated. Disruptions of these neurodevelopmental pathways may subsequently affect future brain function, as reflected by cognitive and intellectual impairment and increased the risk for neurodevelopmental and psychiatric disorders later in life (7).

Here, we compile the currently available evidence arising from observational studies supporting the concept of a developmental origin of brain disorders. We further outline cornerstones of brain development in mice and humans and discuss the effect of prenatal challenges, primarily maternal distress and infections, on maternal immune-endocrine adaptation to pregnancy. Lastly, we introduce novel concepts on how an altered maternal immune-endocrine adaptation to pregnancy can impact on offspring's brain development and subsequent mental health.

Developmental Origin of Neurological Dysfunctions and Psychiatric Disorders

Pregnancy is characterized by significant adaptational processes of the maternal immune and endocrine system in order to ensure its progression until term, which is required for adequate fetal development. These adaptational processes are highly responsive and vulnerable to challenges, such as high maternal stress perception or maternal infections. In this context, chronic stress states (e.g., depression or anxiety) affect approx. 10–15% of pregnant women worldwide (8). Moreover, negative life events may pose a significant threat to maternal wellbeing during pregnancy. **Table 1** provides a comprehensive overview of published evidence largely arising from observational studies that reveal a significant association between various types of maternal distress perception to which the mother was exposed to at specific gestational periods and the risk for psychiatric disorders in the offspring later in life, i.e., during childhood or adolescence. Disorders observed in the offspring include autism spectrum disorder (ASD) (9), depressive symptoms (10–12), anxiety, borderline personality disorder, eating disorders (23) and attention-deficit/hyperactivity disorder (ADHD) (3, 14–20, 22, 45). Interestingly, whilst a sex-specific risk is well known for psychiatric disorders, only very few studies paid attention whether prenatal stress perception skews the risk for such disorders in a sex-specific way. One study describes a sex-bias for ADHD upon prenatal stress, mirrored by a higher risk in daughters (20). Moreover, the timing of the prenatal challenge may be pivotal, as the risk for neurodevelopmental diseases appears to be differentially affected by the trimester of exposure (**Table 1**). In fact, surges of maternal IL-6 levels during the third trimester—which may result from distress or infections—showed a strongest impact on working memory performance in children. These behavioral changes were associated with alterations of brain regions tightly associated with working memory, as identified by functional MRI (46).

Besides high stress perception, maternal infection during pregnancy can interfere with fetal neurodevelopment and increase the risk for neurological dysfunctions and psychiatric disorders in the offspring (**Table 2**). Here, most of the studies focus on the distinct pathogens that have led to maternal infection during pregnancy (1, 35, 37, 42, 43). For example, maternal infection with influenza A or B virus has been associated with an increased risk for developing schizophrenia, although findings between studies are highly ambiguous and hence, hotly debated. Some studies report that influenza infection during the first trimester may trigger the risk for schizophrenia, whilst such effect could not be confirmed in studies focusing on infection at mid to late pregnancy (29–31, 47). The latter includes observations arising from Scandinavian registry analyses, where the query for prenatal influenza infection was solidly based on International Classification of Diseases (ICD)-coded diagnoses (48, 49). A recent meta-analysis confirms that evidence is insufficient to support gestational influenza as a risk factor for schizophrenia and bipolar disorder in the offspring (50). Besides these viral infections, also bacterial infections during pregnancy have been linked to an increased risk for schizophrenia in the offspring in adulthood (36, 44). For instance, 13% of all children surviving the maternal listeria monocytogenes infection were suffering from meningitis in early childhood (38) and this in return is significantly associated with developing schizophrenia and psychotic episodes later in life (39).

Milestones of Brain Development in Mice and Humans

Given that some of the observational studies summarized above highlight that the time point at which challenges occur prenatally may be crucial to trigger changes in fetal neurodevelopment, we here provide a brief summary of key aspects of fetal brain development that commence or are completed at distinct periods of gestation. We also include the brain development of mice, as mouse models have become pivotal in understanding how prenatal challenges affect neurodevelopment and brain functions later in life, as outlined in the subsequent paragraphs.

In humans and mice, brain development shows similar developmental processes (**Figure 1**). Noteworthy, sex-specific differences occur as a result of a faster cerebral maturation in girls (55), which leaves boys at higher risk for challenges-induced disruptions due to the greater window of vulnerability. Some developmental steps continue after birth in mice, which are already completed at birth in humans. This reduces the time window in mice during which vertically transferred maternal biological mediators can impact on offspring's brain development, a confounder which should be considered when

TABLE 1 | Summary of human studies examining the effect of prenatal maternal distress on offspring's mental health.

Proxy for prenatal maternal distress	Offspring's age at outcome evaluation	Offspring's outcome	Reference
INCIDENCE DURING THE FIRST TRIMESTER			
Exposure to a natural disaster	Childhood-preadolescence	ASD symptoms	(9)
Questionnaires-based evaluation of stress perception	Adolescence-adulthood	Internalizing and externalizing problems Depressive symptoms	(10–12)
Self-reported stressful events	Birth-adulthood	No risk for psychosis	(13)
INCIDENCE DURING THE SECOND TRIMESTER			
Exposure to a natural disaster	Childhood-preadolescence	ASD symptoms	(9)
Questionnaires-based evaluation of depression	Childhood-adolescence	Internalizing and externalizing problems Anxiety symptoms Depressive symptoms Hyperactivity Borderline personality disorder	(3, 14–18)
Self-reported stressful events	Childhood-adolescence	Internalizing problems Depressive symptoms ADHD symptoms No association to total psychiatric problems No risk for psychosis	(13, 19–21)
Questionnaires-based assessment of anxiety	Child-preadolescence	Total psychiatric problems Internalizing and externalizing problems Anxiety symptoms Depressive symptoms Hyperactivity	(14, 22)
Saliva cortisol	Preadolescence	ADHD symptoms especially in boys	(21)
INCIDENCE DURING THE THIRD TRIMESTER			
Exposure to a natural disaster	Childhood-preadolescence	ASD symptoms Eating disorder symptoms	(9, 23)
Questionnaires-based evaluation of depression	Preadolescence-adolescence	Externalizing problems Hyperactivity	(18, 24)
Self-reported stressful events	Birth–adulthood	ADHD symptoms No association to total psychiatric problems No risk for psychosis	(13, 20, 21)
Questionnaires-based assessment of anxiety	Childhood-adolescence	Internalizing problems Depressive symptoms Hyperactivity	(3, 14, 15)
Saliva cortisol	Preadolescence	ADHD symptoms especially in girls	(21)
Physician-based diagnoses of depression	Childhood-adulthood	Depressive symptoms ADHD symptoms	(6, 25)
ICD-based diagnoses of anxiety	Childhood	ADHD symptoms	(6)
INCIDENCE DURING PREGNANCY WITHOUT FURTHER TRIMESTER SPECIFICATION			
Self-reported stressful events	Childhood-adulthood	Eating disorders ASD symptoms Schizophrenia in male offspring	(26–28)

discussing the biological relevance of findings on prenatal challenges in mice. Hence, when evaluating the impact of maternal markers on fetal development in mice, research endeavor should focus on milestones that are completed prior to birth, such as neurulation, neuronal migration and microglia invasion, as well as synaptogenesis and neurogenesis, the latter being largely completed at birth.

Effect of Prenatal Challenges on Maternal Immune-Endocrine Adaptation to Pregnancy

In mice and humans, healthy brain development is crucially dependent on an endocrine and immunological homeostasis of the mother-fetus dyad. Here, the balance between pro- and anti-inflammatory cytokines is crucial, as neurogenesis, migration, differentiation and apoptosis are well known to be responsive to cytokine challenges (**Table 3**). Besides cytokines, chemokines are also important in modulating neurodevelopment and the risk for psychiatric diseases. Since these interactions have been addressed in recent reviews (80–82), we refrain from including them here. Similarly, glucocorticoids, which are initially largely maternally derived during gestation in the fetus, can interfere with fetal brain development in mice and humans. Moreover, additional factors such as maternal immune cells can affect fetal brain development. Prenatal stimuli and challenges, including maternal stress perception and infection, have been show to interfere with the

TABLE 2 | Overview of studies examining the effects prenatal infection and related maternal immune activation on offspring's mental health in human.

Gestational time point of infection	Proxy used to identify prenatal infection	Offspring's age at evaluation	Offspring's outcome	Reference
VIRAL INFECTIONS DURING PREGNANCY				
Each trimester	Maternal antibodies against Influenza virus A and B	Adulthood	Increased risk for schizophrenia	(29–31)
End of pregnancy	Maternal antibodies against cytomegalovirus, rubella virus, human parvovirus B19, herpes simplex virus 1 and 2	Lifetime	Increased risk for schizophrenia Increased risk for psychosis	(32, 33)
Newborn's blood (5–7 days old)	Maternal antibodies against herpes simplex virus 2	Lifetime	Increased risk for schizophrenia	(34)
Pregnancy	Retrospective estimation infection	2–5 years	Maternal fever, but not influenza, increases the risk for schizophrenia	(35)
BACTERIAL INFECTIONS DURING PREGNANCY				
End of pregnancy	Maternal antibodies against chlamydia trachomatis	Lifetime	Increase risk for schizophrenia	(32)
Pregnancy	Retrospective estimation	32–34 and 45–47 years	Increased risk for schizophrenia	(36)
Pregnancy	Maternal C-reactive protein	ASD diagnosis	Increased risk for ASD	(37)
Pregnancy	ICD-based registry queries	–	Increased risk for schizophrenia and psychotic episodes via meningitis in childhood	(38, 39)
PARASITIC INFECTIONS DURING PREGNANCY				
Each trimester	Maternal antibodies against toxoplasma gondii	24–30 years	Increased risk for schizophrenia	(30, 40)
End of pregnancy	Maternal antibodies against toxoplasma gondii	Lifetime	Increased risk for schizophrenia	(41)
NON-CLASSIFIED PRENATAL INFECTIONS				
Pregnancy	ICD-based registry queries	Lifetime	Increased risk for ASD Increased severity of ASD symptoms compared to non-infected ASD offspring	(1, 42, 43)
Pregnancy	ICD-based registry queries	Lifetime	Maternal infection before and after pregnancy increased the risk for schizophrenia	(44)

endocrine and immunological hemostasis in the mother. This includes the activation of the sympathetic nervous system and the hypothalamus-pituitary-adrenal (HPA) axis, subsequently leading to an excess secretion and availability of free, biologically active glucocorticoids (83, 84). Pregnancy itself is considered to be a state of "hypercortisolism," which is an essential requirement to meet the maternal demand for increased metabolism and energy generation. The fetus is also critically dependent on the transfer of maternal glucocorticoids, which is controlled for by placental enzymes such as 11ß-Hydroxysteroid-Dehydrogenase (11ß-HSD)-1 and -2 (83, 84). Maternal glucocorticoids ensure structural and functional development of fetal organs, as the fetus is not capable of producing glucocorticoids until late in development. However, elevated glucocorticoid concentrations in the context of maternal stress may negatively impact on fetal brain development (83).

Besides the effect of prenatal challenges on maternal glucocorticoid levels, the maternal immune response may also be skewed toward inflammation in the context of stress in human pregnancy or infection (85, 86). Similarly in mice, maternal stress challenges have been shown to increase levels of pro-inflammatory cytokines in dams (87) and decrease tolerogenic markers such as $CD4^+$ regulatory T cells (88). Similarly, prenatal infection e.g., with influenza A virus in mice leads to an increased type 1 response, along with an increased production of pro-inflammatory cytokines, compared to non-infected pregnant mice (89). Equally, the use of "danger signals" such as lipopolysaccharide to induce an inflammatory response in pregnant mice resulted in a collapse of immune tolerance toward the fetus (90).

Maternal cytokines, glucocorticoids as well as maternal immune cells can cross the placenta. Whilst it is difficult to determine if cytokines are maternally derived or produced by the fetus, maternal immune cells can be clearly identified in the offspring. These maternal microchimeric cells can persist in the offspring long after birth (91). Hence, opposed to the vertical transfer of maternal cytokines and glucocorticoids, maternal microchimeric cells may be capable to continuously modulate brain function in the offspring even after birth.

Impact of Altered Maternal Immune-Endocrine Adaptation to Pregnancy and Offspring's Brain Development and Mental Health

The vertical transfer of maternal immune and endocrine markers is increasingly recognized to modulate fetal neurodevelopment and future mental health of the offspring. As mentioned above,

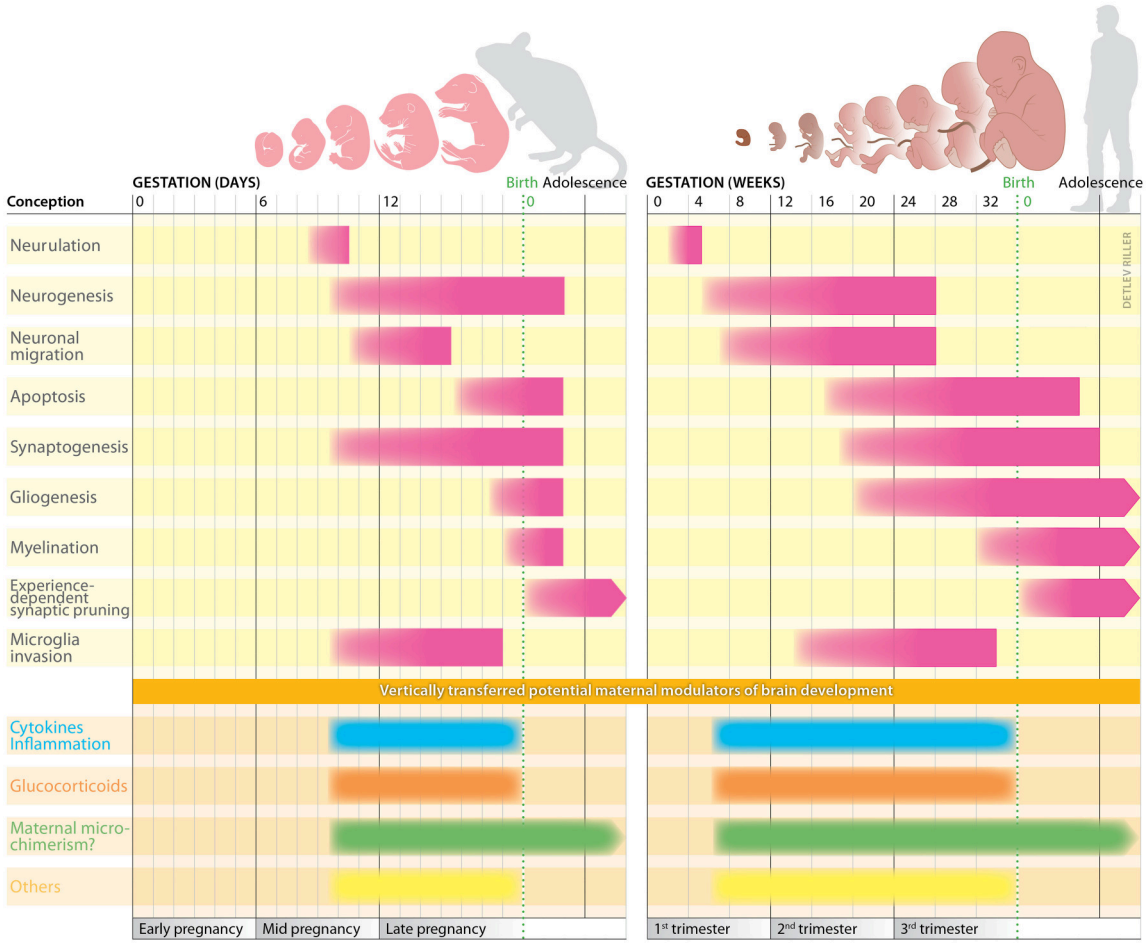


FIGURE 1 | Milestones of brain development in mice and humans. In **both species,** brain development commences with neurulation, a process creating the neural tube. This provides the prerequisite for the subsequent production of neuron from neural stem cells, a process defined as neurogenesis. Early during human development, at gestation week 4, the anterior part of the neural tube begins to form into distinct regions. The forebrain, midbrain and hindbrain are defined as the anterior part; the spinal cord is located at the posterior part. Two weeks later, the neural tube can be clearly divided into the brain regions that are present at birth. Some of the previously produced neurons now start to migrate to distinct brain regions, a process that continues until approx. week 26. Earlier by week 11, the cerebrum has developed—more rapidly than other structures—and largely covers the entire brain, except cerebellum and medulla oblongata. Due to its progressive development within the cranium, the cerebrum is forced to convolve itself resulting in gyri and sulci (51). During the second trimester of pregnancy, several processes start to define brain connectivity. These include synaptogenesis, gliogenesis, and apoptosis. Simultaneously, microglial cells invasion begins. By mid-third trimester, the fine-tuning of neuronal connectivity starts with proliferation of myelin sheaths throughout the neurons of the central nervous system (52). Shortly after birth, the previously established neuronal connections are reduced based on neuronal activity, meaning a reduction of neuronal connections to the ones often used. Since murine gestation is much shorter compared to human pregnancy, some developmental steps continue to proceed after birth. In mice, neural development begins during mid-pregnancy, followed by neurulation and formation of the neural tube (53). Subsequently, production of neurons, their migration and the formation of synapses occur almost simultaneously. Also, yolk sac-derived microglial cells invade the fetus starting on day 9 of pregnancy (54). The developmental milestones underlying brain development in mice and humans are highly susceptible to challenges and can be modulated by maternal markers vertically transferred during pregnancy. Hereby, the time point and intensity of the challenges clearly determine the impact and damage they may cause.

the impact of cytokines on fetal brain development has been well studied and it is widely accepted that milestones of physiological fetal brain development are modulated by cytokines (**Table 3**). Hence, exposure to an imbalanced cytokine response during fetal life may disturb fetal brain development, thereby increasing the risk for neurodevelopmental disorders (92).

In mice, fetal exposure to an altered, pro-inflammatory maternal cytokine response can affect brain morphology, mirrored by e.g., an increased pyramidal cell density or reduced neurogenesis (93–96). Similarly, prenatal maternal treatment with the immunostimulant polyinosinic:polycytidylic acid (poly I:C) to mirror some effects of a viral infection has been shown to result in reduced axonal size, myelin thickness and cortical volume of the hippocampus and amygdala in rodent offspring (97, 98). A general maternal immune activation during pregnancy has also been shown to cause presynaptic deficits in hippocampus (99), pro-inflammatory activation in hippocampal microglia (100) or an increase of microglial cell frequencies in the fetal brain (101). Hence, a wealth of studies has shown that maternal immune activation during pregnancy adversely affects

TABLE 3 | Key cytokines influencing neural cell development.

Cytokine	Functional outcome	Reference
IL-1α	Supports astrocyte lineage commitment	(56)
	Increases neurogenesis	(57)
	Increases microglia activity	(58)
IL-1β	Inhibition of neurogenesis	(59–61)
	Supports astrocyte lineage commitment	(58, 59)
IL-6	Supports establishment synaptic connectivity	(62, 63)
	Suppresses astrocyte development	(64)
TNF-α	Inhibition of neural progenitor cell proliferation and differentiation (via TNF receptor 1)	(65)
	Supports cell survival (via TNF receptor 2)	(65)
	Neuroprotective functions	(66)
IL-2	Increases differentiation of neural progenitor cells	(67)
	Increases neurogenesis	(68)
IFN-γ	Supports differentiation, migration and neuronal outgrowth (via microglia activation)	(69)
IL-10	Increases neural progenitor cell survival, differentiation and neuronal myelination	(70)
	Supports oligodendroglia progenitor cell survival Increases neural progenitor cell migration	(71) (72)
IL-4	Neuroprotection (via microglia activation)	(69)
TGF-β	Excessive concentration leads to declined neurogenesis	(73)
LIF	Promotion of cell growth (via inhibition of differentiation)	(74)
	Supports neural stem cell self-renewal	(75, 76)
IL-17	Inhibition of adult neurogenesis and synaptic function, decreases neural stem cell numbers, proliferation and differentiation	(77–79)

fetal brain development on multiple levels. Yet, it is difficult to comprehensively pinpoint distinct pathways, as the studies performed to date have been rather diverse with regard to the species, the gestational time point or the cause of maternal immune activation (102). In humans, a great deal of research focused on the role of maternal levels of interleukin-6 as a proxy for a prenatal inflammatory challenge. Key observations include that maternal IL-6 levels affects offspring's structural and functional connectivity already at the time of birth (103), delaying the development of sensory and cognitive processing (46, 104).

Similar to cytokines, fetal exposure to elevated concentrations of maternal glucocorticoids has been proposed to exert long-lasting, partly sex-specific effects on offspring's brain morphology and function in rodents but also in humans. This includes decreased dendritic morphology and neuronal volume in hippocampi of both sexes in mice, whereas an increased dendritic branching has been detected only in females (105). Another study using mouse models reports an increased spine density, dendritic length and other morphological features in pyramidal neurons of prenatally stressed males, which was decreased in females (106).

In humans, elevated maternal cortisol concentrations in early pregnancy have been associated with larger amygdala volumes, an altered neural connectivity, along with affective symptoms and internalizing problems in girls (107). Interestingly, moderately elevated maternal cortisol levels in late pregnancy could be associated with greater cortical thickness primarily in frontal regions and enhanced cognitive performance in children (108). These findings point to the importance of considering the moderating role of sex and timing of exposure to glucocorticoids during pregnancy.

Since the experimental or study designs differ with regard to stress paradigms/glucocorticoids applications, time point of prenatal interventions or postnatal analyses, species and read-out parameter (109–111), it is not yet possible to comprehensively summarize the outcome of these studies beyond the statement that prenatal stress and related glucocorticoid surges induce sex- and brain area-specific differences in neuronal complexity and neurogenesis. This may subsequently lead to altered cognitive functions later in life.

Microglial cells, which are the resident macrophages of the CNS, have also been extensively studied in response to prenatal stress or prenatal glucocorticoid application. Due to their phagocytic phenotype, microglia have a functional role in remodeling, shaping and pruning of synapses (112). And indeed, prenatal corticosterone application increased the microglial density in the embryonic brain overall and promoted their amoeboid phenotype (113). Interestingly, this result was supported by isolating and culturing P1-2 microglial cells, showing that they are more likely to acquire an amoeboid phenotype (114). At postnatal age, stress increased the total glia cell number in the hippocampus of female but not male offspring (115). One study showed an increased number of microglia cells in the dentate gyrus, suggesting an adverse effect on neurogenesis and simultaneously no changes in the CA1 of the hippocampus (116, 117). The early stress exposure seems to support phagocytic microglia function in the early brain and potentially stimulate their activity throughout early murine lifetime. Their role as a potential mediator between prenatal stress and early neurobiological changes remains vague, especially in other important brain areas such like the PFC.

The barrier between periphery and the brain is an essential interface for communication between both compartments. Even though the blood brain barrier is supposed to be well established during early development, its function in stress and cytokine-related diseases is poorly investigated. However, its permeability is a key component in how maternal markers may influence the offspring's brain development. This topic is excellently described in an comprehensive review elsewhere [see review, (118)] and thus not explained here.

In **Figure 2**, we depict key effects of cytokines/inflammation and glucocorticoids on microglia cells and neurons in the prefrontal cortex, hippocampus and amygdala of the fetal brain. We deliberately focused on these specific brain regions due to their specific relevance for the mental health problems that have been described in the context of maternal stress and infection (122).

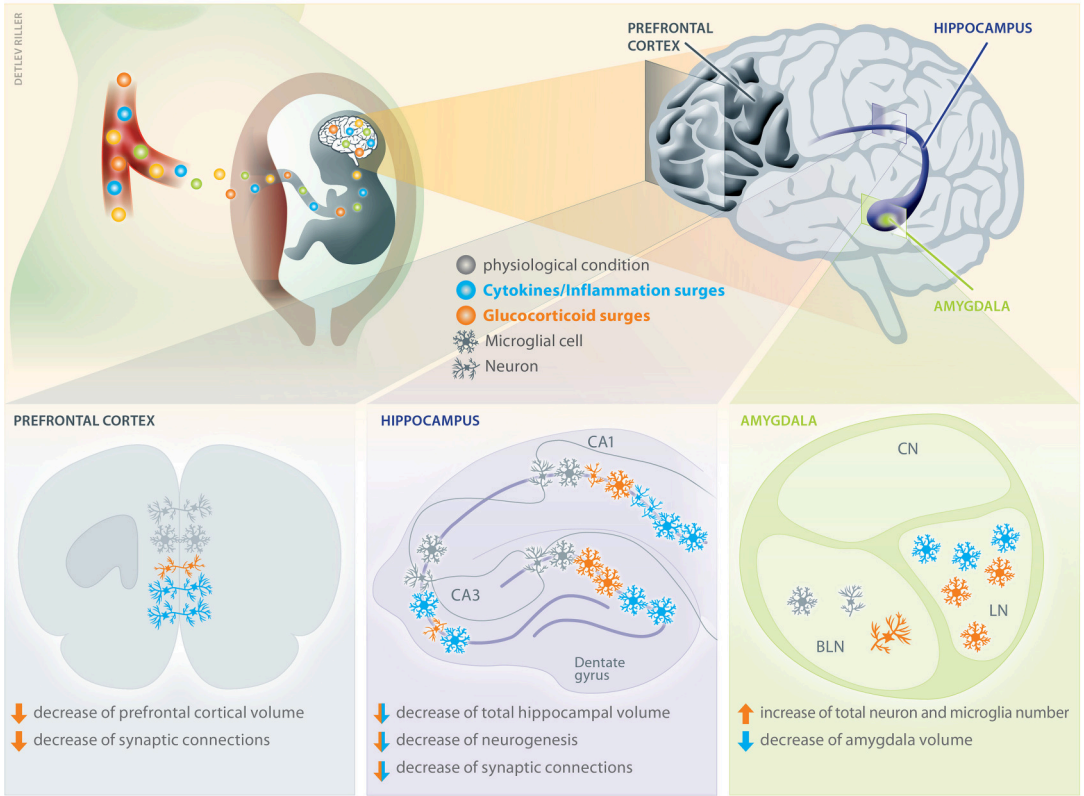


FIGURE 2 | Consequences of cytokine and glucocorticoid surges on distinct areas of the offspring's brain. Maternal cytokines and glucocorticoids can transplacentally cross into the fetus and differentially affect offspring's brain development by interfering with e.g., cell differentiation, axonal growth, and synaptic connectivity. The brain regions depicted here are of pivotal relevance for mental health due to their involvement in cognitive functions. Compared to physiological conditions, prenatal surges of maternal cytokines increase the number of neuronal connections in a subdivision of the prefrontal cortex, whereas glucocorticoids decreases them (105, 119). Additionally, prenatal glucocorticoid exposure decreases prefrontal cortical volume. In the hippocampus, an increase of cytokines is known to increase the number of microglial cells in the corn ammonis area 1 (CA1), whilst simultaneously reducing dendritic arborizations and neuronal complexity (111). Similarly, in the CA3 and dentate gyrus (DG) of the hippocampus, the microglia density increases after cytokine exposure. Also glucocorticoid surges deteriorate the neuronal complexity in CA1 and CA3 (109, 110) and have been shown to increases the number of microglia in DG. Both, cytokines and glucocorticoids can decrease total hippocampal volume, neurogenesis and synaptic connections. Prenatal cytokine surges can also decrease the amygdala volume, whereas glucocorticoids have been shown to increase the number of neurons and microglia. There is no evidence that the central nucleus (CN) of the amygdala is affected, but an increased microglia density after prenatal cytokine and glucocorticoid exposure has been detected in the lateral nucleus (LN) (120, 121). Contrarily, an increased number of neuronal connections and microglia was detectable upon glucocorticoid challenge in the basolateral nucleus (BLN).

Besides the effect of glucocorticoids and cytokines on neurogenesis, synaptogenesis, axon growth etc., these mediators can also exert indirect effects on these fundamental processes of fetal brain development, i.e., by altering the concentration or availability of neurotransmitters. The primary excitatory neurochemical in the central nervous system, glutamate, which acts through different types of metabotropic glutamate receptors (mGluR) has been investigated in a number of studies (123–125). Indeed, a variant of the Kozak sequence of exon 1 of the mGluR 3 could be associated with e.g., bipolar affective disorder (126). Moreover, a reduced expression of mGluR receptors in the hippocampus has been observed in response to prenatal stress (127–129). Brain functions such as mood, satiety, sleep, body temperature and nociception are also critically dependent on the serotonergic system, and prenatal challenges have been shown to interfere with the rate of serotonin synthesis (130), the number of serotonin positive cells (131) and its receptor density (132) in the offspring's brain. Further, the cholinergic system is important in regulating brain function (e.g., learning and short-term memory) (133) that has been shown to be impacted in the offspring in the context of maternal stress and infection during pregnancy. Some evidence from rodent models support the notion that prenatal stress challenges interfere with the release of cholinergic factors in the offspring, such as acetylcholine (134), whereby changes of behavior have not been evaluated in this study.

Introducing a Novel Pathway in the Developmental Origin of Neurocognitive Functions and Psychiatric Disorders: the Impact of Maternal Microchimeric Cells

Maternal microchimeric cells, which are vertically transferred from mother to fetus during pregnancy and—at least in

part—during lactation (135–137), can be detected in the offspring's brain during fetal and adult life. Hence, maternal microchimeric cells have the potential to modulate brain development and the risk for neurodevelopmental disorders. To date, no insights are available on the role of maternal microchimeric cells in brain development and their potential ability to tailor the nervous system individually. Moreover, brain structures where maternal microchimeric cells may abundantly populate have not yet been identified. However, since maternal microchimeric cells have been identified as maternal immune cells of the innate and adaptive immune response, they hold the strong potential to shape neurons by acquiring a phagocytic phenotype, akin to offspring's microglial cells. Future studies should aim at elucidating the functional role of maternal microchimeric cells on the developing brain and to understand whether they may modulate the risk for brain-related disorders.

Given the plethora of mediators that may be functionally involved in shaping brain development and subsequent function, whilst being altered upon maternal stress, infection and other

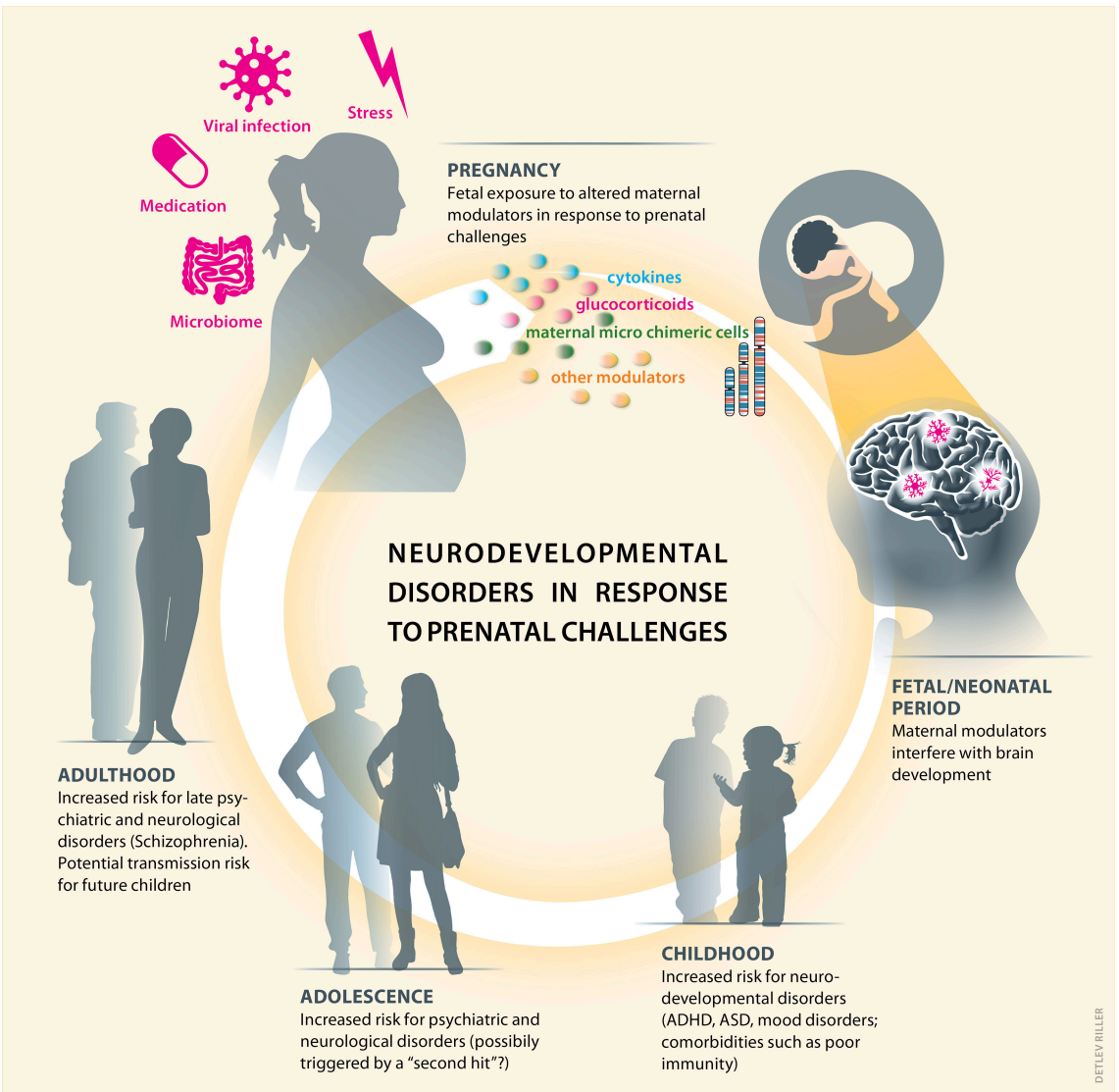


FIGURE 3 | Prenatal challenges and related alterations of immune and endocrine markers can prime postnatal neurodevelopmental disorders. Maternal well-being and health can be challenged during pregnancy, e.g., by distress or infection. This subsequently leads to increased cytokines and glucocorticoids levels and potentially to altered frequencies or phenotypes of maternal microchimeric cells in the offspring. Upon entering the fetal brain, such vertically transferred maternal modulators can significantly interfere with physiologically occurring brain development. A combination of genetic susceptibility and disturbed brain development can subsequently increase the risk for neurodevelopmental disorders in childhood. Subsequent postnatal environmental challenges —drug abuse, trauma, infection, others—may perpetuate such prenatally triggered risk for neurodevelopmental disorders, psychiatric and neurological diseases during adolescence and adulthood, which can also be passed on to the next generation.

prenatal conditions (138), it is not surprising that we are far from fully understanding the developmental origin of neurocognitive functions and brain disorders. Also, it seems unlikely that single mediators determine a clear-cut "good or bad" outcome. It is more likely that the mediators we here proposed act synergistically in modulating brain development and subsequent function with an advantageous or disadvantageous outcome. Hypothetically, this synergistic cross talk could involve the expression of glucocorticoid receptor on maternal microchimeric cells or the release of cytokines from maternal microchimeric cells entering the fetal brain. The longevity of such cells would surpass the short-term effect that could result from the potential transplacental transfer of cytokines itself, as cytokines are rapidly metabolized.

Functional Impact of Vertically Transferred Maternal Markers on the Developing Brain

In response the environmental challenges, altered levels of maternal markers that cross the placental barrier may affect the developing brain by inducing epigenetic alterations of somatic cells (139–142) Persistent epigenetic differences triggered by the prenatal exposure to stress challenges in humans include increased and decreased methylation of insulin-like growth factor 2 or the glucocorticoid receptor gene (NR3C1) in brain cells, depending on the timing of exposure (143, 144). Prenatal distress has been associated with hyper- as well as demethylation of specific regulatory sites in key genes involved in stress processing, such as the glucocorticoid receptor (144–146). Similarly, findings arising from mouse models on maternal immune activation during pregnancy include the observations of a hypoacetlyation of e.g., genes modulating neuronal development, synaptic transmission and immune signaling in the cortex region in exposed offspring (147), as well as sex-specific DNA hypomethylation in the hypothalamus of females (148), specifically affecting the promoter region of methyl CpG-binding protein 2, which is associated with neurodevelopmental disorders (149). Interestingly, prenatal immune activation in mice could be linked to hypermethylation of glutamic acid decarboxylase 1 and 2 in the brain (150), associated with altered behavior.

Strikingly, mouse models have revealed that alterations of brain function can be passed on to the next generations (151), suggesting that underlying epigenetic alterations triggered by prenatal challenges may be intergenerationally inherited. This notion could provide an explanation for the increasing incidence of behavioral disorders (152). Moreover, it implies that exposure of the mother to environmental challenges during pregnancy may not only directly interfere with fetal brain development, but also affects fetal primordial germ cells (153), which may subsequently interfere with brain development in the generation of grandchildren. Primordial germ cells undergo sequential epigenetic events, which are distinct from fetal somatic cells, hereby preserving the plasticity required for the generation of gametes (154–157). Once the offspring reaches adulthood and such oocytes are fertilized, the resulting zygote again undergoes significant epigenetic reprogramming, which includes the demethylation of the maternal and paternal genome, followed by a genome-wide *de novo* methylation (158).

Animal data indicate the possibility of transmission of behavioral traits mediated by epigenetic modifications through the maternal, as well as paternal germ line (159–162), implying the generation of oocytes and sperm may be equally affected. Intriguingly, how epigenetic changes induced by environmental challenges can be maintained throughout the multiple epigenetic reprogramming events physiologically occurring during reprogramming of primordial germ cells and the zygote is still largely elusive. Insights from mouse studies provide a first glimpse, as they reveal that certain regions of the genome, i.e., differentially methylated regions, are resistant to zygotic reprogramming (158). However, future research is required to identify pathways of intergeneration epigenetic inheritance of altered brain function in the offspring, aiming also to differentiate between *de novo* acquired epigenetic alterations from those inherited through the germ line. Besides such intergenerational inheritance of epigenetic marks, the possibility of transgenerational inheritance of epigenetic changes to one further generation of descent, the great-grand generation, has been considered. The primordial germ cells of the forth generation would not have been directly exposed to the environmental challenges or mediators released by the great-grandmother during pregnancy. However, to date, convincing evidence of transgenerational inheritance of epigenetic marks is only available from botany research using plants, whilst confirmation in mammals is somewhat elusive (163).

Besides such epigenetic pathways, the brain as target tissue for prenatal challenges may be affected in its electrical synchrony, which is defined as the coordinated oscillatory activity and neural firing rate between connected brain areas. These links are a prerequisite to execute cognitive tasks (164, 165). Interestingly, prenatal exposure to maternal inflammation or stress impairs oscillatory synchronicity (166), which commenced already during developmental stages in a mouse model of neuropsychiatric disorders (167–169) and affected spatial memory tasks (170, 171).

Outlook

Higher cognitive functions such as planning, self-regulation, memory, learning, and emotional processes result from a complex, tailored, and precisely shaped large-scale communication of neuronal networks (172). These neuronal networks begins to develop prenatally and disturbances of such developing neural systems during pregnancy can disrupt brain development via the vertical transfer of maternal markers, such as cytokines, glucocorticoids or microchimeric cells of the maternal immune system. Subsequently, the risk for mental disorders and diseases can increase in the offspring (**Figure 3**). As most of the studies are correlative, future research should aim to investigate causalities between maternal factors and children's health outcome. Clearly, adverse postnatal childhood experiences can further aggravate such cognitive and behavioral

dysfunctions (173–178) and thus, should be considered in experimental designs and observational studies.

AUTHOR CONTRIBUTIONS

SS and PA developed the structure of the review article, SS provided the first draft, which was amended by CB on aspects including prenatal cytokines and epigenetic pathways, by IH-O by insights on brain development and by PA on issues related to maternal immune adaptation to pregnancy. All authors have been involved in the interpretation of published evidence, critically revised the manuscript and gave their final approval of the version to be published.

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REVIEW
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Hormonal Regulation of Physiology, Innate Immunity and Antibody Response to H1N1 Influenza Virus Infection During Pregnancy

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In 2009, the H1N1 swine flu pandemic highlighted the vulnerability of pregnant women to influenza viral infection. Pregnant women infected with influenza A virus were at increased risk of hospitalization and severe acute respiratory distress syndrome (ARDS), which is associated with high mortality, while their newborns had an increased risk of pre-term birth or low birth weight. Pregnant women have a unique immunological profile modulated by the sex hormones required to maintain pregnancy, namely progesterone and estrogens. The role of these hormones in coordinating maternal immunotolerance in uterine tissue and cellular subsets has been well researched; however, these hormones have wide-ranging effects outside the uterus in modulating the immune response to disease. In this review, we compile research findings in the clinic and in animal models that elaborate on the unique features of H1N1 influenza A viral pathogenesis during pregnancy, the crosstalk between innate immune signaling and hormonal regulation during pregnancy, and the role of pregnancy hormones in modulating cellular responses to influenza A viral infection at mid-gestation. We highlight the ways in which lung architecture and function is stressed by pregnancy, increasing baseline inflammation prior to infection. We demonstrate that infection disrupts progesterone production and upregulates inflammatory mediators, such as cyclooxygenase-2 (COX-2) and prostaglandins, resulting in pre-term labor and spontaneous abortions. Lastly, we profile the ways in which pregnancy alters innate and adaptive cellular immune responses to H1N1 influenza viral infection, and the ways in which these protect fetal development at the expense of effective long-term immune memory. Thus, we highlight advancements in the field of reproductive immunology in response to viral infection and illustrate how that knowledge might be used to develop more effective post-infection therapies and vaccination strategies.

Keywords: pregnancy, H1N1 influenza virus infection, animal models, sex hormones, maternal immune response

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INTRODUCTION

Influenza viruses are segmented, negative-stranded enveloped RNA viruses that cause respiratory infections, fever, malaise, coughing, and mucus production. Influenza viruses are divided into A, B, C, and D types; while all A, B, and C can be infectious in humans with influenza A viruses (IAVs) causing the most widespread disease, influenza D virus is not known to infection humans (1, 2).

Influenza A viruses are further classified by the antigenicity of their surface proteins hemagglutinin (HA) and neuraminidase (NA), which are encoded on individual segments of viral RNA, and define the host range of each virus (1, 3). Through a process unique to influenza and other segmented genome viruses, co-infection of different viral subtypes in human, swine, avian or other animal hosts can result in reassortment leading to antigenically unique novel viruses that may take advantage of an immunologically naïve host species (4). This process of reassortment resulted in the emergence of the four major influenza virus strains causing pandemics; the 1918 H1N1 Spanish influenza, the 1957 H2N2 Asian influenza, the 1968 H3N2 Hong Kong influenza, and the 2009 H1N1/09 swine influenza, that infected up to 50% of the global population and caused a significant increase in mortality (2, 3). While infection can occur year-round, the epidemiology of influenza virus infection is seasonal, causing peak illness in November through March in the Northern Hemisphere and approximately 200,000 hospitalizations and 36,000 deaths annually in the United States (3). Currently, the most common circulating influenza A subtypes are H1N1 and H3N2, which are included in quadrivalent vaccines together with the type B influenza lineages Yamagata and Victoria (5). Due to the wide variety of circulating viruses and the frequency of genetic reassortment between subtypes, vaccination is required annually to provide immune protection during each influenza season although it may not be complete if there is mismatch between predicted strains included in the vaccine and the resultant circulating strains in the following season.

Seasonal influenza infections during the second and third trimester of gestation increase the morbidity of pregnant women with higher hospitalization rates than the general population and mortality (6). Pregnant women have been particularly vulnerable to pandemic influenza viruses showing up to 45% increased morbidity and mortality, and they were at an increased risk of higher cardiopulmonary complications and gestational abnormalities during the four major influenza pandemics in the past 100 years (7–9). While pregnant women typically represent 1% of the American population, during the 2009 H1N1 pandemic they comprised 6.4% of all hospitalizations and 4.3% of all deaths (10, 11). Over half of those women hospitalized for H1N1 influenza virus infection had another pre-existing condition, such as asthma, high blood pressure, and diabetes; women with asthma represented 43.5% of deaths from influenza virus infection during pregnancy, which is part of a larger phenomenon of enhanced viral pathogenesis and severe outcomes among asthmatic adults (11, 12).

Influenza infection-related complications in fetuses and neonates have been associated with increased risk of miscarriage, pre-term birth, stillbirth, neonatal death, and low birth weight (6, 9, 13, 14). Clinical reports of influenza-like illness (ILI) during pregnancy have been correlated with a five-fold increase in perinatal morbidity and mortality (15). The incidence of pre-term birth increased from 7 per 1,000 births to 39 per 1,000 births and the incidence of stillbirth from 6 to 27 stillbirths per 1,000 births in the 2009 pandemic (9, 10, 16). Specifically, pregnant women who tested positive for the 2009 swine-origin H1N1 virus were more likely to deliver low birthweight infants than pregnant women who delivered following ILI that was not caused by the pandemic strain (17). There is historical evidence that the 1918 and 1957 pandemics produced similar clinical outcomes for pregnant women; however, modern diagnostic procedures employed during the 2009 H1N1 pandemic allow for more direct linkage between influenza viral infection of pregnant mothers and poor outcomes for maternal and neonatal health (9).

The mortality rates reported for both women and their newborns led to initiatives by the Centers for Disease Control and Prevention (CDC) and the World Health Organization (WHO) to increase influenza vaccination coverage in pregnant women (5, 18). Maternal vaccination during the second or third trimester of pregnancy with seasonal trivalent influenza vaccine substantially reduced the incidence of ILI in both mothers and their newborns (19–21) and has not been associated with pre-term delivery or an increase of adverse outcomes for mothers or their infants. In a review of 7 clinical studies, Bratton et al concluded that maternal vaccination reduced the likelihood of stillbirth compared to unvaccinated pregnant mothers (22–24). However, while the conventional intramuscular vaccination has been determined to be reasonably safe for routine use during pregnancy, promoting the transplacental transfer of anti-influenza virus antibodies from mother to fetus, clinical data is inconclusive regarding the efficiency of immune responses to the vaccine when compared to those induced in non-pregnant women (18, 19, 25–27).

Vaccination is widely recommended during pregnancy for the benefit of mother and child; however, vaccination coverage among pregnant women in the United States remains around 50% (6). Research into the specific mechanisms by which H1N1 influenza virus infection causes pregnancy complications and how pregnancy hormones modulate the immune response to infection and vaccination may reveal improved routes of therapy for women infected with influenza A virus during pregnancy. This review will discuss how H1N1 influenza virus infection disrupts maternal lung and placental function as well as the role of pregnancy hormones in shaping the innate and cellular immune responses to H1N1 influenza virus infection.

The Physiology of Influenza A Virus Infection and Immune Responses

Human influenza A virus is typically transmitted through respiratory droplets and inhaled into the nasopharynx. Initial virus infection occurs when hemagglutinin (HA), a surface protein on influenza virions, binds to α2,6-linked sialic acids that are widely expressed on the surface of ciliated airway epithelial cells throughout the upper respiratory tract (28, 29). Viruses are then endocytosed primarily via a clathrin-mediated pathway; upon acidification of the vesicle containing influenza virions, the HA protein is triggered to fuse viral and cellular membranes, releasing the viral genome into the cell (30–32). The eight negative-stranded RNA segments of the viral genome are then translocated to the nucleus, where they replicate using the associated viral RNA-dependent RNA polymerase through a complementary RNA (cRNA) replication intermediate (33). In

parallel, viral transcripts are generated using stretches of capped cellular RNA molecules as primers ("cap-snatching"). After translation of the viral transcripts in the cytoplasm, the eight genome segments are packaged into virions which are released from the apical cellular membranes to infect nearby cells and thus result in viral amplification (32, 34). Viruses shed from the nasopharynx may be inhaled further into the lower respiratory tract causing severe pulmonary infections or transmitted through respiratory droplets from the upper respiratory compartment to the next person. People infected with 2009 H1N1 influenza A virus were contagious as early as 12 hours post-inoculation and typically began experiencing symptoms approximately 2 days following infection (35).

The release of viral RNA within the cell activates innate immune signaling pathways, especially toll-like receptors (TLRs) and retinoic-acid-inducible gene 1 (RIG1), which induce the expression of the pro-inflammatory cytokines interferon-α (IFN-α) and interferon-β (IFN-β) (36, 37). The secretion of these cytokines activates the surrounding epithelial cells to express antiviral genes that hamper viral entry and replication and recruit innate immune cells to the site of infection (37–39). Natural killer (NK) cells and neutrophils kill infected cells, and additional cytokines expressed by activated airway epithelium and innate immune cells induce fever and mucus production, which in turn results in coughing and rhinorrhea to shed the virus and cellular debris from the lungs and the nasopharynx (38, 40). These infection-induced responses are the hallmark of influenza illness symptomatology, and in clinically vulnerable populations, chest congestion due to viral infection and the ensuing immune response can lead to bronchitis, pneumonia, and secondary bacterial infections (41–43). Viral clearance finally occurs around 8 to 10 days after the onset of symptoms when the adaptive immune response mounts virus-specific clearing of infected tissue (38, 44). Viral antigen is taken up and processed by dendritic cells which migrate upon activation to draining mediastinal lymph nodes and prime naïve resident T cells to respond to the infection (45). Humoral memory is developed when naïve B cells are primed by soluble antigen and costimulated by CD4+ T follicular helper (TFH) cells to mature into plasmablasts that will then traffic to the site of the infection and secrete virus-specific neutralizing antibodies (46). Ultimately, following costimulatory help from CD4+ T cells, CD8+ cytotoxic T lymphocytes will traffic to the lungs and eradicate virus-infected cells (38, 46, 47). Upon resolution of disease, alveolar macrophages clear cellular debris, and basal stem cells regenerate airway epithelium to restore healthy tissue (48, 49). In animal models for influenza viral pathogenesis, it was demonstrated that virus-specific $CD103^+$ $CD8^+$ tissue resident memory (TRM) T cells in the lungs could provide rapid response upon the next infection and memory B cells persisted in the mediastinal lymph nodes to secrete virus-neutralizing antibody into circulation upon restimulation (50–52).

Research into the specific mechanisms of H1N1 influenza A virus binding, entry, RNA replication, transmission, and induction of the host immune system has been extensive since the 1918 Spanish influenza pandemic; however, investigations into how these mechanisms manifest in disease in immunologically unique populations, such as infants, the elderly, HIV^+ or asthmatic patients, and pregnant women have been limited.

Hormonal Regulation of Pregnancy and Immune Signaling Are Delicately Balanced to Protect Fetal Development

Female reproduction is regulated predominately via estrogen, progesterone, luteinizing hormone (LH), and follicular stimulating hormone (FSH). Estrogen receptors (ERs) and progesterone receptors (PGRs) are typically expressed within the cytosol and translocated to the nucleus upon ligand binding to induce a suite of genes encoding immunomodulators, regulators for tissue remodeling, mammary gland development, metabolism, lung physiology and function (53–55). LH and FSH are synthesized in the anterior pituitary gland and coordinate the decidualization of the uterine endometrium as well as the release of oocytes from mature ovarian follicles into the uterus for fertilization (56). A fertilized oocyte develops into a blastocyst, and then the outer layer of the blastocyst forms a polarized structure called the trophectoderm (57). The trophoctoderm layer implants in the uterine wall to become syncytiotrophoblasts that secrete human chorionic gonadotropin (hCG) and develop into fetal placental chorionic villi (57). Placental hCG expression signals the maternal corpus luteum to produce progesterone, which maintains the appropriate thickness and vascularization of the endometrium to support embryonic growth (56).

Insufficient progesterone production has been associated with infertility and recurrent spontaneous abortions, indicating that variations in progesterone levels as a result of infectious disease are not well tolerated by maternal physiology and may result in miscarriage or pre-term birth (58–60). Sex hormones play a crucial role in organizing endometrial granulated lymphocytes (EGLs) in the innermost layer of epithelial tissue in the uterus and populations of uterine natural killer (NK) cells, dendritic cells, macrophages, and memory and regulatory T cells are tightly controlled throughout the first, second, and third trimesters of pregnancy (56, 61, 62). Estrogens are expressed in several major forms, mainly estradiol (E2) and estriol (E3); each can have biphasic effects in stimulating pro-inflammatory signaling via mitogen-associated protein kinases (MAPKs) and NK activation at low concentrations or enhancing the expression of PD-L1 on T cells and the synthesis of TGF- β and IL-10 at high concentrations (62). Progesterone receptors are expressed broadly on most immune cell subsets and are produced in higher levels in females (62–64). In the uterus, progesterone induces the transition of naïve Th0 cells into IL-4, IL-5, and IL-6 secreting Th2 memory cells upon antigen recognition; these Th2 cells are critical for coordinating immune tolerant cytokine crosstalk between the maternal and fetal sides of the placenta and preventing intrauterine NK cell activation against fetal trophoblasts (61). The expression of IL-4 and IL-6 then promotes hCG secretion from the corpus luteum, which in turn releases more progesterone, creating a positive feedback loop for the amplification of hormone-mediated Th2 polarization (61). This phenomenon has been shown to be important for maintaining immune tolerance, and recurrent miscarriage is associated with

a predominance of Th1 memory cells in the endometrium (65). Estrogens have also been implicated in inducing $CD4^+$ $CD25^+$ T regulatory cells (Tregs) and are critical for maintaining tolerance within the maternal-fetal interface (66). Progesterone also upregulates the activity of uterine Tregs, which act as suppressors of inflammatory immune subsets, particularly NK cells and macrophages resident to the endometrium (62, 64). In this way, estrogens and progesterone coordinate an environment in which both uterine epithelial cells and innate immune cells resident to uterine tissue will tolerate the implantation of a fertilized oocyte and the development of a placenta and fetus.

The structure and cellular composition of placenta is critical to maintaining fetal growth and development as well as protection from inflammation. Fetal placenta develops from the cells in the implanted blastocyst as it transitions into the trophoblast which differentiates into cytotrophoblasts and synciotrophoblasts. Both cell types contribute to the development of chorionic villi that form an interface with the uterine decidua (57). Here, maternal blood makes direct contact with fetal cells, allowing for gas, nutrient, and waste exchange but also providing a potential door for entry of bacteria, viruses, and parasites to a fetus with an undeveloped immune system (67). Few pathogens can cross the placental barrier from the mother to the fetus. TORCH pathogens [*Toxoplasma gondii*; other pathogens including, human immunodeficiency virus (HIV), varicella zoster virus (VZV), malaria-causing *Plasmodium* species, *Listeria monocytogenes*, *Treponema pallidum*, parvoviruses B19, enteroviruses, and recently, Zika virus; *r*ubella virus; *c*ytomegalovirus (CMV); and *h*erpes simplex virus 1 and 2 (HSV)] are associated with fetal and neonatal morbidity and mortality from CNS abnormalities, microcephaly, blindness, deafness, premature birth or low birth weight (67, 68). However, there is limited evidence that influenza A virus crosses the maternal-fetal barrier. Despite the demonstrated ability of the 2009 pandemic strain to infect fetal trophoblasts, the development of chorionic villitis and the widespread reports of increased risk of maternal and fetal mortality, there were few conclusive cases of vertical transmission via the placenta (69, 70). Thus, poor fetal outcomes during pregnancy are likely due to indirect exposure to maternal inflammatory cytokine expression and dysregulation of pregnancy-supportive hormones.

In addition to preventing pathogen entry into the fetal bloodstream, it is also critical that cytokines that make it across the placental syncytiotrophoblast layer into the fetal circulatory system do not cause inflammation or immune cell activation that interrupts fetal growth and development (71, 72). Clinical reports of maternal inflammation and infection during pregnancy have been associated, although inconclusively, with the development of autism, bipolar disorders, and schizophrenia in children born to mothers infected with influenza A virus during pregnancy (73–75). Peripheral blood mononuclear cells (PBMCs) isolated from healthy pregnant women and co-cultured with 2009 pandemic influenza A virus subtype H1N1 or circulating rhinovirus strains (HRV43 and HRV1B) had significantly reduced IFN-α and IFN-γ responses, indicating increased susceptibility to severe outcomes of viral infection during pregnancy (76, 77) A shift away from inflammatory Th1 cytokines (TNF-α, IFN-γ, IL-2) can limit potential cytotoxic damage to the fetus and placenta (61, 62). Sex hormones coordinate this shift by activating transcriptional factors via transmembrane and intracellular receptors which activate a suite of anti-abortive, pro-pregnancy genes (63). For example, progesterone activates progesterone-induced binding factor (PIBF) in lymphocytes, which in turn promotes the synthesis of IL-3, IL-4, and IL-10, while reducing the expression of IL-12 (78, 79). PIBF also inhibits NK cell degranulation, and decreased PIBF expression is linked to recurrent spontaneous abortions (79, 80). Thus, hormone-mediated suppression of inflammatory cytokine production and cellular activation is critical to successful pregnancy in the short-term by protecting the placenta from inflammation that could trigger pre-term birth or neurodevelopment damage; however, proper inflammatory signals must still be activated to recruit innate immune cells and $CD8^+$ T cells in order to clear virus-infected tissue.

While pre-term birth and low birth weight neonates have been well-documented outcomes of the 2009 H1N1 influenza virus infection in pregnant women, a mechanism for this phenotype is unclear, though placental transmission of inflammatory cytokines, dysregulated hormone signaling, and oxygen deprivation due to maternal respiratory distress have all been implicated (62, 81, 82). The effect of the hormonal millieu during pregnancy on innate immune responses is complicated, and *ex vivo* modeling of a single subset of cells may not depict the entire story of hormonal, cytokine and immune cell signaling between lung, fetus, and placenta in an infected pregnant woman. Clinical samples from pregnant women are limited to blood, post-partum placenta, and post-mortem tissues, leaving research questions about maternal lung function and immune responses to non-fatal influenza viral infection unanswered.

Rodent models, particularly mice, are a commonly accepted experimental tool for preclinical research studies due to their hemochorial placental structures, recapitulation of influenza viral pathogenesis seen in humans, and their cost effectiveness over multiple time points (29). One approach for the elucidation of these mechanisms is to expose healthy non-pregnant female mice to low doses of sex hormones comparable to birth control or high doses comparable to those of pregnancy. Pazos et al. implanted female C57BL/6 mice with degradable 17β-estradiol (E2 in mice) pellets to yield serum E2 levels of third trimester pregnancy and infected them with H1N1 PR8 virus; mice implanted with E2 exhibited reduced type I IFN signaling and impaired $CD8^+$ T cell function compared to infected non-implanted female mice (83). Robinson et al proposed that 17β-estradiol has protective effect during pregnancy; ovariectomized and E2-implanted female C57BL/6 mice infected with H1N1 PR8 influenza virus exhibited enhanced recruitment of neutrophils and virus-specific T cells, which promote viral clearance (84). In contrast, studies involving pregnant mice demonstrated that while individual expression of estrogen or progesterone may limit inflammation, the condition of pregnancy resulted in elevated inflammatory responses to influenza virus infection compared to the immune responses of infected non-pregnant female mice (85–87). Pregnant mice infected with a mouse-adapted, 2009 H1N1 influenza virus expressed elevated levels of IL-1α, IL-6, granulocyte-colony stimulating factor (G-CSF),

monocyte chemotactic protein (MCP-1), CXCL1, and RANTES and experienced more severe pathology and mortality when compared to non-pregnant mice (88). These cytokines were highly expressed in humans who died as a result of 2009 H1N1 influenza A virus (87, 89). These differences in immune responses between hormone-treated mice and pregnant mice infected with influenza virus highlights how immune and endocrine crosstalk between mother, fetus, and placenta has far-reaching consequences beyond classical reproductive tissues and complicates our understanding of typical H1N1 viral pathogenesis.

The genetic background of mouse strain is also significant in the selection of a pregnant mouse model. C57BL/6 mice classically tend toward Th1-type immune responses while mice with BALB/c genetic backgrounds tend toward Th2-type immune responses (90, 91). Differences in genetic background have been shown to cause variability in viral pathogenesis, inflammatory cytokine response, pulmonary microRNA expression, alveolar macrophage viability following intranasal infection with 2009 H1N1 pandemic influenza virus strains (92–94). Strain differences also affect the physiological response to influenza viral infection during pregnancy. Recent findings in C57BL/6 mice have highlighted that pregnancy significantly enhances lung function by increasing respiratory compliance and total lung capacity and that influenza virus infection does not alter lung tidal volume, minute ventilation, diffusing capacity, and compliance as shown in non-pregnant infected mice. The authors observed less inflammation in the lungs of infected pregnant mice and suggested that this is a protective mechanism against maternal respiratory damage during pregnancy (95). However, we and others have shown in the BALB/c mouse model that pregnancy increases lung inflammation and expression of stress-induced prostaglandins (PGs) and cyclooxygenase-2 (COX-2) prior to infection and that IAV infection enhances immunopathology in the lungs of pregnant mice relative to non-pregnant mice (86–88). Oxidative stress interferes with lipid raft clustering and has been shown to inhibit the ability of PIBF to bind its transmembrane receptor and IL-4R to induce the STAT6 signaling pathway; this interference reduces the sensitivity of cells to PIBF (96, 97). Thus, influenza viral infection and subsequent oxidative stress may interfere with the unique lung and mucosal physiology tightly regulated by sex hormones toward successful pregnancy and fetal development.

Humoral Immune Responses Following Infection and Vaccination During Pregnancy

The natural outcome of infection is the development of immunological memory to prevent re-infection and future cellular damage. As discussed previously, soluble viral antigen released from infected cells in the lungs primes naive B cells in the proximal draining lymph nodes by binding to the B cell receptor (BCR), crosslinking several BCRs in the process and amplifying an activation signal (46, 98–100). Additional costimulation by $CD4^+$ helper T cells responding to processed viral antigen in MHC class II proteins on the B cell's surface is required to fully activate B cells and provides a second activation signal, resulting in clonal proliferation and amplification of antibodies specific for influenza viral antigens (46). Selection for B cells with BCRs with highest affinity for the viral antigen occurs in the germinal centers found in secondary lymphoid tissues such as the spleen. In the latter, cells undergo somatic hypermutation, a process by which DNA encoding hypervariable Ig regions is broken by activation-induced deaminase (AID) and uracil-DNA glycosylase (UNG) and repaired by MSH2/6 and REV1. The accumulating mutations may result in the generation of antibodies with an increased affinity to viral antigens (101, 102). Immunoglobulin class switching increases the range of functions by recombining antibody variable regions encoding specificity for influenza viral proteins with constant regions encoding receptors for various innate immune cells and intercellular trafficking (102). Ultimately, most antibody-secreting cells (ASCs) will undergo apoptosis following viral clearance. Only a small percentage of these high-specificity B cell clones will become plasma cells that secrete low levels of antibody into the serum for months, or memory cells that reside in the bone marrow, and can be reactivated to provide antibody responses to a subsequent infection (46, 103).

This system-wide coordination of B cell activation and survival in response to foreign antigen delicately balances the pregnant mother's serum antibody levels to both provide the benefits of transplacental immunity to the fetus and avoid the development of fetal-reactive antibodies. The competing priorities of fetal antigen tolerance and the production of antibodies that can be transplacentally conferred to the fetus to promote neonatal immunity are tightly regulated by pregnancy hormones. Clinical evidence has long documented that the symptoms of autoimmune diseases arising from the generation of antibodies against self-antigen tend to recede during pregnancy and resurge after parturition and breastfeeding, indicating that pregnancy hormones play a role in coordinating immune tolerance at the local uterine and systemic level (62, 64). The development of autoimmune disorders such as multiple sclerosis, rheumatoid arthritis, and systemic lupus erythematosus (SLE), which are more prevalent in women, have been linked to the effects of estrogen on B cell activation and function (104). Estradiol (E2) has been shown to upregulate Bcl-2, inducing survival of autoreactive B cells and changing signaling thresholds required to induce apoptosis (105, 106). In contrast, progesterone has been established as negative regulator of B cell lymphopoiesis (107–109). Reduced expansion of B cells within a pregnant mother may help establish allotolerance to the fetus by preventing antibody recognition of fetal antigen, which might result in inflammation, lymphocyte cytotoxicity, and complement activation (63). Healthy pregnancy has been shown to suppress B cell lymphopoiesis in BALB/c mice, which could be reversed by the exogenous addition of IL-7 (107, 110, 111). These data suggest that pregnancy may reduce or redirect activated B cells during their migration to the lungs or bone marrow. Differential recruitment of IgA^+ plasmablasts to the murine mammary glands after parturition and during nursing has been demonstrated, but specific homing receptors have not been identified, suggesting a role of local chemoattractants such

as E-selectin (112, 113). In this way, maintaining immunological tolerance to fetal antigen that reaches the maternal circulatory system may require that B cell activation be altered in order to prevent the proliferation of anti-fetal antibodies.

Understanding how pregnancy impacts the development of immune memory is of clinical significance. Immunization has been reported to reduce hospitalization and ILI of pregnant women and their newborns during the flu season with no record of increased adverse events due to vaccination between this group and the unvaccinated population (21, 23, 114, 115). Clinical trials of seasonal trivalent inactivated influenza vaccination (TIV) in Bangladesh showed improved transplacental transfer of influenza-specific antibodies from mother to child (116). However, there are mixed results in how pregnancy affects humoral immunity following vaccination. Schlaudecker et al. reported that pregnant women seroconverted at the same rate as non-pregnant women following TIV but generated lower geometric mean titers (GMTs) against H1N1 (A/California) and H3N2 (A/Perth) viruses (26). Serological analysis from a cohort of influenza A virus (IAV) vaccinated healthy pregnant and non-pregnant women in California showed similar seroconversion rates and numbers of plasmablasts (18). Thus, while influenza vaccination during pregnancy has been demonstrated to be safe and to reduce the incidence of influenza-induced hospitalization and pre-term birth, further research into antibody functionality and expression is still needed.

Pregnancy hormones may coordinate the down-regulation of class-switching or post-translational modifications (i.e., glycosylation, fucosylation, sialylation, etc.) of the antigen-binding (Fab) or receptor binding (Fc) regions of antibodies in order to attenuate potentially inflammatory or anti-fetal immune responses. There have been reports that pregnant women infected with H1N1 pandemic virus in Shenyang, China in 2009 produced an imbalanced proportion of anti-H1N1 IgG1, IgG2, IgG3, and IgG4 antibody subtypes compared with infected non-pregnant women in the same hospital (117). Interestingly, IgG1 is preferentially transported from maternal circulation across the placenta compared to other IgG classes, especially IgG2, although this phenomenon has not been directly linked to influenza infection and vaccination (118–120). While preferential transport from mother to fetus is linked to neonatal Fc receptor (FcRn) expression on placental syncytiotrophoblasts, how pregnancy shifts expression from virus-specific IgG2 to IgG1 requires further investigation.

Antibody isotype classes and generation of specificity are governed by the class-switching of Ig genes and somatic hypermutation of their variable chain-encoding regions (102, 121). Variability is induced primarily by activation-induced cytidine deaminase (AID) and uracil-DNA glycosylase (UNG) that selectively damage DNA and repair it randomly (102, 121, 122). B cells that have complementarity-determining regions (CDRs) that bind best to antigen are selected for by T follicular helper cells (Tfhs) and clonally amplified to flood the circulatory system with virus neutralizing antibodies (123, 124). Estrogen and progesterone seem to work in opposition to each other on the regulation of AID: estrogen receptors bind the *HoxC4* promoter to induce AID activation, while progesterone receptors can bind directly to the AID promoter to inhibit activation (125–127). Glucocorticoids have also been described as negative regulators of AID activation (128). These phenomena are typically described in the context of autoimmune disease regulation and have not been described in the multi-hormonal environment of pregnancy.

Asymmetric glycosylation, or the single glycosylation of one side rather than both sides of the Fab or Fc antibody chains, can result in fine-tuned interactions with antigen and Fc receptors, and these binding affinities are important for antibody-dependent cellular cytotoxicity (ADCC) (129, 130). Pregnancy has been shown to increase the serum and placental concentrations of asymmetrically glycosylated IgG and may provide an explanation for the reduced avidity and virus-binding capability following viral infection during pregnancy (131, 132). Human and murine placental expression of IL-6 has been shown to induce asymmetrical glycosylation of IgG from hybridomas (133, 134). Trophoblast-produced asymmetric antibodies have been documented throughout the placenta (132, 135). However, whether these signaling effects can extend outside the uterus has yet to be determined and would be a major finding in maternal immunity. By reducing binding specificity for antibody effector cells via asymmetric glycosylation, the maternal immune system may be able to mitigate the negative effects of any anti-fetal antibodies that may have developed while still maintaining a population of semi-functional or selectively-functional antibodies that can neutralize pathogens and non-self-entities (131).

CONCLUSIONS

Influenza viral illness causes significant socioeconomic and clinical burden each year (136). While most research focused on the consequences of influenza A (H1N1) virus infection during pregnancy, there is evidence that influenza B virus can also cause significant maternal and fetal complications following mid-gestation infection (137, 138). It remains unclear if seasonal type A (H3N2) virus infection during pregnancy causes similar poor clinical outcomes compared to the severity of complications following type A (H1N1) or type B virus infection during pregnancy (138–141). Lastly, the recently identified highly neurotropic avian H7N9 and H5N1 influenza A reassortants, which could potentially cause pandemics, have been shown to cause severe disease during pregnancy (142–147). The knowledge gained through research of the 2009 pandemic swine-derived influenza A (H1N1) virus may provide the clinical and research community with an improved capacity for the early detection of a novel pandemic virus entering a naïve pregnant population. These studies we have reviewed demonstrate the vulnerability of pregnant women to infectious diseases and the fact that neonatal health is directly dependent on maternal health, doubles the significance of research that results in improved therapies and treatment strategies.

Respiratory infection during pregnancy is of broad interest. While influenza A virus has generated some of the highest

morbidity rates following maternal infection, coronavirus outbreaks have also been associated with similar outcomes in mothers and neonates following mid-gestation infection. Infection with severe acute respiratory syndrome (SARS) coronavirus and Middle Eastern respiratory syndrome (MERS) coronavirus have been associated with spontaneous abortion, fetal growth retardation, and maternal and neonatal mortality (148–150). Mid-gestation infection with respiratory syncytial virus (RSV) has been described in rare severe adult cases and has also been associated with pre-term birth and low birth weight in a cohort in Nepal (151, 152). High rates of mortality among infants and toddlers infected with RSV highlights the need for improved understanding of maternal immunity to RSV infection and vaccination during pregnancy, and there is hope that vaccination of mothers during pregnancy can provide passive immunity that will protect the fetus for months after birth (153). None of the previously mentioned viruses were transmitted transplacentally to fetuses, and yet respiratory infection during pregnancy induced significant maternal illness, pre-term labor, low birth weight, or spontaneous abortion.

Early antiviral therapy following H1N1 influenza A virus infection during pregnancy has been shown to significantly reduce pre-term birth, hospitalization in intensive care units (ICUs), and maternal death (11, 154). Seasonal H1N1 influenza A virus induced increased levels of cyclooxygenase-2 (COX-2) and prostaglandin-F2α in the lungs and placenta, providing a mechanism for lung immunopathology and pre-term labor in pregnant mice (88). The anti-inflammatory potential of COX-2 inhibitor therapy has already been proposed for decreasing disease severity caused by the highly pathogenic avian influenza strains H5N1 and H7N9 (155, 156). In addition, COX inhibitor treatment has demonstrated to attenuate the lung expression of granulocyte colony-stimulating factor (G-CSF) and keratinocyte-derived (KC) cytokines elevated in pregnant mice infected by H1N1 A/Brisbane/59/2007 and H1N1 A/California/07/2009 (157, 158). However, while non-steroidal anti-inflammatory drugs (NSAIDs) have been shown to be safe during pregnancy, COX-2 specific inhibitors may induce pre-term labor and musculoskeletal defects (159–161). Viral load was negatively associated with progesterone concentration, and reduced progesterone expression was correlated with pre-term labor in influenza virus-infected pregnant mice (88). Administration of progesterone to female mice following influenza A(H1N1) virus infection reduced immunopathological changes and improved lung epithelial cell regeneration, although it did not reduce viral load (162, 163). Hence, limiting viral replication should be one of many aims for anti-influenza therapy during pregnancy, including the limiting of immunopathology caused by cytokine dysregulation and promoting the healing of damaged airway epithelium following viral clearance.

The connections between viral pathogenesis and reproductive endocrinology makes the field of infectious disease in pregnant women complicated, exciting, and clinically significant. Investigations into the immunological components of infertility, recurrent miscarriage, and preeclampsia have yielded a wealth of information regarding the requirements of immune tolerance and rejection, and this information can provide a platform for understanding healthy pregnancy and how inflammation and hormonal dysregulation will impact maternal health and fetal development. Development of accurate animal pregnancy models across a range of species in coordination with broader clinical sampling from influenza-infected or -vaccinated pregnant women will provide an effective platform for validation of experimental studies and improved therapeutics and treatment for pregnant women and their offspring.

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The Immunogenetic Conundrum of Preeclampsia

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Pregnancy is an immunological challenge to the mother. The fetal tissues including the placenta must be protected from activation of the maternal immune system. On the other hand, the placental tissue sheds into the maternal circulation and must be adequately identified and phagocytized by the maternal immune system. During a healthy pregnancy, numerous immunosuppressive processes take place that allow the allograft fetus to thrive under exposure to humoral and cellular components of the maternal immune system. Breakdown of immune tolerance may result in sterile inflammation and cause adverse pregnancy outcomes such as preeclampsia, a vascular disease of the pregnancy with unpredictable course and symptoms from several organs. Immunological incompatibility between mother and fetus is strongly indicated in preeclampsia. Recently, genetic factors linking immunological pathways to predisposition to preeclampsia have been identified. In this mini-review genetic variation in immunological factors are discussed in the context of preeclampsia. Specifically, we explore immunogenetic and immunomodulary mechanisms contributing to loss of tolerance, inflammation, and autoimmunity in preeclampsia.

Keywords: preeclampsia, genetics, complement, major histocompatibility complex, FLT1, autoimmunity, pregnancy

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INTRODUCTION

Preeclampsia is a heterogeneous vascular disease of the human pregnancy that presents in a previously normotensive woman during the second half of the pregnancy with hypertension and proteinuria, or preeclampsia-associated signs in the absence of proteinuria (1, 2). Preeclampsia occurs in 3% of pregnancies (3), and it is one of the most important causes of maternal and fetal morbidity and mortality worldwide. The etiology of preeclampsia is incompletely understood, but it has its origins in early pregnancy and delivery of the placenta is the only cure (4). Two distinct subtypes has been frequently used in the literature based on the timing of the disease onset/delivery: early-onset $<34+0$ and late-onset ≥ 34 weeks of gestation. However, better understanding of the etiology and different subtypes is needed for early recognition and preventive measures.

Preeclampsia is considered a two stage-disease in which poorly perfused placenta produces factor(s) leading to systemic vascular disease and the clinical manifestations of preeclampsia (5).

At 8 weeks of gestation, the trophoblast cells invade from the placenta into the maternal tissue and into the uterine arteries. These endovascular trophoblast cells facilitate the remodeling of spiral

uterine arteries, which is essential for a healthy pregnancy. In order for the placentation process to be sufficient to support a healthy pregnancy, the extravillous trophoblast cells must avoid detection by the alternative pathway, subsequent complement activation, and immune response ((4, 6); **Figure 1**). Immunogenetic susceptibility to preeclampsia may have effect in the early stages of pregnancy whereby through loss of maternal tolerance toward the fetal components, the process of placentation is impaired. On the other hand, during the third trimester, underlying immunogenetic predisposition may aggravate sterile inflammation, which is exacerbated by systemic endothelial dysfunction in the mother's vasculature and result in progression of preeclampsia ((10) **Figure 2A**).

Data from epidemiological studies suggest that maternal and paternal genes through fetus affect the risk of preeclampsia, and its genetic basis is polygenic. Heritability of preeclampsia has estimated 55% with greater maternal (35%) than fetal (20%) contribution (12). Genes coding for components of the immune system are among the more important candidates in the quest to pinpoint clinically relevant genetic association. This is also evidenced by numerous non-genetic studies and observations involving components of the immune system (13–15). Activation of alternative pathway of complement activation has been shown to coincide with the critical weeks of placentation (16, 17). The role of decidual monocyte populations in healthy and pathological pregnancies have been reviewed elsewhere (18).

Genetic risk profile for preeclampsia is currently poorly characterized. The genetic studies have suffered from non-reproducibility and been lacking functional validation. Recently, in a large genome wide association study of preeclampsia a first robust association in the fetal genome was found in the common variant near Fms related tyrosine kinase gene (*FLT1*) encoding anti-angiogenic factor Fms-like tyrosine kinase 1 (FLT1) (19). Our group has published protective maternal low-frequency variants in the same gene (20).

In this mini-review we explore the immunogenetic role of *FLT1* in preeclampsia and selected genetic studies implicating loss of immune tolerance in early pregnancy or late pregnancy inflammation in preeclampsia. Dysregulation of complement system and autoimmunity are discussed in detail as potential causes of loss of maternal tolerance, while obesity is considered a possible cause of inflammation.

IMMUNOGENIC FLT1? AN EVOLUTIONARY PERSPECTIVE

The anti-angiogenic factor, soluble FLT1 is also known to have an anti-inflammatory function (21). FLT1 is expressed on inflammatory cells in addition to endothelial and trophoblast cells (22). In areas of Africa, where *Plasmodium falciparum* malaria is endemic, first pregnancies share a particular risk of not only preeclampsia but also of placental malaria (23). In placental malaria, the fetal tissue will express an excess of sFLT1 apparently in an attempt to regulate the maternal inflammatory response thereby reducing the rate of spontaneous abortions (24). Consequently, positive selection on a genetic variant with capacity to resist placental malaria by increasing sFLT concentration may have influenced *FLT1* allele frequencies within the general population enough to introduce a novel risk to preeclampsia (25).

Soluble FLT1 is conserved across vertebrates. The human FLT1 protein contains two tyrosine kinase catalytic (TyrKc) domains, three domains from the immunoglobulin (Ig) cell adhesion molecule (cam) subfamily (Igcam), one Ig-like domain, and one true Ig domain (26). In a detailed molecular evolutionary analysis, in contrast to other related proteins, in FLT1, only the TyrKc domains located at amino acids 819-933 and 991-1157 were found to be conserved across vertebrates (26). Large degree of variance between related proteins may be a reflection of recent evolutionary selection pressure on the FLT1. Malaria is known to be a potent source of immunological selection. Together this evidence is in support of possible thus far poorly understood immunological roles of the FLT1.

The major contributor to sFLT1 load in human pregnancy is the recently evolved isoform sFLT1-e15 (27). Overexpression of the primate specific isoform sFLT1-e15a is also associated with preeclampsia suggesting, that this novel isoform harbors thus far unexplained fitness advantages (27, 28). Assuming that sFLT1 is pathogenic, it is thereby possible that in non-primate mammals' conditions that lead to pregnancy-associated pathological rise in sFLT1 do not exist. On the other hand, it is also possible that the sFLT1 in humans has evolved specific functions, patterns of expression, or regulatory mechanisms that are essential for development of preeclampsia (25).

Further evidence of the immunological interactions of FLT1 is derived from a murine model, where increase in complement activation resulted in increased levels of FLT1 (29). Monocytes can be stimulated to express an excess of FLT1 when exposed to complement activation products C3a and C5a *in vitro* (29). Additionally, nuclear factor of activated T-cells (NFAT) transcription factors are involved in expression of mRNA of inflammatory cytokines, sFLT1-e15, and FLT1, as well as, and secretion of sFLT1 from primary human cytotrophoblasts (30). NFAT transcription factors may in further studies prove to be another link between FLT1 and immune response. Furthermore, angiogenic dysregulation may play a role in activation of the classical pathway in the kidney in a murine model of preeclampsia as evidenced by C4 deposition in the tissue in presence of excess sFLT1 (31).

FLT1 3'UTR dinucleotide repeat polymorphism have been shown to influence the expression of FLT1 and fetal outcome in the context of placental malaria with possible immunomodulatory effect (23). As far as we know, the distribution of these repeat polymorphisms in preeclampsia has not been explored.

TOLERATING OFFSPRING: DUAL ROLE OF COMPLEMENT SYSTEM IN RECOGNIZING AND CLEARING OF FETAL MATERIAL

Complement system is an ancient part of innate immunity, which consists of cell surface-bound and freely circulating

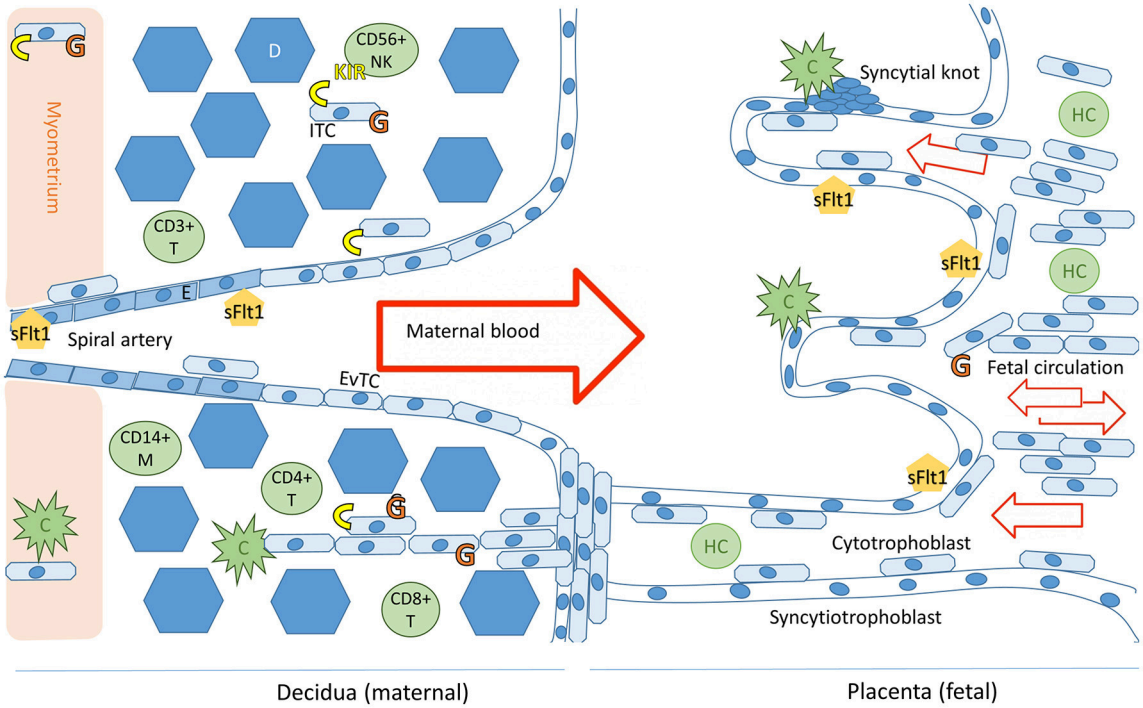


FIGURE 1 | Schematic representation of the maternal-fetal interface and its immunologicalplayers. Trophoblast cells (fetal) and endothelial cells (maternal) express sFlt1. The placental villi are shown in the right side of the image and decidua on the left. Invading trophoblast cells will encounter maternal complement system (C) in the decidua and in the intervillous space. Invading extravillous trophoblasts express HLA-C and HLA-G receptors (in yellow and orange, respectively), expression of HLA-G by villous trophoblast cells in the placenta decreases during the course of the pregnancy (7). Successful trophoblast invasion will extend to the vascular layers of myometrium and invade the uterine spiral arteries, where endovascular trophoblast cells (EvTC) will replace endothelial cells (E) causing remodulation and relaxation of the spiral artery to allow for non-turbulent high volume low pressure circulation into the intervillous space. Interstitial trophoblast cells (ITC) will remain in the maternal tissue creating tolerance of the fetal tissue in the maternal immune system. Hofbauer cells (HC) are the predominant immune cell population in the villi throughout placental development. The decidual immune cell population consists of macrophages (M), natural killer cells (NK), and populations of T-cells (T). Tolerance inducing Treg and Breg cells in particular are essential for a healthy pregnancy (8). Figure adapted from Lokki 2017 PhD thesis (9).

proteins that interact in a cascade of activation and regulation. Complement system has the capacity to discriminate between self- and non-self-cells and particles, and thereby maintain tolerance, or activate adaptive immunity. Complement activation can lead to inflammation, cell death, and tissue destruction. However, complement system also has a crucial role as a facilitator of phagocytosis thereby clearing debris and altered cells, in addition to removing pathogens. To protect own tissues from complement-mediated destruction and death, pathways of complement activation must be carefully regulated. Complement system has been studied extensively in preeclampsia, but genetic association studies linking components of the complement system to preeclampsia are not as plentiful.

Preeclampsia has previously been likened to thrombotic microangiopathies (TMA), which are caused by inadequate regulation of the complement system. In TMA, complement attacks against endogenous tissue structures such as endothelial cells and blood cells causing vascular damage and kidney failure. Pregnancy can act as a trigger of TMA syndromes. Atypical hemolytic uremic syndrome (aHUS) is a complement disease of the kidney with a TMA mechanism (32). Dysregulation of alternative pathway of complement system is indicated in aHUS (33). Similarly, most preeclampsia associations in complement system are found in the alternative pathway (34, 35).

The component C3 is in the core of the complement system. It can be activated by three different pathways. C3 can also become spontaneously activated in the human serum (32). Via the alternative pathway of complement activation, C3 is spontaneously activated and cleaved into activation products C3a and C3b in the absence of complement regulators. According to the sequence context, a haplotype spanning the active domains of C3 may predispose or protect from severe preeclampsia in a Finnish population (36, 37). We found a haplotype of 16 SNPs spanning the functionally critical sections in the middle of the gene. In this haplotype, three SNPs have most robust independent associations to severe preeclampsia further supporting its functional significance (36).

The results of this and other studies indicate that parallel to mice, C3 also plays a central role in the healthy human pregnancy (38, 39). The mechanism of haplotype association to severe preeclampsia is unclear but the effect may be due to functional or regulatory attributes of this region. For example, functional effect may affect the extravillous trophoblasts' capacity to evade complement activation by C3 binding, thereby

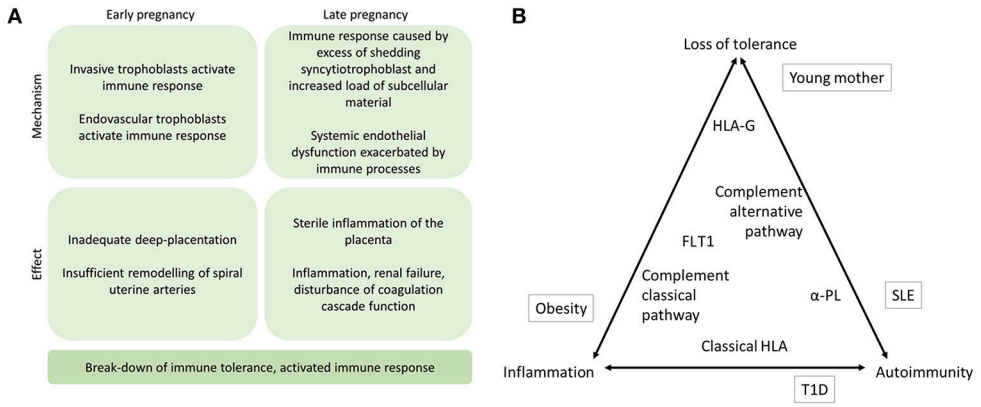


FIGURE 2 | (A) The proposed mechanisms of immune response in the etiology of preeclampsia. Immune mechanisms contribute to preeclampsia in early and late stages of the pregnancy. Specifically, if alternative pathway (AP) of complement system fails to recognize the invading trophoblast cells at the placental bed or in the uterine arteries, placentation may remain superficial and maternal uterine spiral artery remodeling may be compromised. In later stages of the pregnancy, complement activation aiming to facilitate phagosytosis of excess syncytiotrophoblast debris may induce sterile inflammation of the placenta locally. Concurrently, systemic endothelial dysfunction including disturbed coagulation function may be aggravated by immune activation and result in inflammation and shifting of Th2 to Th1 helper cell as well as disturbance of Treg and Th17 cell balances thereby further contributing to activation of the maternal immune response including erroneous antigen presenting in patients with applicable HLA alleles (11). **(B)** The triad of immune mechanisms in preeclampsia. The placement of contributing factors represents their suggested role in contributing to loss of tolerance, inflammation, and autoimmunity. Environmental risk factors outside in boxes, and the immunogenetic factors discussed in this review are inside the triangle. α-PL, anti-phospholipid antibodies; HLA, human leukocyte antigen; FLT1, Fms-like tyrosine kinase 1; T1D, type 1 diabetes.

compromising deep placentation and spiral artery remodeling in early pregnancy. Inflammation caused by excess complement activation may also be involved in a later stage of preeclampsia influencing severity of symptoms such as hypertension (40).

Immune complexes or opsonisation of the target surface by C1 complex triggers the activation of the classical pathway of complement system. Clearance of placenta-derived particles is crucial when preeclampsia symptoms develop in later pregnancy. Classical pathway activation results in cleavage of C4, which is a homolog of C3. C4 is present in two proteins, C4A and C4B, which are coded by usually two copies of each gene. While copy number variation of both *C4* is common, zero copies of both resulting in complete *C4* deficiency are very rare (41). Results of a pilot study conducted on mother-infant pairs with early-onset (delivery $<$ 34 weeks of gestation) or late-onset (delivery $\geq$ 34 weeks of gestation) preeclampsia and non-preeclamptic controls suggest that deficiencies in *C4* may predispose to preeclampsia (14). *C4A* or *C4B* deficiencies were found almost twice as often in women with early-onset preeclampsia than in healthy controls. *C4A* deficiencies are observed in 16% of general population in Finland (42). In preeclampsia, *C4A* deficiencies were found in 40% (2/5) of women with late-onset preeclampsia and in 43% (3/7) of women with early-onset preeclampsia. None were observed in controls ($n = 7$). The copy number of *C4* seems to decrease with the severity of preeclamptic symptoms (14). *C4A* deficiencies have previously been linked to autoimmune diseases (43). The patients in the preeclampsia study did not suffer from autoimmune diseases. The high incidence of *C4A* deficiency in preeclampsia supports the importance of classical pathway of complement system in preeclampsia.

Membrane co-factor protein (MCP, CD46) has the capacity to regulate both alternative and classical pathways of complement activation by binding to C3b or C4b and acting as a co-factor to the inactivator enzyme Factor I. MCP is a widely expressed type 1 membrane bound protein. aHUS, can be caused by mutations in *MCP* (44–47). Proteinuria is one of the cornerstone symptoms of preeclampsia, but its degree varies between patients. Renal dysfunction due to uncontrolled complement activation has been suspected to be the underlying link between preeclampsia and kidney diseases. To investigate whether sequence variants in the *CD46* might predispose to preeclampsia, we sequenced the *MCP* gene in preeclamptic women with severe proteinuria and in non-preeclamptic controls (37, 48). The results of this study do not corroborate the previously reported association of A304V to severe preeclampsia in a cohort of autoimmune pregnancies (49). We observed similar minor allele frequency (MAF) of ∼6% in cases and controls. We also found one control woman, who was homozygous to 304*V allele. Heterozygosity for another functional single nucleotide polymorphism (SNP) K32N (rs150429980) was found in one preeclamptic woman and one control. Thereby results are inconclusive. It is possible, however, that MCP plays a part in a particular subtype of preeclampsia, due to the heterogenous nature of the disease.

The complement co-factor I (coded by *CFI*) is a serine protease that inactivates C3b and C4b in the presence of a co-factor protein, such as complement factor H (FH) and MCP. In the PROMISSE cohort consisting of patients with anti-phospholipid antibodies or systemic lupus erythematosus (SLE), two severe preeclamptic patients with a history of complicated pregnancies were found to carry the loss-of-function mutation I398L in *CFI* (49). Furthermore, a

mutation in the *CFH* the gene coding for FH with unknown functional consequence was found in another patient (49). While FH is mainly effective in inhibition of the alternative pathway, MCP and factor I have the capacity to regulate both alternative and classical pathways of complement activation.

IMMUNOGENETIC PREDISPOSITION FOR COMPROMISED TOLERANCE

Major histocompatibility (MHC) in chromosome 6 (6p21.3), is the most polymorphic region of the human genome as a result of diverse and shifting immunological selection pressures. Many of the genes in the MHC code for proteins with immunological function. Genes coding for C4A and C4B are located in the MHC as are the genes coding for human leukocyte antigen (HLA) receptors.

At least two autoimmune diseases exist that harbor an increased susceptibility to preeclampsia. Among other immunological defects, aberrant NK cell biology has also been implicated in both, SLE and Type 1 Diabetes. Together these observations might shed light to the disease mechanisms in pregnancy complications.

The proportion of natural killer (NK) cells increases markedly in the uterus/endometrium during implantation and they likely have an important function during early stages of placentation (50, 51). Accordingly, genotypes of KIR receptors on the NK cells in combination with genotypes of their ligands, HLA-C on fetal trophoblast cells have been under investigation in preeclampsia (52–54).

SLE is characterized by a diminished number of NK cells with variety of functional abnormalities (55). SLE shares many symptoms with preeclampsia, including hypertension, proteinuria, and thrombocytopenia. SLE carries a 2- to 4-fold increase in risk of preeclampsia during pregnancy. In a Swedish population-based registry study, the risk for severe preeclampsia in SLE patients was 4.3% (56). Among the MHC loci, the HLA-DRB1*15:01, one of the strongest susceptibility loci for SLE in European-descent populations, is also associated to reproductive failure, i.e., recurrent pregnancy loss and secondary recurrent pregnancy loss (57, 58).

Among pregnant women with Type 1 diabetes (T1D), 15–20% develop preeclampsia (59), and nephropathy further increases the risk of preeclampsia to up to 42–52% (60), which raises questions of shared pathologies. NK cells have a crucial role in trophoblast invasion and spiral artery remodeling in the early stages of pregnancy, as well as in the recognition of the allograft fetal cells. Diverse aberrations of NK cell function are widely evidenced in T1D [reviewed in (61)]. It has been shown, that during the diabetic pregnancy, NK cells adhering to normal decidual endothelium are diminished in comparison to the non-diabetic control pregnancies suggested reduced number of NK cells homing to decidua in the diabetic pregnancy (62). Furthermore, the peripheral blood CD56bright NK cells from pregnant T1D patients expressed very low levels of selectin L (SELL) and alpha 4 integrin (ITGA4), which are important receptors for homing to the uterus (63). CXCL10 and CXCL12 chemokines are produced by the decidua. Their receptors CXCR3 and CXCR4, respectively, were expressed in lower levels on NK cells from T1D patients (63). Furthermore, the expression of activating receptor CD335 in the NK cell is increased during pregnancy in T1D patients. Aberrant NK cell function may result in the increased Th1/Th2 ratio and enhanced activation of intermediate and non-classical monocytes (64), both of which have been observed in preeclampsia, as well as T1D. This may contribute to the underlying mechanism of higher incidence of preeclampsia in T1D patients.

While the villous trophoblasts in the placenta are HLA null, the invasive extravillous trophoblasts (EVT) express genes belonging to the MHC, namely HLA-E, -F, -G, and -C genes (**Figure 1**). Trophoblast cells do not express HLA class II on the placental surface but syncytiotrophoblast, the outer most layer of placental villi, contains intracellular HLA class II antigens (65). Therefore, compatibility of maternal and fetal HLA genotypes may also influence the immune response in late pregnancy when fetal components are released from the disintegrating placenta. In this context, HLA-A, -B, -DR, and -DQ gene groups may also be relevant in preeclampsia, but thus far, this hypothesis has received little attention.

HLA-G is considered to be protective and tolerogenic during pregnancy. HLA-G is present in semen, which suggests that immune tolerance induction starts already before conception. There are several studies suggesting low or reduced levels of sHLA-G in preeclampsia patients and reduced levels of HLA-G mRNA has been observed in placentas of preeclamptic women (66–69). In a study of genetic polymorphisms, a 14-bp ins/del polymorphism in the 3'UTR of exon 8 of the HLA-G gene was associated with mRNA stability and overall HLA-G production (70). The role of HLA-G polymorphism in preeclampsia is still unresolved.

ANTIANGIOGENIC SFLT1 AND INCREASED INFLAMMATORY RESPONSE IN ESTABLISHED PREECLAMPSIA

Inappropriate maternal immune responses to trophoblast in early pregnancy may lead to abnormal placentation and set the stage for clinical preeclampsia later in pregnancy. Established preeclampsia is characterized by endothelial dysfunction and systemic inflammatory response to placental oxidative stress [**Figure 2A**; (71)].

It has been suggested that antiangiogenic sFLT1 sensitizes endothelial cells to pro-inflammatory factors (72). The first genome wide association study of offspring from preeclamptic pregnancies reported that common variants near *FLT1* on chromosome 13 were associated with preeclampsia (19). Incidence of preeclampsia is known to be increased in pregnancies with fetal Trisomy 13 (73) suggesting that increased placental production of sFLT1 has a role in preeclampsia susceptibility. Furthermore, the importance of the *FLT1* in the disease is evidenced by the recently discovered protective variants in the gene (20).

Normal third-trimester pregnancy is characterized by activation of peripheral blood leukocytes, which is further increased in preeclampsia (74). Increase in the maternal circulating levels of proinflammatory cytokines tumor necrosis factor alpha (TNF α), interleukin (IL)-6, and also the anti-inflammatory cytokine IL-10 in the third trimester of pregnancy in women affected by preeclampsia have been demonstrated in a meta-analysis (75).

Obesity increases the risk of preeclampsia 2- to 3-fold (76–78), but the underlying mechanisms are not fully understood. Obesity is a state of uncontrolled inflammatory responses leading to systemic low-grade inflammation and increased insulin resistance (79). Even modestly overweight women have vascular endothelial dysfunction assessed by brachial artery ultrasound flow-mediated vasodilation (80). The secretion of pro-inflammatory cytokines including TNFα and IL-6 is increased in hypertrophic adipocytes (79). Herse and coworkers have also shown that TNFα decreased sFLT1 expression in mature adipocytes (81). We and others have found lower concentration of sFLT1 in obese compared to normal-weight preeclamptic women, but not in respective normotensive pregnant women (82, 83). Associations between maternal body mass index and proinflammatory cytokines TNFα and MCP-1 in maternal plasma have been demonstrated (84). Thus, the production of sFLT1 and proinflammatory cytokines by placenta and extraplacental sources may be different in obese and normal-weight women during pregnancy and in normal and complicated pregnancies.

CONCLUSION

The mechanisms regulating the immune response are central in normal pregnancy and the development of preeclampsia (**Figure 2A**). Mechanisms of autoimmunity, loss of tolerance, and inflammation in preeclampsia are evidenced in this mini-review (**Figure 2B**). Angiogenic proteins, continuous subclinical inflammation, and insulin resistance in preeclamptic women have been suggested to result in increased cardiovascular risk that with additional risk factors may result in cardiovascular disease (85–87). Thus far, investigation into the genetic background of the immunological pathogenesis of preeclampsia has mostly concentrated on the genes coding complement components and MHC. While both pathways are relevant to the early pregnancy and later clinical manifestations of preeclampsia, studies addressing other immunological mechanisms will be a welcome contribution to increase our understanding of the complex immunological interactions in the disease.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Regulation of Placental Extravillous Trophoblasts by the Maternal Uterine Environment

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During placentation invasive extravillous trophoblasts (EVTs) migrate into the maternal uterus and modify its vessels. In particular, remodeling of the spiral arteries by EVTs is critical for adapting blood flow and nutrient transport to the developing fetus. Failures in this process have been noticed in different pregnancy complications such as preeclampsia, intrauterine growth restriction, stillbirth, or recurrent abortion. Upon invasion into the decidua, the endometrium of pregnancy, EVTs encounter different maternal cell types such as decidual macrophages, uterine NK (uNK) cells and stromal cells expressing a plethora of growth factors and cytokines. Here, we will summarize development of the EVT lineage, a process occurring independently of the uterine environment, and formation of its different subtypes. Further, we will discuss interactions of EVTs with arteries, veins and lymphatics and illustrate how the decidua and its different immune cells regulate EVT differentiation, invasion and survival. The present literature suggests that the decidual environment and its soluble factors critically modulate EVT function and reproductive success.

Keywords: placental development, extravillous trophoblast, decidual immune cells, trophoblast invasion, uterine natural killer cells, decidual macrophages

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INTRODUCTION

Development of the human placenta, its distinct epithelial trophoblast subtypes and their interplay with maternal cells and growth factors of the pregnant uterus are crucial for a successful pregnancy. After implantation the trophectoderm, the outermost cell layer of the blastocyst, gives rise to mononuclear cytotrophoblasts (CTBs) forming placental villi through branching morphogenesis. During the first weeks of gestation primary villi, consisting of proliferative CTBs, transform into secondary mesenchymal villi and mature tertiary villi, the latter undergoing vasculogenesis and angiogenesis (1–3). At term these tree-like structures of the human placenta display a surface area of ~15 m^2, completely covered with multinuclear syncytiotrophoblasts (STBs). STBs are generated by cell fusion of villous CTBs (vCTBs) and fulfill a vast range of functions such as production of pregnancy hormones, transport of oxygen and nutrients from the maternal blood stream to the growing fetus and clearance of fetal waste products (4, 5). However, early placental development and fetal growth occurs in the absence of maternal blood and oxygen and are likely supported by growth factors and proteins secreted from endometrial glands (6). As soon as the utero-placental circulation is established between 10th and 12th week of pregnancy placental villi are bathed in maternal blood, and hence are termed floating villi.

Whereas, STBs of floating villi represent the transport units of the human placenta, anchoring villi of the placental basal plate form another differentiated trophoblast type, the so called invasive extravillous trophoblast (EVT). Upon attachment of villi to the maternal decidua, the endometrium of the pregnant uterus, proliferative proximal cell column trophoblasts (pCCTs) develop which further differentiate into distal CCTs (dCCTs) ceasing their mitotic activity (**Figure 1**). EVTs are formed upon detachment from the distal cell column. These cells deeply migrate into the maternal decidua and the first third of the underlying myometrium (7). Already 2 weeks after fertilization two types of EVTs can be discerned within the maternal uterine compartment, interstitial CTBs (iCTBs), colonizing the decidual stroma, and endovascular CTBs (eCTBs), penetrating the maternal spiral arteries (8). Stepwise modification of these vessels is regarded as a critical step in placentation. In the first weeks of pregnancy, EVTs plug the spiral arteries, likely to prevent precocious onset of blood flow to the developing placenta, hence protecting against early placental damage through oxidative stress and fetal loss (6, 9). However, as the embryo switches from histiotrophic to haemotrophic nutrition after the 10th week gestation, plugs dissolve and the endothelial layer of the spiral arteries is replaced by eCTBs (8, 10). The latter are thought to arise by luminal migration into the myometrial segments of spiral arteries (11). Moreover, iCTBs accumulate in the muscular vessel wall promoting its elastolysis and degradation, where decidual macrophages and uterine natural killer cells (uNKs) also contribute to this process (12). Notably, uNK cells, increasing in numbers during the secretory phase of the menstrual cycle and early pregnancy (13), initiate remodeling by inducing apoptosis of vascular smooth muscle cells, whereas iCTBs are thought to complete this process (14). These modifications transform the spiral arteries into highly dilated vessels ensuring low-pressure blood flow to the placenta and the developing fetus. Both iCTBs and eCTBs upregulate adhesion molecules mimicking an endothelial phenotype which could be instrumental during invasion and for the replacement of maternal endothelial cells (15). Defects in vessel remodeling, in particular in the myometrial part of the spiral arteries, have been reported in various pregnancy complications, such as preeclampsia, fetal growth restriction, preterm labor, abortions, and stillbirth (9, 10, 16–19). Failures in immunological acceptance of the placenta, decidual function and/or abnormal trophoblast invasion and differentiation could be underlying causes (20–23). During the first weeks of gestation EVTs, originating from the trophoblastic shell (24), also migrate into decidual lymphatics and veins, already before arterial remodeling occurs (25–27). Number of EVTs in lymphatic and venous vessels is lower in recurrent abortions suggesting that, along with defects in spiral artery remodeling, failed interactions of EVTs with other types of uterine vessels could contribute to pregnancy complications (26). Similarly, EVTs also invade into the decidual glands which could promote early histiotrophic nutrition (28).

In the decidua basalis, iCTBs communicate with diverse cell types of the fetal-maternal interface, such as decidual stromal cells (DSCs) and different immune cells (**Figure 1**). Amongst those, uNK cells and macrophages have been delineated as the most abundant cell types (29). The role of uNK cells has been extensively investigated throughout the years. Besides their role in the immunological tolerance of the semi-allogenic fetus, uNK cells are thought to affect decidual angiogenesis and EVT function (22, 30). Expression of human leukocyte antigen C (HLA-C) on EVTs, interacting with killer cell immunoglobulin-like (KIR) receptors on uNK cells, could play a role in pregnancy outcome as certain combinations of fetal HLA-C and maternal KIR alleles might increase the risk of developing preeclampsia and recurrent miscarriage (31, 32). It is anticipated that unfavorable HLA-C/KIR interactions impair trophoblast invasion and as a consequence spiral artery remodeling. The role of KIRs and their effects in allorecognition of EVTs has been subject of numerous reviews (22, 33–36) and will be only briefly discussed herein. Instead, we review how uNK cells influence EVTs in a paracrine manner. Further, we will also focus on the other maternal cell types of the decidua and summarize how they might affect cell column growth of anchoring villi, EVT formation and motility. Factors secreted by EVTs, controlling trophoblast migration and invasion in an autocrine manner, have been extensively discussed elsewhere (37, 38), and will not be presented herein. Likewise, the paracrine effects of EVT-secreted factors on decidual immune cell function will not be a topic of this review.

DEVELOPMENT OF THE EVT LINEAGE AND ITS DIFFERENT SUBTYPES

EVTs originate from distal cell columns of anchoring villi at distinct contact sites with the maternal decidua. Numbers of the latter are a consequence of the frequency of villous branching (5). Different to growth of vCTBs, that form a double-rowed epithelium after lateral cell division and fuse into STBs, pCCTs break through the overlying STB layer and form multiple layers of proliferative trophoblasts (**Figure 1**). Similar to early phases of tumor formation, pCCTs detach from the basal membrane and lose their polarity. However, in contrast to cancer cells, growth and invasion of trophoblasts is highly organized and precisely controlled in a spatiotemporal manner. At distal sites of anchoring villi, pCCTs differentiate into non-proliferative dCCTs. Similar to iCTBs which have deeply migrated into the decidua, dCCTs express numerous EVT markers such as HLA-G (39), T-cell factor 4 (TCF-4) (40), integrin α5 (ITGA5) and β1 (41), Notch2 (42), proteoglycan 2 (26). and ErbB2 (43). Hence, formation of dCCTs represents the first step of EVT differentiation. In comparison to dCCTs, iCTBs undergo further differentiation by inducing/upregulating specific proteins, for example ITGA1 (41), matrix metalloproteinase (MMP) 12 (44, 45) or diamine oxidase (DAO) (46). The latter is predominantly expressed in EVTs surrounding decidual vessels and was shown to be decreased in serum samples of early-onset preeclamptic women (46). *In vivo*, DAO is only detected in ∼20 and 45% of iCTBs and perivascular CTBs, respectively, providing some evidence for the existence of different iCTB subtypes. Similarly, different EVT populations, identified by single-cell RNA-Seq, have recently been suggested (47). However,

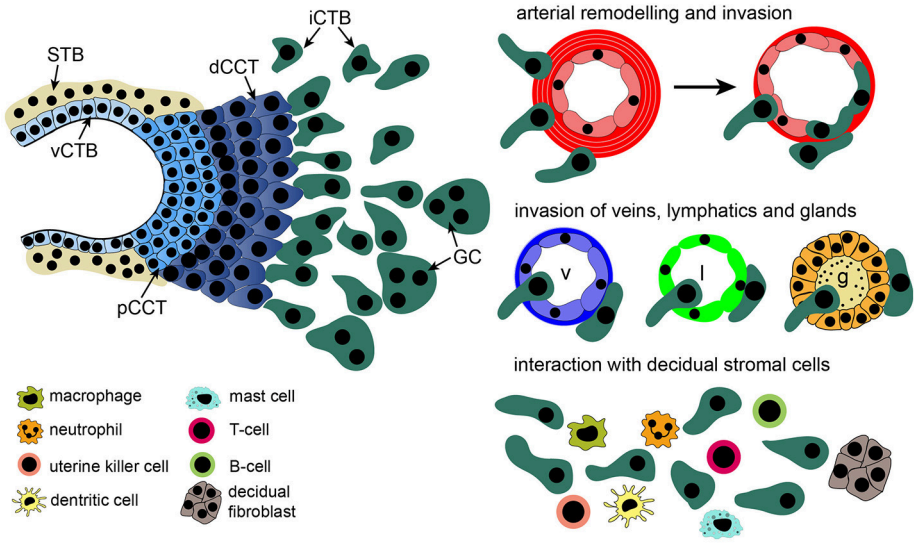


FIGURE 1 | Structure of a placental anchoring villus and its different trophoblast subtypes. Precursors, residing in the villous cytotrophoblast (vCTB) layer either differentiate into multinuclear syncytiotrophoblasts (STBs), surrounded by maternal blood, or give rise to proliferative proximal cell column trophoblasts (pCCTs) upon attachment of villi to the maternal decidua. After differentiation into distal cell column trophoblasts (dCCTs) extravillous trophoblasts (EVTs) develop, breaking through the overlying STB layer. EVTs detach from distal cell columns, migrate into the decidual stroma and the maternal spiral arteries replacing maternal endothelial cells. In the decidual stroma interstitial CTBs (iCTBs) interact with macrophages, B- and T-cells, mast cells, dendritic cells, neutrophils, decidual stromal cells, and uterine natural killer (uNK) cells. Moreover, iCTBs approach the vessel walls of spiral arteries and promote remodeling from outside. Also, veins (v), lymphatics (l) and glands (g) are invaded by these cells forming multinucleated trophoblast giant cells (GC) as an end-stage of differentiation.

it remains largely unknown if variations between iCTBs are specified by the intrinsic genetic program of the placental anchoring villus or determined by the diverse decidual structures. Likewise, the exact route of eCTB migration and the mechanisms specifying these cells have not been unraveled (11). The different phenotypes of EVTs could eventually be influenced by the decidual environment. For example, it was shown that abnormal gene expression of preeclamptic CTBs was reverted back to normal physiological levels when cultured *in vitro* (21). On the other hand, EVT development *per se* occurs independently of the decidual environment and its growth factors. Purified CTBs and villous explant cultures, seeded on extracellular matrix, undergo spontaneous EVT differentiation upregulating dCCT, and iCTB markers in a kinetic manner (48–50). In preeclampsia this endogenous EVT differentiation program could be disturbed (51). Anchoring villi and detaching EVTs of tubal pregnancies show the same pattern in integrin switching as EVTs invading the decidua basalis (52). Similarly, EVTs migrating from implanted villous explants and invading the kidney capsule of SCID mice, were shown to induce HLA-G expression (53).

Although the genome-wide expression profiles of non-migratory CTBs and invasive EVTs have been unraveled (54, 55), mechanisms promoting cell column formation and CTB commitment toward the EVT lineage have been poorly elucidated. Recently, Notch1 has been detected in a subset of proliferative pCCTs, indicating that this particular receptor could mark EVT progenitors (56). Indeed, the active Notch1 intracellular domain promoted pCCT survival and marker expression, but suppressed stemness markers of vCTBs suggesting that Notch1 could convert CTB precursors into EVT progenitors (57). Low oxygen levels, occurring during early phases of placental development (58), were shown to trigger Notch1 expression in primary CTBs (57). Hence, low oxygen could promote expansion of EVT progenitors and promote early stages of EVT differentiation and invasion (59). However, the current literature about the specific role of oxygen in trophoblast biology is controversial, has been extensively discussed (60–63), and will not be subject of the present review. Moreover, changes of the self-renewing conditions of long-term expanding 3-dimensional cytotrophoblast organoid cultures promoted outgrowth of Notch1-positive progenitors and EVT formation (64), further supporting the view that development of different trophoblast subtypes is largely determined by the intrinsic differentiation program of the placenta.

THE IMPACT OF THE DECIDUA ON EXTRAVILLOUS TROPHOBLASTS: GENERAL ASPECTS

In a few species, spontaneous uterine transformation commences during the second half of the menstrual cycle. This process, preceding implantation, is exclusively observed in mammals with menstruation and deep, haemochorial placentation, such as humans and higher primates (65, 66). Shortly after implantation the pregnant uterus undergoes dramatic morphological changes including extracellular matrix remodeling, vascularization, increase in uNK cell numbers and secretory activity of glands

as well as transformation of stromal fibroblasts into polygonal decidual cells (67). Decidual glands secrete glycoproteins, such as glycodelin A, carbohydrates and other metabolites nourishing the embryo during the first weeks of pregnancy (68–70). During this phase of histiotrophic nutrition glandular cells also produce various growth factors likely promoting early placental development such as leukemia-inhibitory factors (LIF), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF) and endocrine gland-derived vascular endothelial growth factor (EG-VEGF) (69, 71–74). Indeed, EGF (see below) and EG-VEGF were shown to augment proliferation of vCTBs/CCTs in villous explant cultures (75). Similarly, VEGF was shown to stimulate growth of trophoblast cell lines and primary cultures (76). In contrast, LIF may be mainly involved in the regulation of implantation and trophoblast invasion (77–79).

Differentiation of uterine fibroblasts, commonly referred to as decidualization, initiates during the luteal phase of the menstrual cycle and requires the combined action of cAMP and progesterone on the estrogen-primed endometrium (80). Besides secretion of growth- and- invasion-controlling factors (see below) numerous other functions have been assigned to decidual fibroblasts (DFs). For example, DFs secrete enzymes clearing reactive oxygen species (67, 81) and thereby might protect the decidua and/or EVTs from adverse stress response when local oxygen levels rise between 10th and 12th week of gestation. Trophoblast-derived human chorionic gonadotrophin (hCG) could further increase resistance of DFs against oxidative tissue damage (82). DFs also express various extracellular matrix proteins, such as fibronectin, emilin-1, decorin, fibulins, collagens and laminins (83–86), potentially controlling EVT motility by binding to trophoblast-expressed adhesion molecules and receptors (87).

In women with placenta accreta, EVTs excessively invade the maternal uterus, mostly as a consequence of implantation onto or close to a scar after preceding cesarean section. It is anticipated that the local absence of decidua facilitates trophoblast invasion into the underlying myometrium (88, 89). From this pathology, one might conclude that the decidua restricts migration of trophoblasts thereby controlling depth of invasion in a temporal manner and preventing aberrant, tumor-like expansion of the placenta. Indeed, former concepts suggested that trophoblast-derived MMPs, known to promote invasiveness, are counter-balanced by tissue-inhibitors of metalloproteinases (TIMPs) present in the decidua (90, 91). Similarly, decidual plasminogen activator inhibitors (PAI) 1 and 2 could control timing and depth of trophoblast invasion by inhibiting the plasminogen activator (PA) system expressed by migratory EVTs. However, physiology of trophoblast invasion is more complex since both EVTs and DFs express MMPs, TIMPs, PAs, PAIs as well as urokinase plasminogen activator receptor (uPAR) (90, 92–98). Hence, the decreasing rate of EVT invasion during pregnancy cannot be merely explained by the reciprocal expression of MMPs/uPA and TIMPs/PAI in EVTs and the decidua, respectively. Moreover, potential changes of inhibitor expression throughout gestation or in superficial vs. deeper regions of the decidua have not been measured. Diminished EVT migration at later stages of pregnancy might also be a consequence of the decreasing growth rates of cell columns. As follows, defects in invasion/remodeling, as observed in IUGR, could at least partly be the result of reduced trophoblast growth in this condition (99).

Other data do not support the concept that the decidua restricts trophoblast invasion. In contrast to anchoring villi of normal uterine pregnancies, distal cell columns of ectopic placental villi, isolated from tubal pregnancies, were extended in size. This suggests that the decidua could facilitate EVT detachment from anchoring villi during physiological development of the placental basal plate (52). Indeed, decidualized endometrial stromal cells express a tissue-specific variant of fibronectin favoring trophoblast invasion (100). Whereas, EVTs, generated by first trimester villous explant culture, migrated superficially on dermal fibroblasts, their co-culture with DFs resulted in promotion of interstitial invasion (100). Therefore, the specific features of the decidua may adapt to different stages of pregnancy and precisely control invasion of EVTs by expressing pro- and anti-migratory matrix proteins and factors (37, 38). In return, DFs have a high migratory capacity and could promote implantation by actively moving toward the blastocyst and provoking encapsulation of the conceptus (101). Indeed, EVT supernatants contain chemotactic signals that promote endometrial stromal cell migration (102). Genome-wide expression analyses revealed that trophoblast-conditioned medium of cultivated CTB preparations, containing a mixture of different CTB subtypes, could induce mRNAs encoding chemokines and angiogenic factors in decidualized endometrial fibroblasts (103). However, compared to EVTs, mixed CTB isolates may elicit different responses. In a recent study EVTs increased numbers of resting FoxP3-positive regulatory T cells (Tregs) upon co-cultivation with $CD4^+$ T cells, whereas vCTBs were ineffective (104).

CAVEATS OF *IN VITRO* STUDIES WITH EXTRAVILLOUS TROPHOBLASTS

Decidua-derived growth factors and their role in trophoblast motility have been investigated in numerous publications. However, many of these studies have drawbacks limiting their scientific value. Access to primary trophoblasts of early pregnancy is generally restricted, hence different trophoblast-like cell lines were utilized in invasion and migration assays. Yet, the specific origin of these cell lines is uncertain and their genome-wide gene expression profiles and HLA status differ considerably from purified CTBs (105, 106). Cell lines proliferate in culture whereas EVTs are non-mitotic cells. Hence, discordant results were obtained between cell lines and primary cells, for example in migration assays under hypoxic conditions (107, 108). Similarly, transforming growth factor β (TGFB), expressed by DFs, uNK cells and uterine glands (109, 110), was shown to either promote or inhibit trophoblast proliferation or invasion (111–115). Besides divergence between cell lines and first trimester CTBs, contaminations with highly migratory placental fibroblasts and variations between different cell isolations and primary model systems might account for multiple discrepancies and the high variances observed in trophoblast-related studies. Invasion

assays are often performed with pooled fractions of trypsinized primary CTBs representing a mixture of CCTs, EVTs and vCTBs. The latter also invade through 8 μm transwells *in vitro*, whilst undergoing cell fusion *in vivo*. Moreover, nuclear size is a limiting factor in invasion/migration assays (116). Indeed, EVTs become polyploid during differentiation displaying increased nuclear diameter (117, 118). Therefore, EVTs hardly pass membranes with 8 μm pores (50), a fact which has not been considered by the majority of trophoblast invasion/migration studies. Moreover, the high complexity of decidual cell types cannot be mimicked *in vitro*. In addition, local concentrations of soluble factors in the tissue and their variations during pregnancy and between uterine cell types are poorly studied. Therefore, *in vitro* assays are usually performed with saturating levels of recombinant factors. As follows, the prime target cell of many decidual proteins remains uncertain since the respective receptors have been identified on several uterine cell types. As a consequence, opposing roles for particular factors were suggested. For example, IL10 was shown to directly impair CTB invasion, but also to abolish the adverse effects of LPS-treated macrophages on trophoblastic cell migration (119, 120). Additionally, results obtained with first trimester villous explant cultures recapitulating attachment, outgrowth and EVT migration *in vitro* (121) are often interpreted differently by authors. For example, depending on the specific analyses, outgrowth was suggested to be indicative of both increased trophoblast motility and elevated proliferative capacity.

Herein, we focus on the abundant decidual factors, cytokines, and chemokines which have been most convincingly proven to affect EVT formation and function in reliable trophoblast *in vitro* model systems. However, these studies should also be interpreted in the light of the above-mentioned limitations.

REGULATION OF EXTRAVILLOUS TROPHOBLASTS BY DECIDUAL FIBROBLASTS

During decidualization fibroblasts upregulate key markers of the pregnant endometrium of which prolactin (PRL) and insulin growth factor binding protein-1 (IGFBP-1) are amongst the most abundantly expressed proteins (122–124). Both proteins likely exert pleiotropic effects on different uterine cell types including regulation of decidualization and EVT migration (125–128). Although PRL, also involved in differentiation of the decidual glandular epithelium (129), was shown to promote motility of first trimester CTBs (130, 131), the role of IGFBP-1 is less clear due to the high complexity of the IGF/IGFBP system. Both migration-activating and -inhibiting effects were attributed to IGFBP-1 upon binding to the EVT-expressed fibronectin receptor ITGA5B1 (132–134). However, one of the main functions of IGFBP-1 could be the regulation of IGF bioavailability at the fetal-maternal interface, possibly triggered by EVT-derived IGF-II (135). Upon secretion of IGF-II from these cells decidual IGFBP-1 might get dephosphorylated and further cleaved by EVT-specific MMP-3 and MMP-9 thereby increasing unbound IGFs, the latter stimulating trophoblast migration (126, 136–139). Yet, proteolytic fragments, generated by trophoblast-derived MMPs, may also restrain trophoblast invasion. Endostatin, a cleavage product of decidual collagen XVIII, was shown to impair IGF-II-induced EVT-motility (50, 140).

Besides the prime markers IGFBP-1 and PRL, other classes of soluble DF-secreted factors were suggested to control EVT motility including chemokines, cytokines and ligands of the EGF and Wingless (WNT) signaling pathways (38, 141). Different CXCL and CCL chemokines have been identified in DFs. Their respective receptors are present on uterine leukocytes and EVTs suggesting a role in immune cell trafficking as well as trophoblast migration, respectively (142, 143). For example, CXCL14 was shown to reduce invasiveness (144), whereas CXCL12 promoted CTB migration and suppressed apoptosis of term trophoblasts through its receptor CXCR4 (145–147). CCL2, expressed by DFs, macrophages and EVTs (148, 149), may recruit T helper 17 cells into the decidua, and interleukin (IL) 17 expressed by these cells could promote trophoblast growth and invasion (150). Other interleukins, shown to stimulate CTB invasion, are IL1B (151, 152) and IL8, secreted from uterine NK cells and DFs (153, 154), whereas IL11 had inhibitory effects (155).

While EGF and heparin-binding EGF (HB-EGF), expressed by the decidua, were shown to stimulate trophoblast invasion and outgrowth from villous explants cultures, proliferation of primary EVTs and trophoblastic HTR-8/SVneo cells was unaffected (156–159). Recently, however, we could demonstrate that these factors increased proliferation of vCTBs and CCTs in villous explant cultures of early placentae (160). Moreover, in contrast to vCTBs and CCTs, EVTs largely lack the EGF/HB-EGF-specific receptors EGFR and ErbB4, and induce ErbB2 and ErbB3 during differentiation (43, 161). Heterodimers of ErbB2 and ErbB3 were shown to interact with neuregulin 1, expressed by DFs, protecting EVTs from apoptosis and thereby retaining their differentiation program (43). Therefore, upregulation of EVT invasion/differentiation by EGF/HB-EGF could largely be a consequence of increased CCT proliferation, while direct effects of these factors on EVTs might be negligible.

Like in other developing tissues WNT signaling has been suggested to play a pivotal role in placental morphogenesis and differentiation (141, 162). Activation of the pathway by secreted ligands stabilizes the key mediator of WNT signaling, β-catenin, and promotes its nuclear recruitment (163). In the nucleus β-catenin binds to DNA-binding proteins of the T-cell factor (TCF) family thereby inducing TCF-mediated gene transcription. Invasive trophoblasts, the secretory endometrium and first trimester DFs express a variety of WNT ligands suggesting autocrine as well as paracrine effects of the particular pathway (164, 165). EVT formation and differentiation is strongly associated with activation of canonical Wnt signaling and nuclear expression of β-catenin, TCF-3 and TCF-4 (40, 166). Indeed, migration and differentiation of EVTs requires TCF-4, whereas survival and proliferation of CCTs is induced by WNT5A involving non-canonical mitogen-activated protein kinase (MAPK) activity (166, 167). Moreover, canonical Wnt signaling might play a dual role in early placental development controlling both long-term expansion of vCTB progenitors and EVT differentiation (64).

IMMUNE CELL DISTRIBUTION IN THE DECIDUA

The pregnant uterus is mainly colonized by cells of the innate immune system, of which the most abundant and by far best characterized cell types are macrophages and uNK cells. Most available literature refers to numbers ranging from 50 to 70% uNK cells, 20–30 % macrophages and 10–15 % T cells of the total $CD45^+$ immune cells in the decidua; only 2 % account toward the less abundant leukocyte populations including dendritic cells or Tregs (30, 168–170). However, the vast majorities of comparative analyses do not consider regional differences (parietalis vs. basalis) in decidual immune distribution and may also miss certain cell populations due to pre-selective isolation methods or due to the lack of appropriate markers to distinguish certain immune cell populations from each other. Hence, before describing uNK cell and macrophage function in detail, we will shortly discuss decidual immune cell populations which in our opinion have widely been ignored in the context of reproductive biology.

Mast cells have been previously described to mainly colonize the uterine myometrium and were shown to localize around decidual vessels. Interestingly, mast cell depletion in a mouse model results in diminished spiral artery remodeling and as a consequence leads to IUGR. The same study demonstrated a close spatial distribution of mast cells and EVTs in the human decidua basalis and reported a mast cell-dependent positive effect on EVT migration (171). Another study showed a role for neutrophils in placentation and trophoblastic giant cell invasion in mice (172). Interestingly, neutrophils seem to accumulate around spiral arteries and develop a pro-angiogenic phenotype toward the second trimester of pregnancy (173). The reason for the oversight of neutrophils might be explained by methodological issues as most protocols to obtain tissue leukocytes involve density gradient centrifugation eliminating all non-mononucleated immune cells, including neutrophils. Nevertheless, it should be taken into consideration that neutrophil accumulation in the decidua may at least be partly a response to blood coagulation and tissue damage occurring during tissue collection. Although mostly ignored, some scientific papers describe the presence of B cells in human term decidua (174). Moreover, a recent study shows that term decidua basalis contains more B cells when compared to decidua parietalis tissues (175). The authors of this study further found that decidua parietalis contains a higher proportion of mature/naive B cells whereas transitional B cells were enriched in decidua basalis. Since altered B cell distributions have recently been associated with preterm labor (174), more studies are needed to determine the role of B cells during pregnancy. In addition, distribution and characterization of B cells in first trimester decidua tissues has not been studied so far. While macrophages are considered to be the main phagocytic and antigen-presenting cell type in the human decidua little is known about dendritic cell distribution and function during pregnancy. One reason for the scarce information concerning decidual dendritic cells is the lack of marker combinations, which could reliably segregate macrophages from dendritic cells, since they develop from a common myeloid progenitor and therefore express common cell surface markers (176, 177). A good example for the problem to distinguish between macrophages and dendritic cells are Langerhans cells. These cells have long been referred to as long-lived dermal dendritic cells and are now considered tissue-resident macrophages with features of dendritic cells such as T cell-stimulation in lymph nodes (178). Despite overlapping cell marker expression and functional similarities in the skin, dendritic cells have unique properties. For instance, DCs homeostatically migrate to draining lymph nodes and are much more potent in antigen cross-presentation to $CD8^+$ T cells (179). Consequently, DCs are likely involved in shaping host immune responses toward the invading EVTs.

PROPOSED FUNCTIONS OF UTERINE NATURAL KILLER CELLS

Unlike conventional peripheral blood (pb) NK that are efficient killers, uNK cells in rodents and humans do not normally mount cytotoxic responses against fetal or placental tissues. Instead, growing evidence highlights the importance of uNK in controlling uterine neo-angiogenesis, spiral artery remodeling, the immune response against fetal antigen, and trophoblast function (30, 180–182). However, recent work shows that aberrant inflammation in pregnancy resulting from infection or fetal-driven alloimmunity programs uNK cells to acquire cytotoxic properties that promote fetal death and/or placental dysfunction (183, 184). Therefore, a contemporary view suggests that appropriate uNK activation is important for promoting healthy placentation, where *inadequate* (not enough) or *inappropriate* (too much) uNK activity contributes to defective placentation and related disorders of pregnancy that may include recurrent miscarriage, preterm birth, and preeclampsia (185, 186). In women, uNK cell numbers rapidly expand during the progesterone-dominant luteal phase of the menstrual cycle (22). Evidence suggests that the decidual environment, enriched with factors like progesterone and transforming growth factor TGFB1, promote the differentiation of NK cell progenitors into mature uNK cells that are defined phenotypically as $CD56^{superbright}/CD16^-$ cells (187, 188). By contrast, the phenotype of conventional pbNK cells is predominantly $CD56^{dim}/CD16^+$. Other distinctive features of uNK include the expression of tissue-residency markers (i.e., CD9, CD69, CD49a) (189, 190) and cytolytic proteins (i.e., perforin, granzyme, and granulysin) (191), and the expression of a distinctive natural killer receptor repertoire (192). In particular, killer immunoglobulin-like receptor (KIR) and natural killer group 2 (NKG2) receptors are robustly expressed by uNK cells (189, 192). These and other natural killer cell receptors, are thought to modulate maternal-fetal recognition, but may also play important roles in controlling aspects of trophoblast biology.

COMMUNICATION BETWEEN INVASIVE TROPHOBLASTS AND UTERINE NATURAL KILLER CELLS

In vivo evidence shows that EVTs directly interact with uNK cells (193), indicating that by EVT- uNK cell interactions these potentially modulate each other's functions (194–196). EVTs, unlike vCTBs that do not express major histocompatibility complex (MHC) type-I molecules, express a unique combination of classical HLA-C and non-classical HLA-E, HLA-F, and HLA-G class-I ligands playing a role in immunological acceptance of the placenta/fetus (30, 197, 198). This unique MHC composition enables EVT to directly interact with and modulate uNK cell processes through specific combinations of natural killer receptors. However, hard evidence for *in vivo* or *ex vivo* natural killer cell receptor-EVT interactions has been challenging to generate, due in large part to the ethical boundaries of working with human samples of pregnancy and to the logistical hurdles of working with primary CTB cultures and uNK cells isolated from tissues of the same pregnancy. As a surrogate for primary EVTs, co-cultivation of trophoblastic HTR-8/SVneo cells with uNK cells were performed demonstrating elevated uNK cell survival and downregulation of the activating NKG2D receptor (199). However, these data have to be interpreted with caution since HTR-8/SVneo cells express a different repertoire of HLA proteins, including HLA-A and HLA-B, which are absent from EVTs (105).

MHC class-I molecules expressed on EVTs can interact with multiple natural killer receptors that transmit inhibitory or activating signals to dampen or promote uNK cytotoxicity and production of cytokines, respectively (30). Perhaps the best-studied uNK cell receptors are the family of polymorphic KIRs that are defined by the presence of either 2 (2D) or 3 (3D) immunoglobulin-like domains and long (L) or short (S) cytoplasmic tails that help initiate inhibitory (L) or activating (S) signals. Inhibitory KIRs expressed on uNK cells include KIR2DL1, KIR2DL2, and KIR2DL3, and these receptors transmit strong inhibitory signals through their immunoreceptor Tyr-based inhibitory motif (ITIM) (35). Activating uNK KIRs include KIR2DS1 and KIR2DS4 (186, 200), however the receptor KIR2DL4, an unconventional KIR that predominantly localizes to endosomes and not the cell membrane, is also capable of transmitting activating signals (104, 201, 202). KIRs bind mainly to HLA-C, expressed by multiple cell types within decidual tissue, including EVTs (203). The number of *KIR* genes in the genome of any given individual varies within the population, as does the expression of haplotypic specific HLA-C, making the immunogenic complexities of uNK cell-EVT responses unique for any given pregnancy (204).

To date, most research has examined the importance of HLA-C in controlling uNK cell-related processes through either uNK-target cell or antibody cross-linking experiments. It is important to note that, the directionality of uNK cell response to HLA-C depends largely on the epitope type, designated broadly as C1 or C2. This designation is based on a dimorphism at position 80 of the α1 domain of HLA-C (205). Overall, in mixed uNK cell populations that express high levels of inhibitory KIR, HLA-C challenge promotes an inhibitory signal regardless of the co-presence of activating KIRs (186). KIR-directed inhibitory signals associate with impaired or blunted degranulation (206) and reduced secretion of EVT-regulatory factors (i.e. IL8, VEGF, placental growth factor (PGF) and CXCL10, also known as IP-10) (153). In uNK cells, expressing activating KIR2DS1 or KIR2DS4, HLA-C promotes uNK cell degranulation and secretion of granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumor necrosis factor (TNF) (186, 200, 207). Production of these factors potentially impacts EVT biology (discussed below). However, the direct effect of endogenous HLA-C expressed by EVTs on uNK cell processes has yet to be determined. Nonetheless, *ex vivo* EVTs physically interact with HLA-C-specific KIR2DL1 and KIR2DS1 (204), indicating that biological functions for these KIR-EVT interaction likely do exist.

HLA-E protein is present in EVTs at the 5th week of gestation but absent from these cells after the 7th week, suggesting a predominant role in implantation and/or early trophoblast development (198). Inhibitory signals elicited by HLA-E are mediated through the dimeric CD94/NKG2A receptor (208). Previous work shows that CD94/NKG2A elicits strong suppressive signals that generally override most activating inputs (209). However, whether EVT-derived HLA-E's sole purpose is to restrain uNK cell cytotoxicity in pregnancy is currently not well understood. For example, CD94 function-blocking experiments do not potentiate trophoblast killing (208), suggesting that CD94/NKG2A may serve other roles within the maternal-fetal interface that have yet to be elucidated.

The role of HLA-G in controlling uNK cells has been studied more so than other MHC class-I ligands, due in part to the relevance of its unique and restricted expression within EVT (197). Previous studies have identified two receptors expressed on uNK cells that interact with HLA-G: leukocyte immunoglobulin-like subfamily B member 1 (LILRB1) (210) and KIR2DL4 (211, 212). Interpretation of previous HLA-G-related findings necessitates caution due to the nature and design of the experimental systems used. For example, most approaches implement forced ectopic expression of HLA-G in HLA-null target cell lines, designed to synthesize either of two major HLA-G isoforms expressed by EVTs, namely membrane-bound HLA-G1 (213) or the truncated soluble HLA-G5 (211, 214). Further, many studies have interchangeably used peripheral blood NK cells or NK-like cell lines as surrogate readouts of HLA-G-uNK cell interaction within the maternal-fetal interface. These assumptions have perhaps contributed to over-interpretation of the role of HLA-G in controlling uNK cell-related processes in pregnancy. Conflicting findings have arisen from these studies, indicating that both membrane-bound and soluble HLA-G enhance the production of pro-inflammatory (IFN-γ, TNF, IL1B, IL6) and angiogenic (IL8) factors in endometrial (i.e., not decidual) (213, 215) or pbNK cells (202, 216). In support of the above findings using pbNK cells, Li et al. demonstrated that HLA-G treatment of *ex vivo*-derived uNK cells induces IL6, IL8, and TNF through processes dependent upon KIR2DL4 (212). In contrast to this, *ex vivo* uNK cells, exposed to membrane-bound HLA-G, resulted in inhibition

or had marginal impact on uNK degranulation and cytokine production (214). Two recent studies, directly examining the function of HLA-G in primary EVTs and uNK cells, indicated that HLA-G does not induce cytokine secretion from uNK cells but instead dampens uNK cell activity (104, 193), a finding that is consistent with the original paradigm that HLA-G likely promotes immune cell tolerance within the fetal-maternal interface.

REGULATION OF EXTRAVILLOUS TROPHOBLASTS BY UTERINE NATURAL KILLER CELLS

Uterine NK are robust producers of cytokines and growth factors, and due to their close proximity to EVTs within the maternal-fetal interface, these cells likely play roles in regulating the diverse functions of trophoblasts. In support of this, uNK cell-generated conditioned media modifies specific biological processes of primary trophoblast, including the promotion of cell invasion (153, 154, 217). Notably, the pro-invasive effect of uNK cell-derived conditioned media is solely attributed to soluble factors produced by uNK cells harvested from later gestational age time-points within the first trimester of pregnancy (i.e., 10–13 weeks' gestation). By contrast, soluble factors produced by uNK cells from the earlier first trimester time-points do not elicit pro-invasive characteristics on EVTs, indicating that the composition of uNK cell factors is influenced by processes related to gestational age, and that the impact of uNK cells on EVT biology is relative to stage of development.

Multiple factors produced by uNK cells have been identified, and importantly, receptors for many of these substances are expressed on primary EVTs. For example, uNK cells produce high levels of IL8, TNF, interferon (INF) γ, TGFB1, CXCL10, as well as the angiogenic factors such as vascular endothelial growth factor A (VEGF-A), VEGF-C and PGF (153). Reciprocally, immunolocalization studies on implantation sites and *ex vivo* studies using primary EVTs provide evidence that invasive trophoblasts populating the maternal-fetal interface produce receptors for these ligands. For example, EVTs express CXCR1 (an IL8 receptor), CXCR3 (an CXCL10 receptor), TNFR1, as well as VEGFR-1 and VEGFR-3, the latter binding VEGF-A and VEGF-C, respectively (110, 153, 218, 219). Notably, supplementation of IL8 and CXCL10 promoted migration of primary CTBs (153). Likewise, inhibition of ligand binding to VEGFR-1 and VEGFR-3 diminished trophoblast invasion (220). Moreover, downregulation of VEGF in uNK cell-conditioned media impaired EVT outgrowth compared to controls (221). In contrast, TNF and IFN-γ inhibited trophoblast migration and invasion by increasing PAI expression and impacting MMP-directed proteolysis, respectively (222, 223). Taken together, factors produced by uNK cells do have the ability to control EVT-related processes *in vivo*. Given the interplay between promoting and restraining invasive characteristics in EVTs, uNK cells could be an important cellular component of the decidua that controls depth of EVT invasion as well as extent of trophoblast-mediated spiral artery remodeling.

CAN THE BREAKDOWN OF MATERNAL-FETAL TOLERANCE BE RELATED TO UTERINE NATURAL KILLER CELLS DYSFUNCTION?

Much research related to uNK-trophoblast interactions has centered on the possibility that uNK cells, activated by infection or inflammation, may mount cytotoxic responses toward the semi-allogeneic fetus and trophoblast, thus contributing to infection-related miscarriage and other pregnancy disorders with aberrant inflammation. Surprisingly, most well designed studies, utilizing primary syngeneic uNK cell and trophoblast co-cultures, provided convincing evidence that trophoblasts (both HLA-G- and non-HLA-G-expressing trophoblasts) are highly resistant to uNK-directed killing (193, 224). Although uNK cells do not target trophoblasts for killing, even when artificially activated, uNK still retain pro-cytotoxic features (i.e. granzyme, perforin) that enable efficient cellular immune responses against virally-infected maternal uterine stromal cells, highlighting that trophoblasts are immuno-privileged (193, 225). Nonetheless, research in mice suggests that uNK cells can adopt anti-trophoblast characteristics in the right context. For example, aberrantly activated uNK cells in response to inflammation induced by bacterial endotoxin (183) or alloimmunogenic responses (184) target fetal tissues, including the placenta, and induce fetal resorption. These uNK-driven processes lead to impairments in uterine artery remodeling and placental sufficiency, and can be reversed through genetic ablation strategies (i.e., $IL15^{-/-}$) (183) or antibody-directed inhibition of uNK cells (184). It has also been suggested that uNK cell numbers are altered in pregnancy complications such as PE and IUGR, although contradictory data have been published. Reduced numbers of uNK cells in pregnancies with IUGR have been consistently demonstrated using different methods (226–229). Some studies also suggest a decrease of uNK cells (229) or of the $CD56^{+}/CD16^{+}$ uNK subset (228) in PE compared to healthy pregnancies. In contrast, others describe an elevated number of total uNK cells or of the $CD56^{+}/CD16^{+}$ subset in PE (230, 231). More recently, research has identified pre-existing health conditions of the mother that associate with low-grade inflammation, like obesity, that potentiate uNK cell activity and modify how uNK cells interact with fetal MHC ligand (207). However, although the *in utero* environment likely shapes uNK cell processes, to date, hard evidence showing that aberrantly activated uNK cells in humans directly target trophoblasts for killing has yet to be clearly demonstrated.

REGULATION OF EXTRAVILLOUS TROPHOBLASTS BY DECIDUAL MACROPHAGES

Next to uterine natural killer cells, macrophages are thought to comprise the second largest leukocyte population within the decidua (168, 232). Besides suggested contributions to spiral artery remodeling and immune modulation (12, 233), the function of decidual macrophages, in particular their

effect on EVT activity, remains largely unknown. Although historically described for their function in immune defense, inflammation, and clearance of apoptotic cells, macrophages have also been recognized to play important roles in the development, homeostasis, and repair of various tissues (234).

Macrophages are usually classified into M1, representing a classical pro-inflammatory, anti-microbial activation or into M2, referring to an anti-inflammatory phenotype promoting wound healing. A body of growing evidence however suggests that M1 and M2 rather represent two extreme poles of a broad spectrum of macrophage polarization (235). The non-exclusivity of M1 and M2 macrophage phenotypes *in vivo* has likely several reasons. Firstly, it has been shown that macrophage activation statuses are reversible when specific stimuli change. Secondly, macrophages are often exposed to opposing activating signals *in vivo* (236). For instance, the co-existence of pro-inflammatory M1 and anti-inflammatory M2 profiles has been demonstrated in various mouse models and during tumor progression (237, 238). In light of these data it is not surprising that decidual macrophages also show a unique activation status with a dominating but not exclusive M2 phenotype. While decidual macrophages express typical M2 markers such as CD209 and CD206 and secrete the anti-inflammatory cytokines IL10 and TGFB, they were also shown to secrete pro-inflammatory cytokines including IL6 and TNF and the neutrophil chemoattractant CXCL8 (IL8) (239, 240). Nevertheless, the anti-inflammatory M2-related cytokines IL10 and M-CSF were shown to be important for inducing a decidual macrophage phenotype in peripheral blood monocytes (240). In addition, decidual macrophages were able to suppress T cell activity and induce Tregs *in vitro* (194, 241), which further strengthens the notion of an M2-dominated function. Two independent studies suggested the presence of two distinct decidual macrophage subpopulations, characterized by the absence or presence of the cell surface markers CD11c and ICAM3 (239, 240). Expression of these markers may at least partly relate to immature macrophages or blood contamination, as high levels of CD11c and ICAM3 are also found in blood monocytes. More recently, a study described three decidual macrophage subtypes, based on the expression or absence of CCR2 and CD11c. Using RNA sequencing and functional assays, the authors identified distinct functional states, including differences in phagocytosis, anti-oxidative, and anti-inflammatory activities, and proximity to EVTs (242). Whether these differences indeed account for unique subpopulations or reflect cellular plasticity and thus relate to phenotypical alterations awaits further clarification.

Due to their high abundance in the decidua, it is conceivable that macrophages markedly influence the local paracrine environment and thus EVT function. As follows, it is interesting to note that decidual macrophages are more abundant at the site of implantation and accumulate at the invasive front of EVTs (243, 244). There is growing evidence for a macrophage-guided growth-promoting function in various epithelia (245–247). Interestingly, both placental and decidual macrophages could promote proximal cell column proliferation by secreting the M2-associated (248, 249) factors IL33 and Wnt5a (167, 250). These data suggest that paracrine activity of decidual macrophages could be important for the initial steps in EVT formation. Furthermore, decidual macrophages have been shown to secrete a range of factors known to alter EVT motility (239), albeit with conflicting evidence as to whether they promote or restrict EVT invasion. For instance, while IL8 (153) was described as pro-invasive factor in the context of trophoblast migration, TNF (222) and IL10 (119) have been shown to inhibit EVT motility. The net effect of these factors on EVTs may be pro-invasive, anti-invasive, or neither. In addition, it is unclear under which circumstances decidual macrophages produce these opposing cytokines in terms of macrophage polarization and EVT response. Phenotypic macrophage polarization is controlled by a complex array of soluble factors provided by the local microenvironment and even dictated by extracellular matrix-dependent cell morphology (251). Moreover, ligand distribution within tissues is limited by a wide variety of factors, including limited diffusion capacities, endocytosis, and interaction with extracellular matrix proteins. It is therefore also important to consider the spatial relationship between macrophages and EVTs.

Unfortunately, there is limited information on the difference in macrophage distribution between decidua basalis and parietalis, and especially on whether these different tissue compartments harbor specific macrophage phenotypes. Immunohistochemical studies provided evidence for an enrichment of macrophages in the decidua basalis (243, 244), which has recently been confirmed via flow cytometric analysis (175), suggesting that macrophages preferentially accumulate in the vicinity of EVTs. In a similar context, it has been shown that binding between HLA-G homodimers and macrophage-associated leukocyte immunoglobulin-like receptor B1 (LILRB1) upregulates secretion of IL6, IL8, and TNF (212). Although it is not clear whether these effects relate to membrane-bound or soluble HLA-G, the presence of EVTs likely influences the macrophage phenotype and thus could substantially influence the paracrine activity of decidual macrophages.

ARE DECIDUAL MACROPHAGES ALTERED IN COMPLICATED PREGNANCIES?

Complicated pregnancies with IUGR or early-onset PE have repeatedly been associated with compromised EVT function. Several studies have tried to decipher whether aberrations in the decidual macrophage population could mediate these EVT defects, albeit with conflicting results. Some studies suggest an increase in macrophage numbers in preeclampsia compared to healthy pregnancies (252, 253). Further, an inverse relationship between macrophage infiltration and invasion of trophoblasts into arteries, with a shift toward macrophage infiltration in preeclamptic pregnancies, was shown (252). On the contrary, other studies point toward a decrease in the number of macrophages in IUGR and PE, compared to healthy pregnancies (229, 254). Still some additional studies found no significant differences in macrophage distribution and activation patterns between preeclamptic women and preterm labor controls (255).

In addition, it is unclear whether aberrant macrophage polarization could be associated with EVT defects in

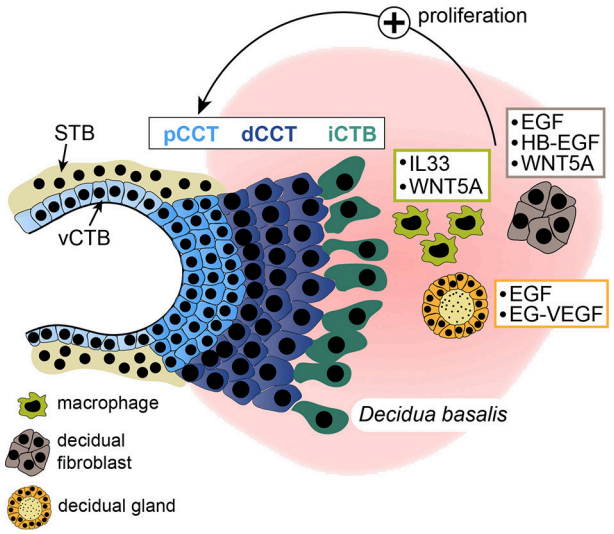


FIGURE 2 | Schematical depiction of soluble factors secreted from decidual macrophages, stromal cells, or glands. Mediators, stimulating proliferation of proximal cell column trophoblasts (pCCTs) in villous explant cultures are illustrated. dCCT, distal cell column trophoblast; STB, syncytiotrophoblast; vCTB, villous cytotrophoblast; iCTB, interstitial cytotrophoblast.

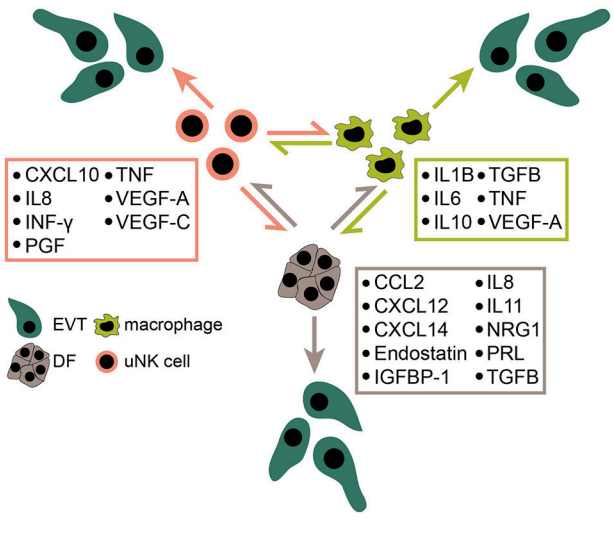


FIGURE 3 | Expression of decidual chemokines, cytokines and other soluble mediators affecting trophoblast migration and/or invasion. Some of the depicted factors likely play numerous roles at the fetal-maternal interface including activation and maturation of immune cells, as well as decidual angiogenesis and spiral artery remodeling. EVT, extravillous trophoblast; uNK cell, uterine natural killer cell; DF, decidual fibroblast.

the development of placental pathologies. In detail, some evidence exists that macrophages could display a more M1-like polarization in cases of PE, resulting in an exacerbated production of pro-inflammatory cytokines adversely affecting EVT function (256, 257). For instance, TNF has been shown to inhibit trophoblast motility in villous explant cultures (222). A study utilizing a rat model, demonstrating that systematic LPS injection results in IUGR and PE-like symptoms, also reported increased levels of TNF and exacerbated numbers of uteroplacental macrophages (258). Unfortunately, the authors did not further elucidate whether LPS-induced systemic inflammation also changes the phenotype of decidual macrophages. Nevertheless, whether a shift toward a pro-inflammatory M1-like macrophage phenotype indeed adversely affects EVT function has not been proven. As mentioned above, M2-related anti-inflammatory cytokines such as TGFB or IL10 were also reported to exert adverse effects on EVTs by restricting their migratory potential. Conversely, macrophages isolated from miscarriages showed reduced expression of IL6 and IL8, the latter with pro-invasive potential toward EVTs (153). In summary, studies investigating the functional interplay between primary macrophage and EVT cultures are scarce. Thus, the particular role of macrophages in the context of EVT function remains unclear both in healthy and complicated pregnancies. Unfortunately, there is also limited availability of suitable human *in vitro* systems to study this interaction. Immortalized monocytic cell lines, such as THP-1 cells, do not represent a useful model system for decidual macrophages due to their massive genomic rearrangements and their phenotypic and functional differences (259). It is still unclear whether the decidua-specific macrophage phenotype can be sustained in isolated primary cells or mimicked by controlled differentiation of peripheral blood monocytes *in vitro*. Moreover, more information is needed on whether macrophages change their polarization and secretory profile depending on their location within the human decidua. Finally, the long lasting paradigm of macrophage differentiation from recruited monocytes has been challenged by numerous studies demonstrating that macrophages are also maintained throughout adult life by a tissue-resident, proliferative population originating from embryonic or yolk sac-derived precursors (260, 261). In mice, tissue-resident macrophage populations, such as liver Kupffer cells (262), epidermal Langerhans cells (263), microglia (264), and pleural macrophages (265), were shown to be able to proliferate and renew independently from the bone marrow. Although very few data have been generated so far to confirm the existence of tissue-resident macrophages in humans, it is interesting to note that decidual, tissue-resident $CD34^+$ stromal cells were described to differentiate into functional $CD56^+$ uNK cells (266). Moreover, a recent study shows that continuous pregnancies induce a pregnancy-promoting memory uNK subset which differentiates from progenitors residing in the post-gravid endometrium (267). Additional studies, confirming the existence of tissue-resident NK cells and T cells in other tissues, strengthen the idea of an organ-specific immunity that is maintained independently of the bone marrow and secondary lymph nodes (268, 269). Intriguingly, several reports describe the proliferative signature of decidual macrophages (270, 271), supporting the idea that tissue-resident macrophages could be maintained in the uterus by local proliferation. On the other hand, both EVTs and decidual macrophages produce monocyte-recruiting chemokines, such as CCL2 (148, 149) or CCL4 (148, 239) suggesting a contribution of monocytes to the pool of decidual

macrophages in early pregnancy. In light of these data it is still not clear whether the endometrium is continuously populated by bone marrow-derived monocytes or whether local progenitors mainly repopulate within the tissue.

SUMMARY

The influence of soluble decidual factors on trophoblast proliferation, migration and invasion has been intensively investigated using different trophoblast cell models including cell lines and primary CTBs containing a mixture of different trophoblast subtypes. However, studies about the effects of secreted proteins on cell column proliferation of the anchoring villus are scarce. Moreover, isolated primary CCTs rapidly cease proliferation in culture and undergo differentiation (57), impairing their usability for proliferation assays. Hence, only few factors were convincingly shown to promote CCT expansion using first trimester villous explant cultures (**Figure 2**). Recently however, self-renewing trophoblast stem cells and organoids have been developed (64, 272). These culture systems should allow more reliable investigations on the role of decidual growth factors in trophoblast progenitor growth, EVT formation and differentiation. Furthermore, DFs, macrophages and uNK cells express a plethora of cytokines, chemokines and soluble factors, some of which are detectable in more than one cell type (**Figure 3**). Although the majority of these proteins likely control trophoblast invasion and/or migration the prime decidual target cell of an individual factor remains elusive. Besides their presumptive role in trophoblast motility, chemokines and cytokines could regulate immune cell recruitment and mutual activation of macrophages, uNK cells and DFs as well as other less abundant immune cells of the fetal-maternal interface.

AUTHOR CONTRIBUTIONS

The manuscript was written by JP, AGB, and MK and edited by SV and JB. JP provided the graphical illustrations.

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Hofbauer Cells: Their Role in Healthy and Complicated Pregnancy

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Hofbauer cells are placental villous macrophages of fetal origin that are present throughout pregnancy. Although Hofbauer cell populations are antigenically and morphologically heterogeneous, their epigenetic, antigenic, and functional profiles most closely resemble alternatively activated macrophages or what are referred to as M2a, M2b, M2c, and M2d polarity subtypes. Consistent with an M2-like profile, these cells play an important role in placental development including vasculogenesis and angiogenesis. During placental inflammation Hofbauer cells may produce pro-inflammatory cytokines or mediators that damage the villous cell barrier, and induce fibrotic responses within the villi as a continuum of chronic inflammation. However, to date, there is no evidence that Hofbauer cells become classically activated or adopt an M1 polarity phenotype that is able to kill microbes. To the contrary, their predominant M2 like qualities may be why these cells are ineffective in controlling most TORCH infections. Moreover, Hofbauer cells may contribute to vertical transmission of various pathogens to the fetus since they can harbor live virus and serve as reservoirs within the placenta. The goal of this review is to summarize what is currently known about the role of Hofbauer cells in normal and complicated pregnancies that involve immunologic disorders, inflammation, and/or infection.

Keywords: Hofbauer cells, placental macrophages, preeclampsia, villitis, chorioamnionitis, Zika, TORCH

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HOFBAUER CELLS IN NORMAL PREGNANCY

Hofbauer Cell Location and Proposed Function

Hofbauer cells (HBC) originally referred to round to ovoid placental macrophages with a small nucleus and abundant vacuolated cytoplasm that could be identified by light microscopy (1). Subsequent ultrastructural studies further identified placental macrophages that were spindle or stellate shaped (2, 3) and that these cells are of fetal origin (4). HBC is now often used to describe any fetal derived placental macrophage that resides within the placental villous core, amnion, and chorionic lavae (5). HBCs are found in human placental tissue as early as 18 days post-conception (6) and remain throughout gestation (1).

HBCs are presumed to play a role in placental morphogenesis and homeostasis. HBCs are typically in apposition to endothelium and trophoblasts where they can mediate the function of these cells through paracrine signals or possibly cell-to-cell crosstalk (7–9). HBCs are pro-angiogenic in that they express large amounts of vascular endothelial growth factor (VEGF) (10, 11), and Sprouty (Spry) proteins Spry 1, 2, and 3 that modulate branching morphogenesis of placental villi (12). It has been suggested that HBC may participate in vasoregulation of placental blood vessels since they have the capacity to produce prostaglandin E_2 and thromboxane *in vitro* (13).

Phenotypic Diversity of HBCs

As is typical of macrophages, HBCs exhibit plasticity and their pleomorphism is likely a reflection of the complex and shifting microenvironment in which they reside (14–16). This has been verified through a variety of techniques including electron microscopy, immunohistochemistry, and flow cytometry. Initial descriptions of HBC pleomorphism came from ultrastructural observations that reported 4 types of macrophages based on their shape (3). Histochemical studies have further classified HBC subtypes by their expression of major histocompatibility complex (MHC) type II, complement receptors, lectins, lipopolysaccharide co-receptor (CD14), and CD68 that vary based on HBC location within the placenta as well as gestational stage (5, 15–19). Using multi-parameter flow cytometry coupled with serial gating of first trimester macaque HBCs, we previously identified two HBC subsets based on whether or not they expressed CD68 (14). To further illustrate this point we reanalyzed the dataset and added additional samples from late second trimester (gestation day 100), and near term (gestation day 160) (20, 21). We specifically measured the expression of HLA-DR, CD14, DC-SIGN, CD68, CD64, and CD163 in HBC by flow cytometry (**Figure 1**; **Table 1**, workflow detailed in **Supplementary Material**). Although our panel was not comprehensive, it included markers previously validated in rhesus macaque HBCs (18), some of which indicate innate immune activation, such as CD14 (23), or immune modulation (DC-SIGN, HLA-DR, CD68) (1, 15, 18, 24). In order to better capture the spectrum of HBC diversity, we used an unbiased approach to analyze the high-dimensional flow cytometric data. Raw flow cytometry data files were first processed with FlowJo, LLC version 10 software (Ashland, OR). Processed datasets from first ($n = 2$), second ($n = 2$), and third ($n = 1$) trimester pregnancies were then imported into Cytofkit (https://bioconductor.org/packages/cytofkit/), normalized, and analyzed with the DensVM computational clustering tool (22). *t*-Distributed Stochastic Neighbor Embedding (*t*-SNE) was used to created 2-dimensional maps of all HBC subsets generated by DensVM (**Figure 1**).

With this approach we identified 10 HBC subsets within macaque placental tissues (**Figure 1**; **Table 1**). The 10 HBC subsets were subsequently validated by serial manual gating (**Table 2**). Both first and third trimester HBC populations were more diverse than the second trimester (8 vs. 5 clusters, respectively). This is not unusual given the physiological events that occur during these stages of pregnancy. For example, during the first trimester, HBCs are thought to participate in placental villous growth and tissue remodeling (13). At the same time HBCs in the vicinity of the placental bed may be affected by the inflammatory processes necessary for decidualization and embryo implantation (25). During the latter part of the third trimester, HBCs scattered through the placenta are exposed to various products released from senescent trophoblasts (26), necrotic cell debris associated with fibrinoid deposits within aging placental villi (13), and inflammatory mediators produced during parturition (27). These may promote the development of specialized subsets of HBCs in response to their microenvironment. Alternatively, they may represent different populations of fetal monocytes that are trafficking to the placenta across gestation.

In order to assess gestation dependent changes in HBC subsets, marker expression heatmaps specific to each gestational stage were generated (**Figure 2**). We found that both CD163 and CD64 appeared to be constitutively expressed in all HBC subsets throughout pregnancy, indicating that these markers may be well-suited for the identification of HBCs in general. However, CD68 which is often used as a single marker to identify HBCs had variable expression over time. Namely, the intensity of CD68 expression peaked in the second trimester and significantly dropped as pregnancy progressed. This temporal expression pattern is similar to previous studies that described human HBC populations as changing in density as pregnancy progressed (1, 24).

As expected (5), there was at least one population of HBC that was negative for HLA-DR (cluster 5). Of the HBC subsets that were positive, HLA-DR expression followed a similar pattern to CD68 in that peak expression was observed in second trimester placenta. However, contrary to human studies (5), we found the proportion of HLA-DR positive cells in first trimester placenta to be greater than third trimester tissue. This may be due to timing of sampling since human studies evaluated tissues collected during 8–10 weeks gestation (5) whereas we examined tissues collected at a later gestational time point that developmentally would be equivalent to 17–18 weeks in human gestation.

DC-SIGN positive HBCs were present in all 3 stages of pregnancy; albeit with varying degrees of DC-SIGN expression. Consistent with Yang et al. (28) we found that DC-SIGN positive third trimester macaque HBCs co-expressed CD14, CD68, and CD163. In contrast to Bockle et al (29), we detected a large population of DC-SIGN positive HBC in third trimester placenta that co-expressed HLA-DR (23% in cluster 6). One caveat is that these cells had dim expression of both markers, which may explain why these cells may not be readily detected by immunofluorescent histology.

Of all the markers that we studied, the proportion of CD14 positive cells and their level of CD14 expression was the most diverse. Midgestation placenta, which is characterized by immune tolerance, had the greatest proportion of $CD14^{Hi}$ positive HBCs that were also $CD163^{Hi}$ (cluster 10). Although increased CD14 expression has been linked to pro-inflammatory HBCs (23), in normal pregnancy CD14 expression may represent an immune suppressed phenotype. $CD14^{Hi}/CD163^{Hi}$ expression as seen in cluster 10, is characteristic of immunosuppressive M2d or tumor associated macrophages (TAM) (30). CD14 positive HBCs also express anti-inflammatory TGF-β and IL-10 (31).

Even though this was a pilot experiment with a small sample size, our results demonstrate the potential of utilizing computational methods to analyze multidimensional data from HBCs. For instance, we obtained a global perspective on how HBC subsets change during pregnancy. Moreover, we gained insights into which macrophage markers may or may not be suitable for the general identification of HBCs by flow cytometric or immunohistochemical approaches.

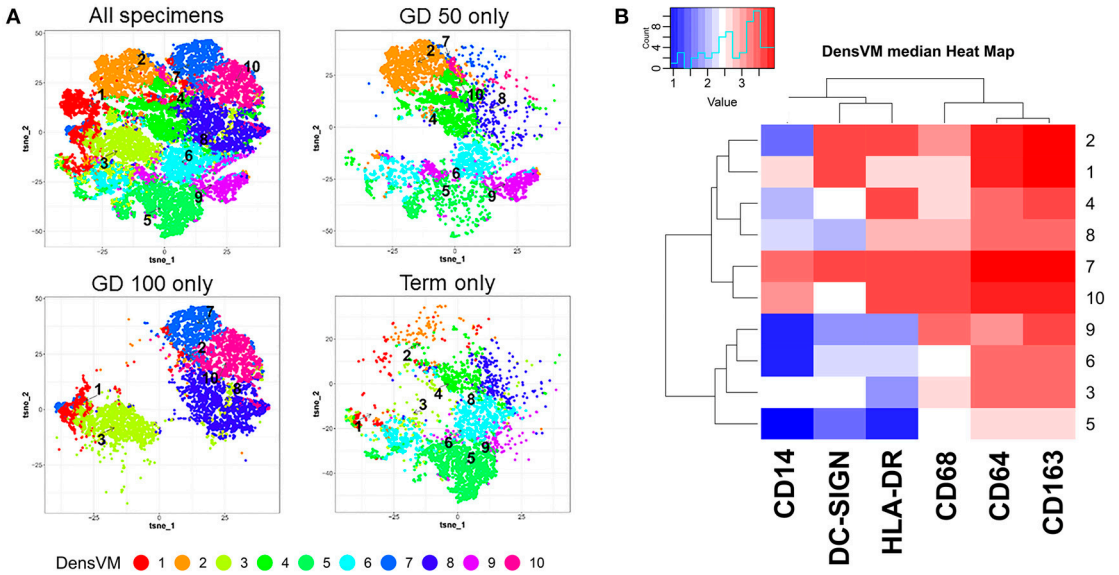


FIGURE 1 | Marker defined HBC subsets within macaque placenta collected at different stages of gestation: Gestation day (GD) 50 ± 2 days ($n = 2$), GD100 ± 2 days ($n = 2$), and Term. **(A)** *t*-SNE visualization of DensVM generated HBC clusters of combined flow cytometry data from all gestation stages (All specimens) and by each individual gestation stage. **(B)** Marker and cluster specific DensVM median heatmap generated with flow cytometry data from all gestation stages. Clustering was ranked by both HBC cluster group (designated by row) and marker median expression (column). Images were created with Cytofkit ShinyAPP (22).

TABLE 1 | Marker profiles of HBC subsets generated with DensVM clustering algorithm (22).

Cluster	1st[a]	2nd[a]	3rd	Marker profile
1	–	5 ± 4	3	$CD163^{Hi}/CD64^{Hi}/CD68^{Lo}/HLA\text{-}DR^{+}/DC\text{-}SIGN^{Hi}/CD14^{Hi}$
2	23 ± 22	–	3	$CD163^{Hi}/CD64^{Hi}/CD68^{Med}/HLA\text{-}DR^{Hi}/DC\text{-}SIGN^{Hi}/CD14^{Lo}$
3	–	24 ± 18	4	$CD163^{Med}/CD64^{Med}/CD68^{Lo}/HLA\text{-}DR^{Lo}/DC\text{-}SIGN^{Med}/CD14^{Lo}$
4	17 ± 1	–	10	$CD163^{Med}/CD64^{Med}/CD68^{Lo}/HLA\text{-}DR^{Hi}/DC\text{-}SIGN^{Med}/CD14^{Lo}$
5	8 ± 6	–	48	$CD163^{Lo}/CD64^{Lo}/CD68^{Lo}/HLA\text{-}DR^{-}/DC\text{-}SIGN^{Lo}/CD14^{-}$
6	11 ± 4	–	23	$CD163^{Med}/CD64^{Med}/CD68^{Lo}/HLA\text{-}DR^{Med}/DC\text{-}SIGN^{Med}/CD14^{-}$
7	2 ± 0.1	16 ± 5	–	$CD163^{Hi}/CD64^{Hi}/CD68^{Hi}/HLA\text{-}DR^{Hi}/DC\text{-}SIGN^{Hi}/CD14^{Hi}$
8	3 ± 1	24 ± 21	6	$CD163^{Med}/CD64^{Med}/CD68^{Med}/HLA\text{-}DR^{Med}/DC\text{-}SIGN^{Med}/CD14^{Lo}$
9	12 ± 3	–	3	$CD163^{Med}/CD64^{Lo}/CD68^{Hi}/HLA\text{-}DR^{Lo}/DC\text{-}SIGN^{Lo}/CD14^{-}$
10	2 ± 2	20 ± 2	–	$CD163^{+}/CD64^{Hi}/CD68^{Hi}/HLA\text{-}DR^{Hi}/DC\text{-}SIGN^{Med}/CD14^{Hi}$

[a]*Values represent the mean ± SD% positive cells per group (n = 2) per gestation group.*

TABLE 2 | Marker profiles of HBC subsets generated by manual gating using FlowJo Software.

Cluster	1st[a]	2nd[a]	3rd	Marker profile
1	–	1.3 ± 0.4	8	$CD163^{Hi}/CD64^{Hi}/CD68^{Lo}/HLA\text{-}DR^{+}/DC\text{-}SIGN^{Hi}/CD14^{Hi}$
2	11 ± 11	–	1	$CD163^{Hi}/CD64^{Hi}/CD68^{Med}/HLA\text{-}DR^{Hi}/DC\text{-}SIGN^{Hi}/CD14^{Lo}$
3	–	20 ± 24	6	$CD163^{Med}/CD64^{Med}/CD68^{Lo}/HLA\text{-}DR^{Lo}/DC\text{-}SIGN^{Med}/CD14^{Lo}$
4	19 ± 4	–	3	$CD163^{Med}/CD64^{Med}/CD68^{Lo}/HLA\text{-}DR^{Hi}/DC\text{-}SIGN^{Med}/CD14^{Lo}$
5	8 ± 1	–	52	$CD163^{Lo}/CD64^{Lo}/CD68^{Lo}/HLA\text{-}DR^{-}/DC\text{-}SIGN^{Lo}/CD14^{-}$
6	9 ± 4	–	17	$CD163^{Med}/CD64^{Med}/CD68^{Lo}/HLA\text{-}DR^{Med}/DC\text{-}SIGN^{Med}/CD14^{-}$
7	1.8 ± 0.4	6 ± 3	–	$CD163^{Hi}/CD64^{Hi}/CD68^{Hi}/HLA\text{-}DR^{Hi}/DC\text{-}SIGN^{Hi}/CD14^{Hi}$
8	2.3 ± 1	17 ± 19	3.2	$CD163^{Med}/CD64^{Med}/CD68^{Med}/HLA\text{-}DR^{Med}/DC\text{-}SIGN^{Med}/CD14^{Lo}$
9	6 ± 4	–	2.5	$CD163^{Med}/CD64^{Lo}/CD68^{Hi}/HLA\text{-}DR^{Lo}/DC\text{-}SIGN^{Lo}/CD14^{-}$
10	0.5 ± 0.7	8 ± 7	–	$CD163^{+}/CD64^{Hi}/CD68^{Hi}/HLA\text{-}DR^{Hi}/DC\text{-}SIGN^{Med}/CD14^{Hi}$

[a]*Values represent the mean ± SD% positive cells per group (n = 2) per gestation group.*

TABLE 3 | General features of macrophage polarity pertinent to HBC (30, 33, 34).

	M1	M2a	M2b	M2c	M2d
Stimulus	IFN-γ + LPS IFN-γ + TNF GM-CSF	IL-4 IL-13 Fungal and parasitic infections	Immune complexes IL-1R	IL-10 TGF-β Glucocorticoids	IL-6 LIF M-CSF Adenosine
Surface markers	CD80 MHC II	CD163 IL-1R MHC II CD206 IL-RN DC-SIGN	CD86 MHC II	CD163 TLR1 TLR8 CD206 CD14	CD163 CD14 CD85
Secreted factors	TNF IL-1β IL-6 IL-12 IL-23 CCL10 CCL11 CCL5 CCL8 CCL9 CCL2 CCL3 CCL4	IL-10 TGF-β IL-1ra	IL-1 IL-6 IL-10 TNF-α CCL1	IL-10 TGF-β CCR2 Pentraxin 3	VEGF MMP-9 IDO IL-10 IL-12 TNF-α TGF-β CCL5 CXCL10 CXCL16
Biological effects	TH1 responses Killing intracellular pathogens Tumor resistance	TH2 responses Killing of extracellular responses	TH2 activation Immune regulation	Immune regulation Matrix deposition and tissue remodeling	Immune suppression Angiogenesis

Functional Diversity of HBCs

Mills et al. (32) were the first to introduce the concept that macrophage function could be polarized based on how the cell metabolized arginine. Arginine conversion to nitric oxide was linked to a macrophage that produced IFN-γ and inhibited wound healing, which was labeled as M1. Arginine conversion to ornithine was linked to a macrophage that produced TGF-β and promoted wound healing, this phenotype was labeled as M2. Over the years, the criteria for defining a macrophage as M1 has expanded (**Table 2**) to include microbicidal activity, and the production of pro-inflammatory cytokines and chemokines that promote cell mediated (TH1 type) responses. M2 polarity phenotype has been divided subsequently into M2a, M2b, M2c, and M2d subcategories (reviewed by Martinez et al. (33)), which are based on their responses to various agonists (**Table 3**). Collectively, M2 subtypes are linked by a dominant TH2 response profile, their development in response to fungal or helminth infections, and their role in tissue remodeling. However, M2b polarized macrophages share some qualities in M1 in that they can be pro-inflammatory by producing TNF, IL-1, and IL-6 along with IL-10, but they lack microbicidal activity.

Since HBC are macrophages, it has been assumed that these cells protect the placenta and fetus from infection (13), which would be consistent with an M1 phenotype. However, there is no experimental evidence that HBCs within the placenta are capable of killing microbes (discussed in the next section of this review). Pro-M1 genes in HBC, such as TLR9, IL1B, IL12RB2, CD48, and FGR are silenced by methylation (35). On the other hand, pro-M2 genes, such as CCL2, CCL13, CCL14, CD209, and A2M are hypomethylated in HBCs and thus available for transcription (35). Collectively, HBCs isolated from term human placenta display M2a, M2b, and M2c characteristics based on cell surface marker profiles and cytokine expression (19, 31, 36). HBCs also share some features with M2d phenotype in that they are immune suppressive, pro-angiogenic, and co-express CD163 and CD14 (7, 10, 12, 36). Moreover, HBCs form multinucleated giant cells that express matrix metalloproteinase genes along with VEGF-C (37), which are also features of M2d. Hypothetically, a normal pregnancy has a balanced blend of HBC subtypes that functionally complement each other in providing optimal vascular development, villous growth and immune tolerance. Conversely, an imbalance in HBC subtypes may bring about or exacerbate pathologic pregnancy.

THE ROLE OF HBCS IN PREGNANCY COMPLICATIONS

Villitis

Villitis is a histopathologic diagnosis with multiple underlying etiologies (38). It can be a consequence of hematogenous infection of the placenta by TORCH organisms that include

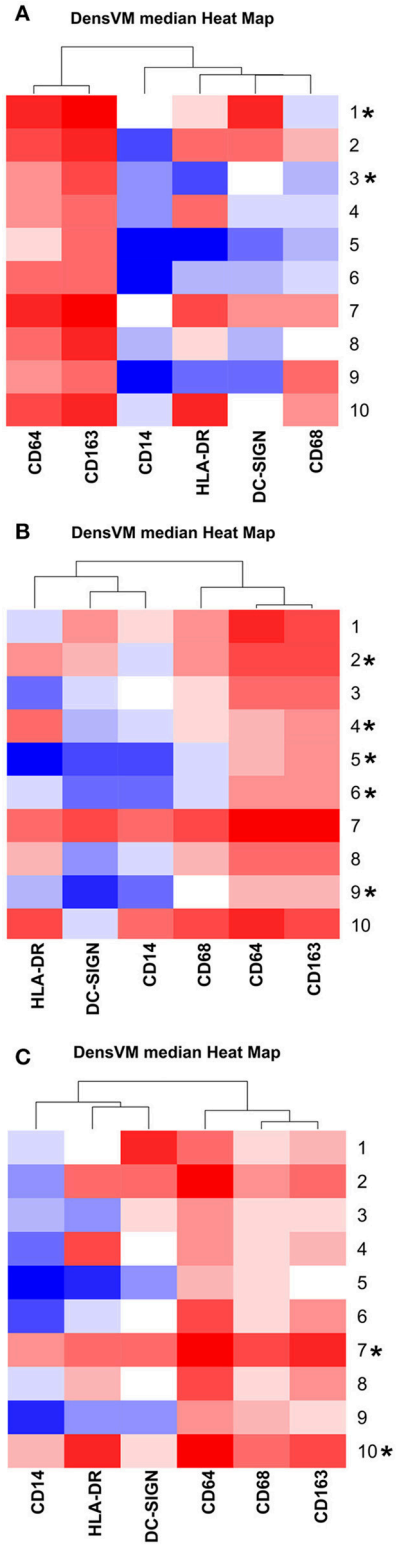


FIGURE 2 | DensVM median heatmaps specific to each gestation stage. **(A)** GD50 ± 2 days, **(B)** GD100 ± 2 days ($n = 2$), and **(C)** Term ($n = 1$). Heatmaps were clustered by median expression of each marker (column). *Denotes HBC clusters that contained 1% of cells for that specific gestation stage. Images were created with Cytofkit ShinyAPP (22).

Toxoplasma, Others (syphilis, varicella-zoster, parvovirus B19), Rubella, Cytomegalovirus (CMV), and Herpes infections (38, 39). However, most cases of villitis are not associated with infection, but may be immune mediated (40, 41). Acute villitis is usually caused by infection and it characterized by polymorphonuclear leukocytic infiltration of the villi with or without necrosis. *Listeria monocytogenes* infection during pregnancy is one of the most common causes of acute villitis (13, 42). Placental infection with *Treponema pallidin* (syphilis) may also present as acute villitis, but chronic villitis is more characteristic of congenital syphilis (43, 44). On the other hand chronic villitis is characterized by infiltration of the tissue by lymphocytes and macrophages. It may be accompanied by cellular proliferation and fibrosis of the villi. Most cases of villitis are multifocal and asymptomatic, but the lesion can be more extensive leading to preterm birth or miscarriage.

Perturbed HBC function is a common occurrence in chronic villitis. HBC hyperplasia or proliferation is seen in chronic villitis caused by infection with CMV, Zika, Herpes virus, Coxsackie, and villitis of unknown etiology (VUE) (38, 45–47). Regardless of the underlying cause, HBCs in chronic villitis exhibit an inflammatory phenotype. Satosar and colleagues showed an increase in the number of TNF positive HBCs with a concomitant decrease in SOCS-1 (suppressors of cytokine signaling) positive HBCs in villitis placentas positive for viral and bacterial infection (45). A similar response is also evident in VUE, which is now recognized to be an immune mediated process that resembles maternal anti-fetal rejection and placental graft vs. host disease (40). In VUE, hyperplastic HBCs are intermixed with infiltrating maternal macrophages and $CD8^+$ T cells with an inflammatory transcriptome that is similar to the biological processes that occur during antigen presentation and immune response (40). In particular, HBCs in VUE are positive for CXCL9, CXCL10, CXCL11, and CXCL13 (40, 45). In this scenario HBCs are thought to be contributing to placental damage. In the case of TORCH infections, it is unknown whether HBCs are contributing to placental damage, or controlling infection. The presence of viral inclusions (CMV, Herpes Simplex, Coxsackie) or parasites (*Toxoplasma* and *Leishmania*) can certainly be found in HBCs, but it is unknown if these organisms are live and replication, or dying within the cell. HBCs are harboring live organisms these organisms are viable in these cells (38, 39).

HBC hyperplasia without villitis has also been observed with placental Zika virus infection (48, 49). This lesion is characterized by enlarged, hydropic chorionic villi, hyperplasia and focal proliferation of HBC, without necrosis or lymphocytic infiltration of the affected villi (48, 49). Some of the proliferating HBCs were found to contain Zika virus (49). Since Zika has been shown to replicate in HBCs (50–52), it has been proposed by several investigators that these cells may serve as a source of infection to the fetus (48, 53). However, the significance of HBCs in releasing infectious Zika virions is questionable since by Gavegnano and colleagues showed that Zika virions released from HBCs were incapable of infecting susceptible Vero cells (54). Whether antiviral responses in HBC are effective or compromised *in vivo* is yet to be determined. Regardless, perturbations in HBCs during placental infection with Zika

suggest that these cells have altered function that may be detrimental to placental morphogenesis.

Preterm Delivery

There are a limited number of studies concerning HBCs in preterm delivery. These have been limited to chorioamnionitis-induced spontaneous preterm birth, severe preeclampsia, and HELLP syndrome. Two independent studies have shown that the density of $CD68^+$ HBCs are significantly reduced in chorioamnionitis (24, 37). The underlying mechanism for a decrease in HBCs during chorioamnionitis is unknown but it has been speculated that these cells may be undergoing apoptosis (37). HBC function, particularly multinucleated giant cells, is altered in placentas with chorioamnionitis. Namely, these cells exhibited decreased tolerogenic activity compared to the same cells retrieved from normal pregnancies (37). The application of high dimensional flow cytometry may allow discernment of the significance of a decrease in the density of $CD68^+$ cells: is CD68 selectively lost from a subset of cells? Does a different population of HBCs arise in these placentas?

Although a subset of preeclampsia patients develop HELLP, HELLP is considered a separate syndrome. Preeclampsia and HELLP have different clinical presentations (55, 56). Classical preeclampsia is characterized by hypertension and proteinuria, whereas HELLP involves activation of the coagulation system (55). Pathologic features within the placenta also differ between preeclampsia and HELLP. Infarction, intervillous thrombosis, and abruption is more common in placentas from preeclampsia patients than patients with HELLP (55). Furthermore, HBC numbers, and their expression of DC-SIGN and IL-10 are significantly reduced in patients with severe preeclampsia (28, 57). It has been suggested that the reduction of HBCs in preeclampsia may be promoting inflammatory damage due to the loss of tolerance-promoting HBCs (28). In contrast to preeclampsia, patients with HELLP exhibit increased numbers of $CD68^+$ HBCs, and it was concluded that this may be due to increased inflammation or an adaptive response (56).

CONCLUDING REMARKS

Our understanding of the role of HBCs in pregnancy is still rudimentary, but current evidence provides a compelling argument that these cells are important in placental development and homeostasis. At least in some pregnancy complications, such as VUE and chorioamnionitis, HBC dysfunction may be contributing to disease pathogenesis. Since HBCs exhibit functional plasticity, they may be ideal targets for therapeutic manipulation during disease states. However, additional studies are needed to better define the functional role of various HBC subsets in both health and disease.

ETHICS STATEMENT

Macaque data presented in this article was obtained with approval from the University of Wisconsin Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

LR conceived of the topic, conducted the research, and wrote the manuscript. TG assisted with non-human primate studies, and contributed to the writing of the manuscript.

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Cross-Reactivity of Virus-Specific CD8+ T Cells Against Allogeneic HLA-C: Possible Implications for Pregnancy Outcome

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Heterologous immunity of virus-specific T cells poses a potential barrier to transplantation tolerance. Cross-reactivity to HLA-A and -B molecules has broadly been described, whereas responses to allo-HLA-C have remained ill defined. In contrast to the transplant setting, HLA-C is the only polymorphic HLA molecule expressed by extravillous trophoblasts at the maternal-fetal interface during pregnancy. Uncontrolled placental viral infections, accompanied by a pro-inflammatory milieu, can alter the activation status and stability of effector T cells. Potential cross-reactivity of maternal decidual virus-specific T cells to fetal allo-HLA-C may thereby have detrimental consequences for the success of pregnancy. To explore the presence of cross-reactivity to HLA-C and the other non-classical HLA antigens expressed by trophoblasts, HLA-A and -B-restricted CD8+ T cells specific for Epstein-Barr virus, Cytomegalovirus, Varicella-Zoster virus, and Influenza virus were tested against target cells expressing HLA-C, -E, and -G molecules. An HLA-B*08:01-restricted EBV-specific T cell clone displayed cross-reactivity against HLA-C*01:02. Furthermore, cross-reactivity of HLA-C-restricted virus-specific CD8+ T cells was observed for HCMV HLA-C*06:02/TRA CD8+ T cell lines and clones against HLA-C*03:02. Collectively, these results demonstrate that cross-reactivity against HLA-C can occur and thereby may affect pregnancy outcome.

Keywords: heterologous immunity, virus-specific T cells, allogeneic HLA, HLA-C, pregnancy

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1. INTRODUCTION

High frequencies of memory T cells against several viruses such as Influenza virus (FLU), Epstein-Barr virus (EBV), Human Cytomegalovirus (HCMV), and Varicella-Zoster virus (VZV) have been described in healthy individuals (1–3). Primary infection or reactivation of these viruses can compromise graft survival after transplantation and during pregnancy result in fetal malformation and pregnancy complications such as preterm birth and intrauterine growth restriction (4–7). A significant proportion of virus-specific CD8+ T cells in healthy (non-HLA sensitized) individuals display alloreactivity against allogeneic human leukocyte antigens (allo-HLA) (8, 9). This phenomenon, referred to as heterologous immunity, enables the same T-cell receptor (TCR) to recognize its autologous virus-peptide presenting HLA allele as well as allo-HLA. The avidity of these cross-reactive T cells for their viral and allogeneic targets depends on the levels of peptide

presentation (10). Within an individual, an HLA-restricted virus-specific T cell response can generate several clonotypes that have different patterns of allo-HLA cross-reactivity (11). Both naïve and memory T cells show alloreactive potential, though memory T cells pose a superior threat (12, 13). Their activation threshold is significantly lower as they have less need for co-stimulation while their cytotoxic function is enhanced (14, 15). To ensure a comprehensive immune response to foreign antigens, this high degree of cross-reactivity is an intrinsic and essential feature of antigen recognition by T cells, of which allo-HLA cross-reactivity is an inherent consequence (16).

In the pregnancy setting T cells have a dual role in mediating tolerance toward the allogeneic fetus and at the same time responding to infections. During gestation, fetal extravillous trophoblasts (EVT) deeply invade the maternal tissues (decidua) where they establish direct contact with the maternal immune cells. EVT do not express the highly polymorphic HLA-A and -B, but do express HLA-C, -E, and -G (17, 18). CD8+ T cells present in the decidua demonstrate a mixed transcriptional profile of T cell dysfunction, activation and effector function. They are not permanently suppressed, but maintain the capacity to respond to proinflammatory occurrences, such as infections (19). Significant numbers of HLA-A and -B-restricted virus-specific CD8+ T cells are found in decidual tissue of term pregnancy (20). Furthermore, in the maternal peripheral blood, cytotoxic T lymphocyte (CTL) responses to paternal allo-HLA (HLA-A/B) and minor histocompatibility antigens (mHag) have been detected during pregnancy (21–23). Thus, maternal CD8+ T cells can respond to viral, fetal and placental antigens during pregnancy but so far no evidence exists on the presence of HLA-C-restricted viral and mHag-specific CD8+ T cells and whether maternal HLA-A and -B-restricted virus-specific CD8+ T cells can cross-react with fetal HLA antigens, leading to possible pregnancy complications. Recognition of fetal HLA-C by both B cells and helper T cells is suggested by the presence of specific HLA-C IgG antibodies in women with recurrent miscarriages (24). Furthermore, HLA-C incompatibility is significantly increased in couples with unexplained recurrent miscarriages when compared to control subjects (25). In addition, certain combinations of maternal killer cell immunoglobulin-like receptor (KIR) genotypes, expressed by decidual NK cells, and fetal HLA-C are associated with pregnancy complications such as preeclampsia (26). These data indicate that fetal HLA-C could play a vital role in guiding the maternal immune response during pregnancy.

Studies on heterologous immunity in transplantation have focused on the cross-reactivity of HLA-A and -B-restricted virus-specific CD8+ T cells with allogeneic HLA-A and -B, with less attention for HLA-C considering its lower cell surface expression levels when compared to HLA-A and -B (27). In the context of pregnancy HLA-C is the only polymorphic antigen expressed on EVT and alloreactivity to HLA-C (and HLA-E and -G) is therefore unique and highly significant. The importance of HLA-C incompatibility in pregnancy complications coupled to the presence of virus-specific CD8+ T cells at the maternal-fetal interface, led us to investigate whether cross-reactivity of virus-specific CD8+ T cells against HLA-C, -E and -G is a common phenomenon in healthy individuals.

2. RESULTS

2.1. Alloreactivity of an EBV B8/FLR CD8+ T Cell Clone 4D5 Against HLA-C*01:02

To investigate the ability of virus-specific CD8+ T cells to cross-react with HLA-C, -E, and -G, 29 HLA-A and -B-restricted human CMV, FLU, VZV, and EBV-specific CD8+ T cell lines and clones (28) were tested against a panel of single antigen expressing lines (SALs) expressing HLA-C, -E, and -G alleles ($n = 11$) (29, 30). An HLA-A2-restricted EBV-specific CD8+ T cell clone isolated from placental decidua parietalis was also included (20). The specificities of the isolated virus-specific CD8+ T cell lines and clones are listed in **Table 1**. Lack of IFNγ production revealed that alloreactivity against HLA-C, -E, and -G is not common **Table 2**. Nonetheless, one HLA-B*08:01-restricted EBV-specific (EBV B8/FLR) T cell clone, 4D5, showed significant alloreactivity against HLA-C*01:02 **Figure 1A**. This T cell clone was isolated from an HLA-C*01:02 negative donor.

To corroborate alloreactivity against HLA-C*01:02, one EBV B8/FLR T cell line and four T cell clones were stimulated with a panel of SALs and EBV lymphoblastoid cell lines (EBV-LCLs) expressing HLA-C*01:02 and HLA-B*44:02 alleles for 24 h after which IFNγ production was measured. Alloreactivity of EBV B8/FLR T cells against HLA-B*44:02 is a commonly described occurrence (31). T cell clone 4D5 reacted against its virus-specific restriction allele HLA-B*08:01 loaded with FLR peptide as well as HLA-C*01:02 expressed by SALs and EBV-LCLs. Its lower alloreactivity against the second EBV-LCL donor expressing heterozygous HLA-C*01:02 may have been a result of low HLA-C expression. T cell clone 4D5 did not show alloreactivity against HLA-B*44:02 **Figure 1B**. T cell clone 4B8 (here shown as a representative example), comprising a different TCR Vα and Vβ usage than 4D5 **Table 3**, displayed no alloreactivity against HLA-C*01:02 and only cross-reacted with HLA-B*44:02 when loaded with the appropriate self-peptide (EEY). The other EBV B8/FLR CD8+ T cells tested also did not cross-react with HLA-C*01:02, but displayed cross-reactivity against HLA-B*44:02. No alloreactivity against HLA-E and -G was discerned **Figure S1**.

Alloreactivity of virus-specific CD8+ T cells can be cell type or tissue-specific (9, 32). Therefore, to further functionally

TABLE 1 | Specificities of isolated virus-specific CD8+ T cell lines and clones.

Virus	HLA	Antigen	Epitope
HCMV	HLA-A*02:01	pp65 (495-503)	NLVPMVATV
HCMV	HLA-B*35:01	pp65 (123-131)	IPSINVHHY
HCMV	HLA-C*06:02	pp65 (201-209)	TRATKMQVI
HCMV	HLA-C*07:02	IE-1 (309-317)	CRVLCCYVL
EBV	HLA-A*02:01	BMLF1 (280-288)	GLCTLVAML
EBV	HLA-B*08:01	EBNA-3A (325-333)	FLRGRAYGL
EBV	HLA-B*35:01	EBNA-3A (458-466)	YPLHEQHGM
FLU	HLA-A*02:01	IMP (58-66)	GILGFVFTL
VZV	HLA-A*02:01	IE-62 (593-601)	ALWALPHAA

HCMV, human Cytomegalovirus; EBV, Epstein-Barr virus; FLU, Influenza virus; VZV, Varicella-Zoster virus.

TABLE 2 | Alloreactivity of virus-specific CD8+ T cell lines and clones against HLA-C, -E, and -G.

Donor	Specificity	TCR Vβ	# T cell lines/clones tested	Allo-HLA-C	Allo-HLA-E, -G	Target cell (s)	Allo-HLA-C, -E, -G cross-reactivity
A	HCMV A2/NLV	2	2	2	2	SALs	No
	HCMV A2/NLV	8	3	2	3	SALs	No
	HCMV A2/NLV	13.1	1	0	1	SALs	No
	HCMV A2/NLV	5.2	1	0	1	SALs	No
	HCMV A2/NLV	#	9	0	9	SALs	No
A	HCMV B35/IPS	2	1	0	1	SALs	No
	HCMV B35/IPS	3	2	2	2	SALs	No
	HCMV B35/IPS	13.2	2	1	2	SALs	No
	HCMV B35/IPS	22	1	1	1	SALs	No
	HCMV B35/IPS	*	2	2	2	SALs	No
B	HCMV B35/IPS	3	3	2	3	SALs	No
C	HCMV B35/IPS	3	4	2	4	SALs	No
A	FLU A2/GIL	17	3	2	3	SALs	No
	FLU A2/GIL	17+18	1	0	1	SALs	No
D	FLU A2/GIL	17	4	1	4	SALs	No
B	FLU A2/GIL	#	3	2	3	SALs	No
E	FLU A2/GIL	#	2	1	2	SALs	No
F	FLU A2/GIL	#	1	0	1	SALs	No
F	VZV A2/ALW	#	1	1	1	SALs	No
G	VZV A2/ALW	#	1	1	1	SALs	No
H	EBV B35/YPL	14	1	1	1	SALs	No
	EBV B35/YPL	21.3	2	2	2	SALs	No
	EBV B35/YPL	*	1	1	1	SALs	No
I	EBV A2/GLC (decidua parietalis)	#	1	1	1	SALs	No
J	EBV B8/FLR	7	2	2	2	SALs, EBV-LCLs	No
K	EBV B8/FLR (4D5)	3	1	1	1	SALs, EBV-LCLs, PHA blasts	HLA-C*01:02
	EBV B8/FLR	4	2	2	2	SALs, EBV-LCLs	No
L	HCMV C*0702/CRV	6	1	1	1	721.221, EBV-LCLs	No
M	HCMV C*0602/TRA	13	2	2	2	SALs, EBV-LCLs	No
	HCMV C*0602/TRA (1A3, 7A12, 10C1)	28	2	2	2	SALs, EBV-LCLs, PHA blasts	HLA-C*03:02

* The TCR Vβ could not be determined with the TCR Vβ kit used.

Not tested.

Summary	
Specificities	9
Donors	13
TCR tested	21
T cell lines/clones tested against HLA-C, -E, -G	34

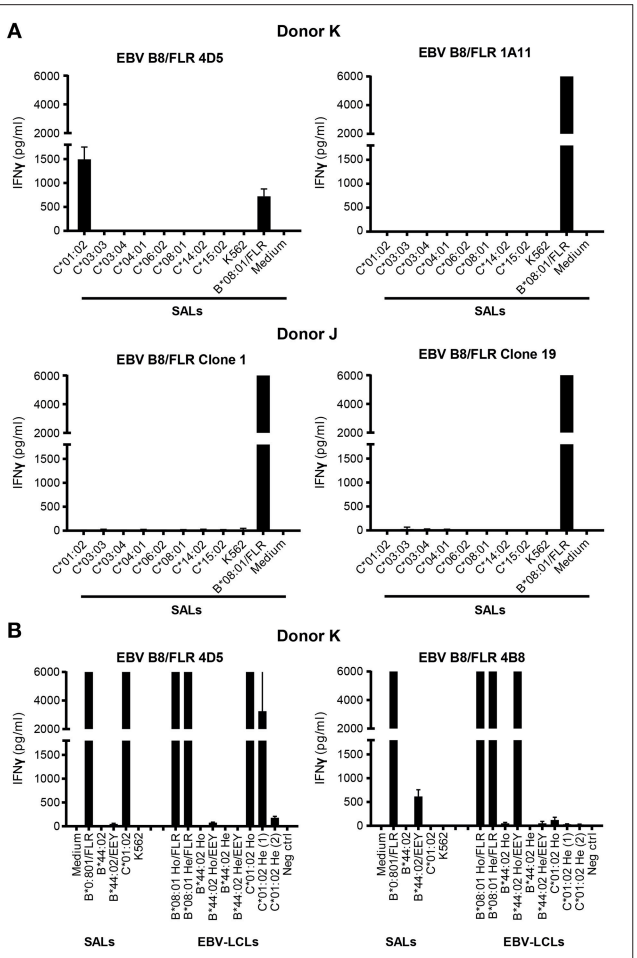


FIGURE 1 | Alloreactivity of EBV B8/FLR T cell clone 4D5 against HLA-C*01:02. **(A)** EBV B8/FLR T cell lines ($n = 9$; 1A11 shown) and T cell clones ($n = 6$; 4D5, clone 1, and clone 19 shown) were stimulated with a panel of HLA-C expressing SALs after which IFNγ production was measured. EBV B8/FLR T cell clone 4D5 showed alloreactivity against HLA-C*01:02. **(B)** One EBV B8/FLR T cell line and four EBV B8/FLR T cell clones (4B8 and 4D5 shown) were stimulated with a panel of SALs and EBV-LCLs expressing HLA-B*08:01, HLA-C*01:02, and HLA-B*44:02 alleles after which IFNγ production was measured. The range of the ELISA standard curve: 5–5120 pg/ml; Ho, homozygous; He, heterozygous. Bars represent duplicate values with standard deviation of the mean.

validate our results, cytotoxicity of the T cell clones 4D5 and 4B8 was investigated against ^{51}Chromium (^{51}Cr) -labeled human umbilical vein endothelial cells (HUVECs), SALs (myeloid origin), EBV-LCLs (B cells) and PHA blasts (T cells) expressing the recognized allo-HLA-C*01:02 allele and the virus-specific restriction allele HLA-B*08:01 loaded with viral peptide as a positive control. Target cells expressing no HLA-B*08:01 and HLA-C*01:02 were included as a negative control. The T cell clones were added to their targets in four effector: target ratios. Specific lysis of HLA-C*01:02 expressing SALs, EBV-LCLs and PHA blasts by T cell clone 4D5 was observed in a ratio-dependent manner. T cell clone 4D5 was however not lytic against HUVECs expressing HLA-C*01:02, presumably the result of the relevant self-peptide not being expressed by this cell type (33) **Figure 2A**. The robust cytolytic response of T cell clone 4D5 against EBV-LCLs expressing HLA-C*01:02 was substantially decreased by addition of an anti-CD8 blocking antibody, while lysis of target cells expressing the virus-specific restriction allele HLA-B*08:01 loaded with FLR peptide was not affected, indicating distinct TCR affinities **Figure 2B**. No specific lysis by T cell clone 4B8 was observed **Figure 2C**. Together, these results demonstrate that alloreactivity of HLA-A and -B-restricted virus-specific CD8+ T cells against HLA-C can occur and is dependent on CD8.

2.2. Characterization of HLA-C*06:02-Restricted HCMV-Specific T Cell Lines and Clones

HLA-C-restricted virus-specific CD8+ T cells have been described in the context of HIV infection where they recognize a highly conserved epitope and in HCMV infection where HLA-C*07:02-restricted CD8+ T cells dominate the T cell response to the immediate-early 1 (IE-1) viral antigen and their levels increase with age (34–36). Given their high allele frequency in the population, we set out to isolate HLA-C*06:02- and HLA-C*07:02-restricted HCMV-specific CD8+ T cells (37, 38) and explore their alloreactivity against HLA-C, -E, and -G. PBMC of HLA-C*06:02+HCMV+ donors ($n = 10$) were stained with an HLA-C*06:02 tetramer containing the HCMV TRA peptide (39) **Table 1**. From a donor with 15% positivity for the HLA-C*06:02/TRA tetramer, CD8+ T cell lines and clones were generated by sorting tetramer positive CD8+ T cells and expanding them *in vitro* **Figure 3A**; **Figure S2**. An established HLA-C*07:02-restricted HCMV-specific CD8+ T cell clone (LH) was included in the analysis (35). To examine the functionality of these HLA-C*06:02/TRA-restricted HCMV-specific T cell lines and clones, as well as the HLA-C*07:02/CRV-restricted HCMV-specific T cell clone LH, IFNγ production was measured after 24 h of co-culture with SALs and EBV-LCLs expressing HLA-C*06:02 or C*07:02 loaded with the appropriate viral peptide. All HLA-C*06:02-restricted T cell lines and clones, and the HLA-C*07:02-restricted clone LH responded against their virus-specific restriction HLA-allele loaded with viral peptide **Figure 3B**. In addition, specific lysis in a ratio-dependent manner of ^{51}Cr-labeled SALs, 721.221 cells expressing HLA-C*07:02, and EBV-LCLs was detected **Figure 3C**. These results confirmed functionality of the generated HLA-C*06:02-restricted T cell lines and clones (HCMV C*06:02/TRA), and the established HLA-C*07:02-restricted T cell clone LH (HCMV C*07:02/CRV).

2.3. Alloreactivity and Cytotoxicity of HCMV C*06:02/TRA T Cell Lines Against HLA-C*03:02

Next, the HCMV C*06:02/TRA CD8+ T lines and clones ($n = 4$), and HCMV C*07:02/CRV CD8+ T cell clone LH were tested against a panel of SALs expressing HLA-C, -E, and -G, in a co-culture system where IFNγ production was assessed. No alloreactivity was observed in this setting **Figures S3A,B**. Subsequently, in a similar manner, alloreactivity against a panel of EBV-LCLs covering the most common HLA alleles was

TABLE 3 | TCR Vα and Vβ usage of CD8+T cell lines and clones.

T cell line/clone	TRαV	TRαJ	CDR3α	TRβV	TRβJ	TRβD	CDR3β	Cross-reactivity
B8/FLR T cell clone 4B8	TRAV1-2*01 F	TRAJ36*01 F	C A V R D Q T G A N N L F F	TRBV4-3*02 (F)	TRBJ2-5*01 F	TRBD2*01 F	C A S S H G L A G I L E T Q Y F	No
B8/FLR T cell clone 4D5	TRAV40*01 F	TRAJ43*01 F	C L L G D N D M R F	TRBV3-1/2*01	TRBJ1-6*02 F	TRBD2*01 F	C A S S Q P P T G R S Y N S P L H F	HLA-C*01:02
C*0702/CRV T cell clone LH	TRAV27*03 (F)	TRAJ33*01 F	C A G G D M D S N Y Q L I W	TRBV6-2/3/6	TRBJ2-7*01 F	TRBD1*01 F	C A S G E V Y E Q Y F	No
C*0602/TRA T cell clone 1F12	TRAV19*01 F	TRAJ26*01 F	C A L S E G G S Y G Q N F V F	TRBV13*01/02 (F)	TRBJ2-1*01 F	TRBD2*01 F	C A S S L R D E Q F F	No
C*0602/TRA T cell line 1A3	TRAV12-2*02 (F)	TRAJ20*01 F (a)	C A V N N D Y K L S N	TRBV28*01 F	TRBJ2-1*01 F	TRBD2*01 F	C A S S S G G L E N E Q F F	HLA-C*03:02
C*0602/TRA T cell clone 10C1				TRBV28*01 F	TRBJ2-1*01 F	TRBD2*01 F	C A S S S G G L E N E Q F F	HLA-C*03:02

investigated **Table 4**. Interestingly, two HCMV C*06:02/TRA T cell lines cross-reacted with EBV-LCL donor 12 of which T cell line 1A3 is shown as a representative example **Figure 4A**. When comparing the HLA typing of all 20 EBV-LCL donors in the panel, HLA-C*03:02 expressed by donor 12 was the only non-overlapping HLA allele candidate. A SAL expressing HLA-C*03:02 is not present in the panel and therefore cross-reactivity against this allele was not picked up in the initial screening **Figures 3SA,B**. A role for HLA class II was ruled out. CD8+ T cell lines and clones cross-reacting against donor 12 disclosed a distinct TCR Vα and Vβ usage when compared to CD8+ T cells showing no alloreactivity **Table 3**. No alloreactivity of the HCMV C*07:02/CRV clone LH was observed **Figure 4A**.

To further gauge the alloreactivity against HLA-C*03:02, HCMV C*06:02/TRA T cell lines ($n = 2$) and clones ($n = 5$) with the same TCR Vβ usage as the two CD8+ T cell lines that cross-reacted with cells from donor 12 (and all isolated from an HLA-C*03:02 negative donor) were stimulated with EBV-LCLs and PHA blasts expressing the recognized allo-HLA-C*03:02 allele. Target cells expressing the virus-specific restriction allele HLA-C*06:02 loaded with viral peptide were included as a positive control. Alloreactivity against HLA-C*03:02 was detected for all HCMV C*06:02 T cells tested, with substantially more IFNγ production against EBV-LCLs than against PHA blasts 1 and 2, obtained from two different donors **Figure 4B**. Differential HLA expression levels on the cell surface may explain increased alloreactivity against PHA blast 3 expressing HLA-C*03:02, obtained from a third donor **Figure S4**.

Having identified IFNγ production against allo-HLA-C*03:02, HCMV C*06:02/TRA T cell lines and clones were tested for cytotoxicity against ^{51}Cr-labeled SALs, EBV-LCLs, and PHA blasts expressing HLA-C*03:02. Target cells expressing the virus-specific restriction allele HLA-C*06:02 loaded with viral peptide were included as a positive control. The CD8+ T cell lines and clone were added to their targets in four effector: target ratios. Specific lysis of HLA-C*03:02 expressing target cells was observed in a ratio-dependent manner **Figure 5A**. Subsequently, the T cell lines and clone were incubated with an anti-CD8 blocking antibody prior to co-culture with the ^{51}Cr-labeled target cells resulting in substantially decreased lysis of target cells expressing HLA-C*03:02. A decrease in lysis was not observed for target cells expressing the virus-specific restriction allele HLA-C*06:02 loaded with viral peptide, indicating distinct TCR affinities **Figure 5B**. These findings highlight the functionality of the isolated HCMV C*06:02/TRA T cell lines and clones and provide evidence that alloreactivity of HLA-C-restricted virus-specific CD8+ T cells against HLA-C is a phenomenon that occurs.

3. DISCUSSION

Alloreactivity of HLA-A and -B-restricted virus-specific CD8+ T cells against HLA-A and -B is common. Eighty percent of virus-specific T cell lines and 45% of virus-specific T cell clones disclosed cross-reactivity against allo-HLA molecules (9). Here, we have shown that alloreactivity of HLA-A and -B-restricted

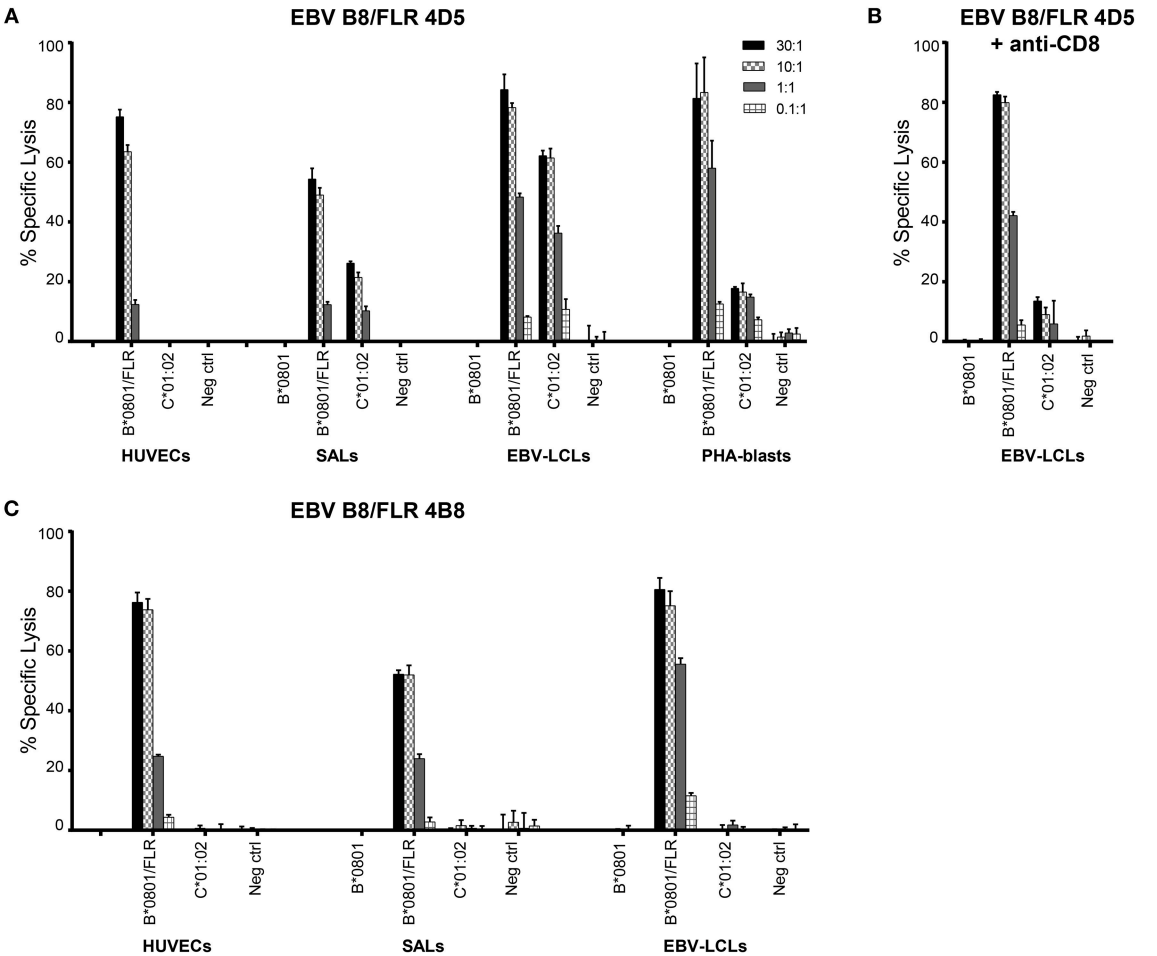


FIGURE 2 | EBV B8/FLR T cell clone 4D5 is cytotoxic against HLA-C*01:02. Cytotoxicity of T cell clones 4D5 **(A)** and 4B8 **(C)** was tested against ^{51}Cr-labeled HUVECs, SALs, EBV-LCLs, and PHA blasts expressing the recognized allo-HLA-C*01:02 allele and the virus-specific restriction HLA allele loaded with viral peptide as a positive control. As a negative control, target cells expressing no HLA-B*0801 and HLA-C*0102 were included. **(B)** T cell clone 4D5 was incubated with an anti-CD8 blocking antibody prior to co-culture with ^{51}Cr-labeled EBV-LCLs and specific lysis was measured. Bars represent triplicate values with standard deviation of the mean.

virus-specific CD8+ T cells against HLA-C can also occur. Amongst the 29 HLA-A and -B-restricted virus-specific T cells tested, one EBV B8/FLR CD8+ T cell clone 4D5 with distinct TCR Vα and Vβ usage displayed cross-reactivity and cytotoxicity against target cells expressing HLA-C*01:02, indicative of a more than 10 times lower frequency within the pool of HLA-A and -B-restricted virus-specific CD8+ T cells tested. This T cell clone did not reveal the classical described cross-reaction against HLA-B*44:02. Our preliminary data suggests that HLA-C cross-reactivity in HLA-C-restricted virus-specific T cells is more common. HLA-C*06:02-restricted HCMV-specific CD8+ T cells were successfully isolated from an HCMV+ donor by means of HLA-C tetramers and deemed fully functional *in vitro*. Alloreactivity of these HCMV C*06:02/CRV T cell lines and clones, with distinct TCR Vα and Vβ usage, was observed against HLA-C*03:02. This alloreactivity was mediated by IFNγ production and cytotoxicity. Viral specificity and alloreactivity are thought to be mediated by the same TCR (9), where in our setting anti-viral reactivity occurred independent of CD8, while allo-HLA-C reactivity was CD8 dependent. Differential recognition of HLA-C on SALs, EBV-LCLs, PHA blasts, and HUVECs, that did not provoke any alloreactivity, is an indication that cross-reactivity is determined by endogenous peptide (11) and supports the anticipation that tissue-specific peptides are presented and recognized. The nature of the endogenous peptide presented in HLA-C*01:02 and HLA-C*03:02, provoking the allo-response, is however unknown. Alternatively, expression of costimulatory and coinhibitory molecules by virus-specific T cells may have an influence on T cell signaling and thereby the extent of the allo-response (40). No alloreactivity against HLA-E and -G was observed. Our initial screening was against a panel of SALs expressing most, but not all HLA-C, -E, and -G molecules and we therefore may have underestimated the allo-response of virus-specific CD8+ T cells against these HLA alleles.

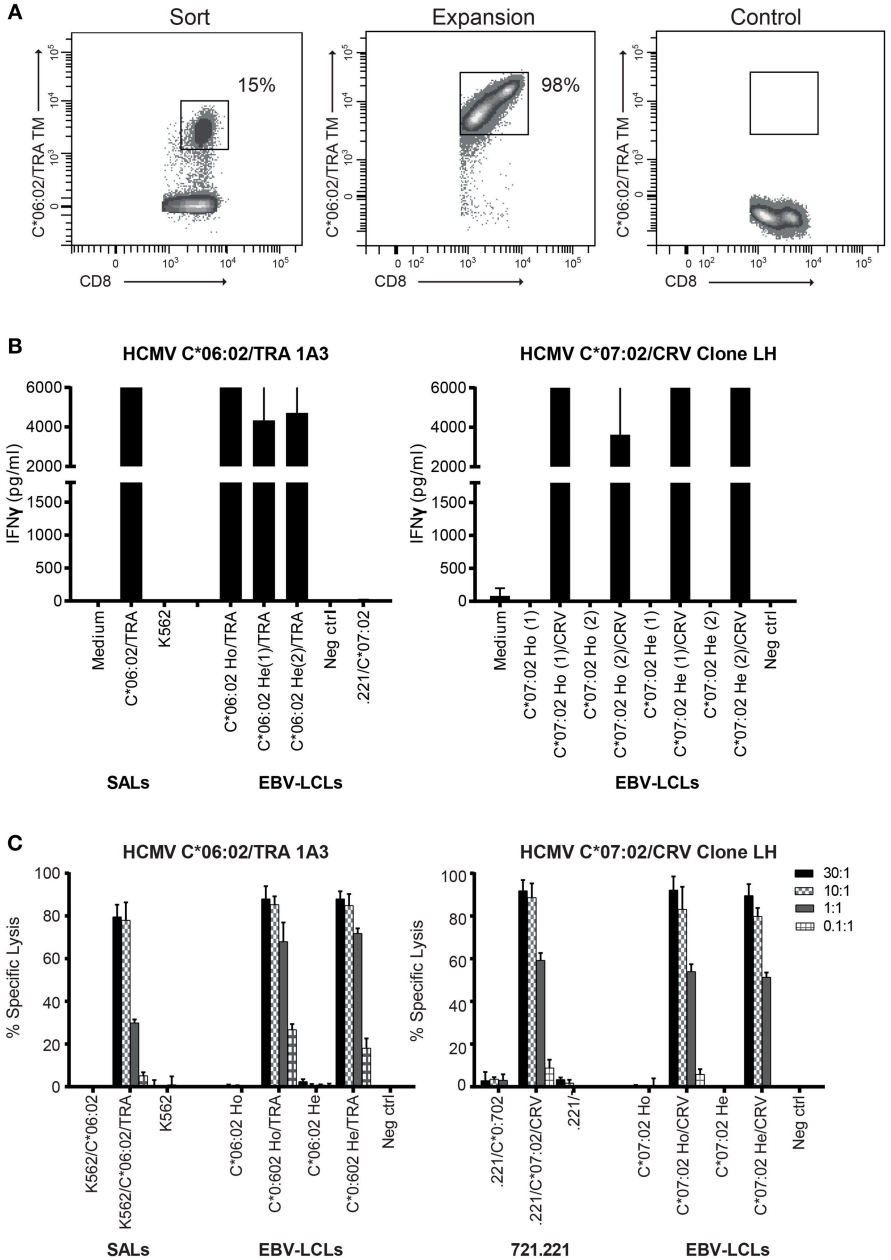


FIGURE 3 | Generated HCMV HLA-C*06:02/TRA T cell lines and clones, and the HCMV HLA-C*07:02/CRV T cell clone are cytolytic against their target cells. **(A)** FACS plots of HLA-C*06:02/TRA tetramer staining at time of sorting and 2 weeks after expansion of the tetramer-positive CD8+ T cells. **(B)** Two HCMV C*06:02/TRA T cell lines (1A3 shown) and two T cell clones, and HCMV C*07:02/CRV T cell clone LH were stimulated with SALs and EBV-LCLs expressing HLA-C*06:02 or C*07:02 loaded with viral peptide. **(C)** Cytotoxicity of one HCMV C*06:02/TRA T cell line (1A3 shown) and two T cell clones, and HCMV C*07:02/CRV T cell clone LH was tested against ^{51}Cr-labeled SALs, 721.221 cells and EBV-LCLs expressing the virus-specific restriction HLA allele alone or loaded with viral peptide. The range of the ELISA standard curve: 5–5120 pg/ml. Ho, homozygous; He, heterozygous. Bars represent triplicate values with standard deviation of the mean.

Variation of HLA-C expression at the cell surface can be a result of microRNA binding and discrepancies in exons that influence the structure of the peptide-binding cleft and the diversity of peptides bound by HLA-C molecules (27, 41). Differential expression of HLA-C has an influence on the ability of CD8+ T cells to mount an immune response. High expression of HLA-C has been associated with protection against infections, yet at the same time correlates with autoimmune disease (42). Nevertheless, when viral peptides are presented in the HLA-C locus, immune responses are still lower than to those presented in the HLA-A and -B loci (43). The lower expression of HLA-C at the cell surface and the differential

TABLE 4 | HLA class I typing of the EBV-LCL panel.

	HLA class I		
Panel ID	**A**	**B**	**C**
1	02:01, 32:01	08:01, 44:05	02:02, 07:01/06/18
2	24:02, 33:01	14:02	02:02/32, 08:02/29
3	03:01/22, 29:02/09	07:02/61/114, 44:03/105	07:02, 16:01
4	11:01/43, 33:03/51	18:01/17N, 52:01	07:02, 07:04
5	11:01/33, 30:01/54	13:02, 35:01/42	04:01, 06:02
6	01:01, 26:01	08:01, 49:01	07:01
7	11:01, 31:01	15:01, 57:01	03:03, 06:02/55
8	02:03, 24:02	38:02, 40:01	03:04, 07:02
9	29:02, 31:01	18:01/17N, 58:01	05:01, 07:18/01
10	24:03	51:01	15:02
11	26:01	38:01	12:03
12	24:02, 30:01	51:01, 58:01	01:02, 03:02
13	02:01, 03:01	15:01	03:03
14	68:01, 68:02	44:02/19N, 55:01	03:03, 07:04
15	24:02, 31:01	39:01, 55:01	03:03, 12:03
16	30:01, 68:02	42:01	17:01
17	01:01	41:01	17:01
18	02:01, 11:01	35:01, 44:03	04:01, 16:01
19	02:01, 25:01	18:01/17N/43, 44:02/19N/55	05:01, 12:03
20	24:02, 31:01/119	07:02/294/298, 07:05	07:02, 15:05

immune responses that it triggers when compared to HLA-A and -B (44) may be an explanation for less frequent alloreactivity against HLA-C.

We speculate that alloreactivity of virus-specific CD8+ T cells against HLA-C may play a role in pregnancy complications where HLA-C is the only polymorphic HLA allele expressed by EVT. Viral infections of the fetus or the placenta can lead to severe birth defects or pregnancy loss (5). Viruses are capable of downregulating surface HLA-A and -B expression upon infection, while HLA-C expression is spared (45). EVT also persistently express HLA-C when infected with HCMV (46). HLA-A and -B-restricted virus-specific CD8+ T cells are present at the maternal-fetal interface (20) and may be capable of cross-reacting with HLA-C under certain pro-inflammatory environmental circumstances and depending on (allo) peptide expression, thereby jeopardizing the success of pregnancy. It is yet to be established whether HLA-C-restricted virus-specific CD8+ T cells are present at the maternal-fetal interface and if so, whether they are capable of mounting an immune response. While the presence of anti-HLA-C IgG antibodies has been described in women with recurrent miscarriages, the competency of virus-specific CD8+ T cells to cross-react with HLA-C raises the question whether allo-HLA-C IgG antibodies are the only player in recurrent miscarriages or whether decidual virus-specific CD8+ T cells with cross-reactive potential also play their part in pregnancy complications.

Cross-reactivity of virus-specific CD8+ T cells against HLA-C can occur and consequently our results lay the foundation for further investigation into this cross-reactivity in the context of pregnancy. Future research will focus on isolating virus-specific CD8+ T cells from the peripheral blood of women with either a healthy pregnancy or recurrent miscarriage. Alloreactivity and differences thereof by these virus-specific CD8+ T cells, obtained after normal pregnancy and miscarriage cases, against target cells expressing allo-HLA-C, -E, and -G molecules can then be investigated. Isolating viable HLA-G+ EVT from first trimester and term placentas that express the correct HLA typing is challenging. Yet, it is important that allo-HLA reactive CD8+ T cells are tested against primary EVT expressing allo-HLA-C, as EVT may have protective mechanisms in place that prevent allo-HLA responses to ensure a successful pregnancy. A recent study described aberrant expression of HLA-DR in syncytiotrophoblasts and syncytiotrophoblast-derived extracellular vesicles (STEVs) in pre-eclampsia but not control placentae, addressing the importance of further examining heterologous immunity of not only decidual CD8+ T cells, but also decidual CD4+ T cells (47).

Not only in the pregnancy setting has HLA-C disparity been described as a possible cause of complication. In transplantation, HLA-C incompatibility has been associated with graft failure after bone marrow transplantation (48). Furthermore, HLA-C mismatches were significantly correlated with acute transplant rejection and increased chronic graft-vs.-host disease (GvHD) after hematopoietic stem cell transplantation (49, 50). Graft loss after solid organ transplantation and GvHD after hematopoietic stem cell transplantation (51) have been associated with heterologous immunity against allo-HLA-A and -B. The proven alloreactivity of virus-specific CD8+ T cells against HLA-C could lead to allo-immune responses and add an additional barrier to tolerance that requires further assessment in transplantation.

In conclusion, alloreactivity against HLA-C occurs and may have pronounced clinical implications in pregnancy, where the only polymorphic allo-HLA antigen expressed by EVT is HLA-C. It remains to be established how often this alloreactivity could lead to the development of pregnancy complications, such as recurrent miscarriages.

4. MATERIALS AND METHODS

4.1. Preparation of Responder and Target Cells

Peripheral blood leukocytes were isolated from buffy coats obtained from healthy blood donors after informed consent, at Sanquin Blood Supply, the Netherlands. PBMC were isolated by standard density gradient centrifugation and cryopreserved until use. Single HLA antigen–transfected K562 cells (SALs) were generated as described previously (52). HLA typing was performed by sequence-specific oligonucleotide or sequence-specific primer genotyping at the Department of Immunohematology and Blood Transfusion, LUMC, the

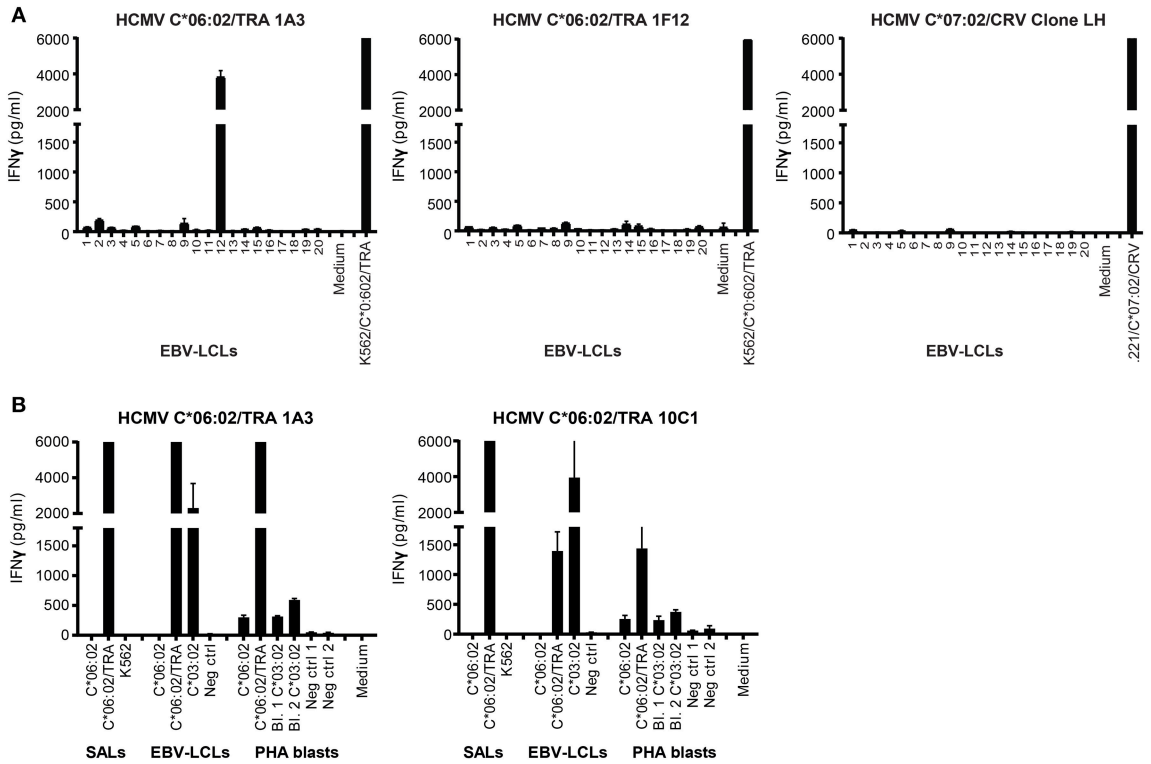


FIGURE 4 | Alloreactivity of HCMV C*06:02/TRA T cell lines against HLA-C*03:02. **(A)** Two HCMV C*06:02/TRA T cell lines (1A3 shown) and two T cell clones (1F12 shown), and HCMV C*07:02/CRV T cell clone LH were stimulated with a panel of EBV-LCLs after which IFNγ production was measured. **(B)** Three HCMV C*06:02/TRA T cell lines (1A3 shown) and five T cell clones (10C1 shown) were stimulated with EBV-LCLs and PHA blasts, obtained from two different donors, expressing the recognized allo-HLA-C*03:02 allele and the virus-specific restriction allele HLA-C*06:02 loaded with viral peptide as a positive control. SALs expressing HLA-C*06:02 loaded with viral peptide were also included as a positive control. The range of the ELISA standard curve: 5–5120 pg/ml. Bars represent duplicate values with standard deviation of the mean.

Netherlands. Epstein-Barr virus transformed lymphoblastoid cell lines (EBV-LCLs) were generated by incubating PBMC with the supernatant of the EBV-producing marmoset cell line B95.8 for 1.5 h at 37°C, and additional culture in RPMI 1640 Medium (Gibco Life Technologies, Carlsbad, CA) supplemented with 10% Fetal Calf Serum (FCS; Sigma Aldrich, St. Louis, Missouri), Penicillin/Streptavidin (Pen/Strep) and L-glutamine (all from Gibco). The 721.221 cell line expressing HLA-C*07:02 was kindly obtained from Professor Anthony W. Purcell (Monash University; cell line originally made by the laboratory of Prof. Andrew Brooks at the University of Melbourne).

Phytohaemagglutinin (PHA) blasts were generated by incubating PBMC for 8 days in RPMI 1640 Medium, Pen/Strep, L-glutamine, 15% human serum (HS, Sanquin, Amsterdam, the Netherlands), IL-2 (60 IU/ml; Novartis, Novartis, Horsham, UK) and PHA (4 μg/ml; Murex Biotech Ltd, Dartford, UK). Human umbilical vein endothelial cells (HUVECs) were cultured in M199 medium supplemented with 10% Newborn calf Serum (NCS), 1% sodium pyruvate, Pen/Strep (all from Gibco), 0,1% β-mercaptoethanol (0.05M, Sigma Aldrich), 1% sodium heparin (400 IE/ml; LUMC, Leiden, the Netherlands), and bovine purine extract (BPE; 100 μl in 20 ml; Invitrogen, Carlsbad, CA).

4.2. Generation of Virus-Specific CD8+ T Cell Lines and Clones

PBMC from EBV+, HCMV+, FLU+, and VZV+ blood donors were stained with phycoerythrin (PE)-labeled viral tetramers HCMV HLA-A*02:01/NLV, HCMV HLA-B*35:01/IPS, EBV HLA-A*02:01/GLC, EBV HLA-B*08:01/FLR, EBV HLA-B*35:01/YPL, FLU HLA-A*02:01/GIL, VZV HLA-A*02:01/ALW (Protein facility, Department of Immunohematology and Blood Transfusion, LUMC, Leiden, the Netherlands), and an Alexa-647-labeled viral tetramer HCMV pp65 HLA-C*06:02/TRA (NIH Tetramer Core Facility, Emory University, Atlanta, GA) **Table 1**. The HCMV HLA-C*07:02/CRV-specific CD8+ T cell clone was generated by CRV peptide stimulation of PBMC from an HCMV+ HLA-C*07:02+ donor. Additional staining with conjugated mouse anti-human monoclonal antibodies CD56, CD14, CD4, CD19 (FITC; BD Biosciences, San Jose, CA), CD45 (PE-Cy5; eBioscience, San Diego, CA), and CD8 (Pacific Orange; ThermoFisher, Waltham, MA) was performed. When staining with the HCMV pp65 HLA-C*06:02/TRA tetramer, CD158

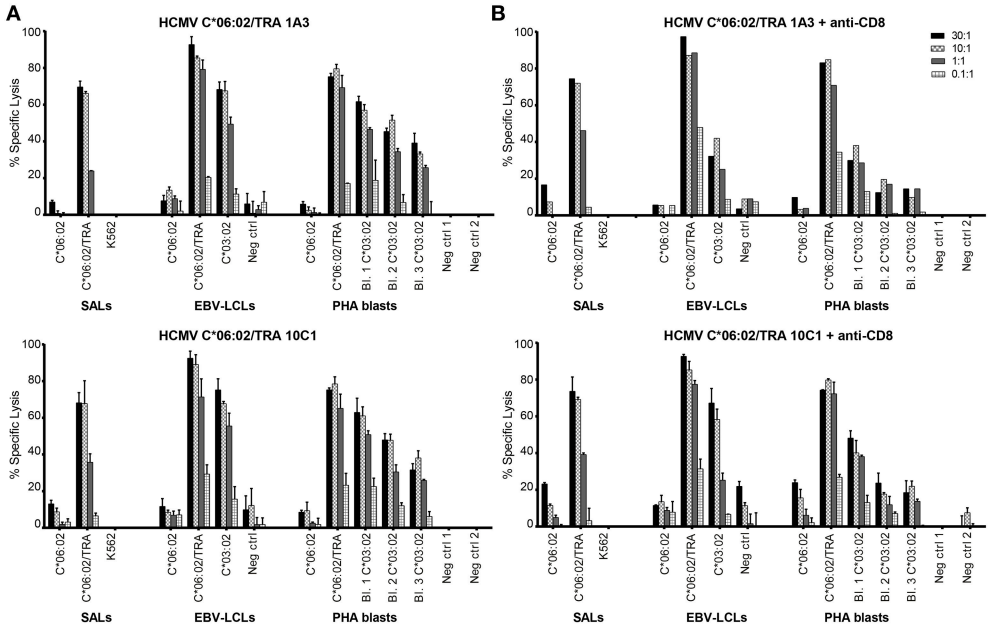


FIGURE 5 | HCMV C*06:02/TRA T cell lines and clones are cytotoxic against HLA-C*03:02. **(A)** Cytotoxicity of two HCMV C*06:02/TRA CD8+ T cell lines (1A3 shown) and one CD8+ T cell clone (10C1) was tested against ^{51}Cr-labeled EBV-LCLs and PHA blasts, obtained from three different donors, expressing the recognized allo-HLA-C*03:02 allele and the virus-specific restriction HLA allele loaded with viral peptide as a positive control. SALs expressing HLA-C*06:02 loaded with viral peptide were also included as a positive control. **(B)** The CD8+ T cell lines and clone were incubated with an anti-CD8 blocking antibody prior to co-culture with target cells and specific lysis was measured. Bars represent triplicate values with standard deviation of the mean.

(KIR2DL1/S1/S3/S5) and CD158b (KIR2DL2/L3) (PE-Cy7; Biolegend, San Diego, CA) were included in the panel to exclude binding of CD8+ T cells to the tetramer through KIR (38). Tetramer-positive CD8+ T cells were purified with FACS sort based on the expression of CD45+CD8+CD19-CD14-CD56-CD4-KIR- tetramer+ cells. CD8+ T cell lines were generated by sorting 10 tetramer-positive cells per round-bottom 96-well and CD8+ T cell clones by sorting 1 tetramer-positive cell per round-bottom 96-well, respectively. After sorting, tetramer-positive CD8+ T cells were expanded in 96-well plates with irradiated PBMC (4,000 Rad) isolated from buffy coats in Iscove's Modified Dulbecco's Medium (IMDM; Lonza, Basel, Switzerland) supplemented with Pen/Strep, L-Glutamine, 5% HS, 5% FCS, PHA (2 µl/ml; Remel, Lenexa, KS), and IL2 (60 IU/ml). TCR Vα and Vβ usage was determined by DNA sequencing using TCR-specific polymerase chain reaction primers (53) followed by use of the BigDye® Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA).

4.3. Cytokine Production Assay

After 8 days of expansion with allogeneic irradiated PBMC, CD8+ T cell lines and clones (5 x 10^3) were incubated with EBV-LCLs and PHA blasts (5 x 10^4) expressing either self-HLA, self-HLA loaded with viral peptide (incubation 30 min at 37°C; washed thrice), or allo-HLA molecules (duplicate; 96-wells) in IMDM supplemented with Pen/Strep, L-Glutamine, 5% HS, 5% FCS, and IL-2 (60 IU /ml). PHA blasts were irradiated (5,000 Rad) before co-culture with T cells. After 24 h at 37°C, supernatants were collected and frozen until further use. IFNγ levels were measured in a standard enzyme-linked immunosorbent assay (ELISA), according to manufacturer's protocol (U-Cytech, Utrecht, the Netherlands). The range of the ELISA standard curve was 5–5120 pg/ml.

4.4. Cytotoxicity Assays

After 8 days of expansion with allogeneic irradiated PBMC, serial dilutions of responder CD8+ T cell lines and clones were incubated with ^{51}Chromium-labeled EBV-LCLs, SALs and/or 721.221 target cells, PHA blasts and HUVECs (responder/stimulator ratio 30:1; 10:1; 1:1; 0.1:1) in round-bottom 96-wells plates for 4 or 20 h at 37°C in IMDM supplemented with Pen/Strep, L-Glutamine, 5% HS, 5% FCS, and IL-2 (60 IU/ml). Where applicable, viral peptide was loaded onto the target cells for 60 min at 37°C, simultaneously with chromium incubation, and washed thrice. In addition, CD8+ T cell lines and clones were incubated with the anti-CD8 blocking antibody FK18 (4.3 µl/ml) for 60 min at 37°C, where after cells were washed twice. Supernatants were harvested for analysis on a gamma-counter (PerkinElmer 2470 Wizard2, Waltham, MA), counts from triplicate wells were averaged, and specific lysis was calculated as follows: (Condition of interest ^{51}Cr release − Spontaneous ^{51}Cr release)/(Maximum ^{51}Cr release − Spontaneous ^{51}Cr release) x 100. Maximum ^{51}Cr release of the target cells was determined in PBS 1% Triton X-100 and spontaneous ^{51}Cr release in medium.

ETHICS STATEMENT

This study was carried out in accordance with the guidelines issued by the Medical Ethics Committee of the Leiden University Medical Center. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

AvdZ, EvdM-P, FC, and SH designed the research and wrote the manuscript. AvdZ, EvdM-P and PvM performed the experiments. HvdH and EvdM-P generated the HLA-A and -B-restricted virus-specific T cell lines and clones. JA performed TCR sequencing analyses and DR provided extensive HLA typing.

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The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02880/full#supplementary-material

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REVIEW
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The Role of Extracellular Vesicles and PIBF in Embryo-Maternal Immune-Interactions

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Pregnancy represents a unique immunological situation. Though paternal antigens expressed by the conceptus are recognized by the immune system of the mother, the immune response does not harm the fetus. Progesterone and a progesterone induced protein; PIBF are important players in re-adjusting the functioning of the maternal immune system during pregnancy. PIBF expressed by peripheral pregnancy lymphocytes, and other cell types, participates in the feto-maternal communication, partly, by mediating the immunological actions of progesterone. Several splice variants of PIBF were identified with different physiological activity. The full length 90 kD PIBF protein plays a role in cell cycle regulation, while shorter splice variants are secreted and act as cytokines. Aberrant production of PIBF isoforms lead to the loss of immune-regulatory functions, resulting in and pregnancy failure. By up regulating Th2 type cytokine production and by down-regulating NK activity, PIBF contributes to the altered attitude of the maternal immune system. Normal pregnancy is characterized by a Th2-dominant cytokine balance, which is partly due to the action of the smaller PIBF isoforms. These bind to a novel form of the IL-4 receptor, and induce increased production of IL-3, IL-4, and IL-10. The communication between the conceptus and the mother is established via extracellular vesicles (EVs). Pre-implantation embryos produce EVs both *in vitro*, and *in vivo*. PIBF transported by the EVs from the embryo to maternal lymphocytes induces increased IL-10 production by the latter, this way contributing to the Th2 dominant immune responses described during pregnancy.

Keywords: pregnancy, progesterone, PIBF, NK cells, cytokines, extracellular vesicles

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INTRODUCTION

Fifty per cent of the antigens expressed by the fetus originate from the father. Therefore, they are recognized as foreign and should be "rejected," yet in spite of all odds, the maternal immune system does not attack the fetus.

The immune system of the mother must comply with two conflicting requirements, i.e., while creating a favorable environment for the developing fetus, it has to be prepared to control possible emerging infections. By establishing a delicate balance, the foeto-maternal unit is able to satisfy the interests of both the mother and the fetus. Progesterone, and its mediator the progesterone-induced blocking factor (PIBF) are important players in this process. In addition to its endocrine effects,

progesterone also acts as an "immunosteroid" (1). Progesterone induces Th2 differentiation of established T cell clones (2) and regulates the homing and activity of uterine NK cells (3), among others, by upregulating HLA-G gene expression (4), which is the ligand for both NK inhibitory and activating receptors. Many of the immunological effects of progesterone are mediated by PIBF.

This review aims to give an overview on the diverse roles of progesterone and PIBF in re-setting the functions of the maternal immune system, and on extracellular vesicles (EVs) as means of establishing the communication between the two sides of the feto-maternal unit.

PROGESTERONE RECEPTORS

The biological activity of progesterone is mediated by genomic and non-genomic pathways. The former depends on two nuclear progesterone receptor (PR) isoforms, PRA, and PRB (5, 6). Both isoforms are the products of the same gene, but their transcription is controlled by two distinct promoters (7).

Mice lacking PRA are infertile (8, 9), while the PRB isoform mediates the effects of progesterone on mammary gland development (10). The reproductive tissue responses to progesterone depend on the relative expression of the two isoforms (11). Progesterone can also signal through membrane-bound PRs or via the MAPK or PI3K/Akt pathway. The latter entirely bypasses the classical PR pathway, signaling either through the JNK pathway or by increasing cAMP (12).

Studies on PR knock out mice revealed, that PRs are required not only for endometrial receptivity and decidualization (13), but also for establishing an appropriate immune environment in the endometrium (14) (**Figure 1**). Several studies using nuclear and cytosol binding assays and immunohistochemistry—indicate, that in certain conditions lymphoid cells might express PRs (15–20).

Peripheral lymphocytes of pregnant women, but not those of non-pregnant individuals express PRs (21, 22). Earlier we demonstrated an inverse relationship between progesterone binding capacity and cytotoxic activity of peripheral human lymphocytes (23). The cytotoxic activity of pregnancy lymphocytes was significantly reduced by progesterone at concentrations comparable to those, present in pregnancy serum, while 100-fold higher progesterone concentrations were required to alter the cytotoxic activity of lymphocytes from non-pregnant individuals (24). These findings already suggested that pregnancy lymphocytes might contain progesterone binding sites, which enable them to respond to progesterone.

The number of PR positive cells increases throughout normal gestation. In women with recurrent miscarriage, or in those, showing clinical symptoms of threatened pre-term delivery, the % of PR expressing cells among peripheral lymphocytes, is significantly lower than in women with uneventful pregnancies (21, 22). These findings suggest, that the presence of PR positive lymphocytes is required for a normally progressing pregnancy.

PR expression in peripheral lymphocytes or lymphoid cell lines has been confirmed by several studies (15–17, 25, 26). Both classical PR isoforms are present in peripheral blood NK cells (18), however, PR expression in decidual NK cells is controversial. Van den Heuvel et al. (3) demonstrated PRs in murine decidual NK cells, while Henderson et al. (27) failed to detect of PRs in purified decidual NK cells. Nevertheless, the majority of decidual NK cells are PIBF positive (28).

Both *in vitro* and *in vivo* activation of human non-pregnancy lymphocytes result in increased PR expression (29, 30). Paternal leukocyte immunization of women with recurrent miscarriage also increases the number of PR expressing lymphocytes (31).

These data indicate that PR expression is a characteristic feature of activated immune cells (**Figure 2**).

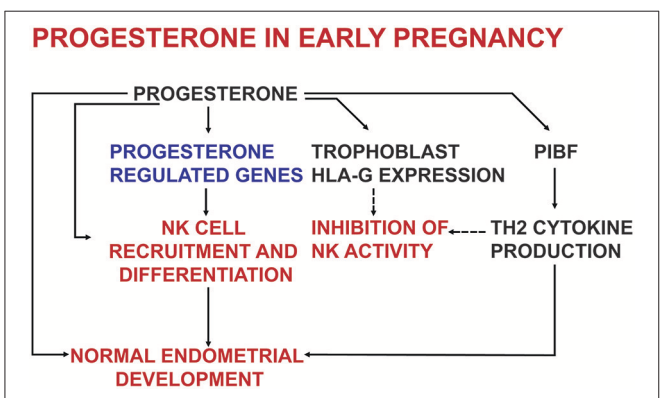


FIGURE 1 | The effects of progesterone on endometrial development and on the immune system in early pregnancy.

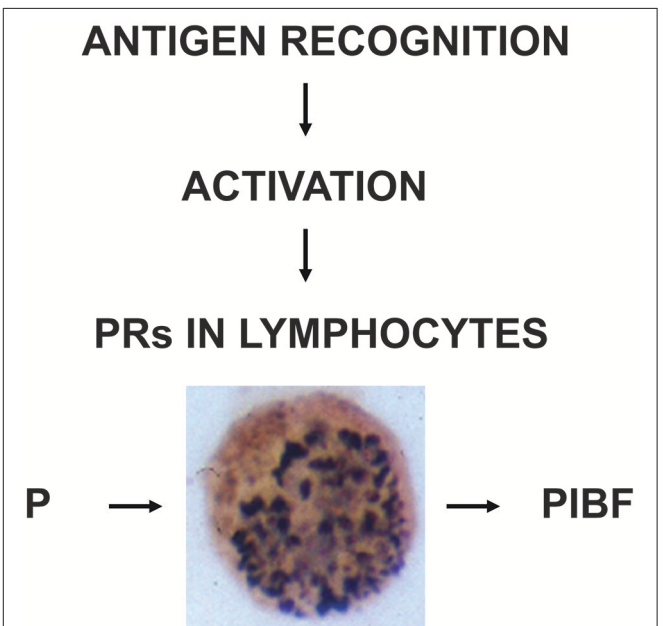


FIGURE 2 | The induction and biological significance of lymphocyte progesterone receptors. Following recognition of fetal antigens, maternal lymphocytes become activated and express progesterone receptors (PR). The presence of PRs enables the cells, to respond to progesterone (P), e.g., by PIBF production.

PROGESTERONE-REGULATED GENES

Among the progesterone-regulated genes, the transcription factors Hox-A10, Hox-A11, and the glican binding protein galectin-1 (Gal-1) are the most relevant for the feto- maternal immunological interaction (32). Hox-A10 deficient mice are characterized by a polyclonal T cell proliferation (33), and impaired decidual NK cell differentiation (24, 25, 34, 35).

Gal-1 expression in the female reproductive system was described in the nineties, and recently, many functional aspects of this lectin during pregnancy have been discovered (36–38). Gal-1 gene expression in the mouse uterine tissues has been shown to be regulated by ovarian steroids during implantation (39). In line with this, Than et al. (40) identified an estrogen response element in the Gal-1 gene.

Altered Gal-1 expression in the placenta has been implicated in several pregnancy pathologies.

Proteomic studies showed that Gal-1 expression is reduced in placental villous tissues from patients with spontaneous miscarriages (41). On the other hand, placental Gal-1 expression was found to be increased in severe preeclampsia (42) as well as in chorioamnionitis (43), possibly representing a fetal response to an exaggerated systemic maternal inflammation.

In pregnant mice, stress-induced Gal-1-deficiency results in an increased rate of fetal loss, which is corrected by progesterone exposure. Gal-1 treatment on the other hand, prevents the stress-induced decrease of progesterone as well as PIBF levels, and restores the resorption rates to a normal level (44). These data suggest a cross-regulation between progesterone and Gal-1 at the foeto-maternal interface.

PIBF is another progesterone-regulated gene. The mouse PIBF1 gene, is transcribed to 16 different mRNAs, the longest of which is 3,677 bp long and includes 18 exons. The predicted protein is a 90 kDa molecule, composed of 756 amino acids (45). The full-length PIBF protein shows a peri-nuclear localization, (46) and has been identified as a component of the peri-centriolar satellite (47), suggesting its role in cell cycle regulation. Alternative splicing produces several smaller isoforms, which are localized in the cytoplasm (45) and are accountable for the immunological effects of PIBF.

In murine pregnancy, embryo resorption as well as term delivery are associated with the absence or lower expression of the N terminal PIBF exons, which might have important functional consequences (48).

The loss of the N-terminal exons results in a significantly reduced production of the full length protein, and also prevents the synthesis of the smaller protein isoforms, which act on the cytokine pattern and NK activity (45).

THE IMMUNO-MODULATING EFFECTS OF PIBF AND THE MAINTENANCE OF PREGNANCY

PIBF was first described as a 34 kDa protein produced by activated pregnancy lymphocytes (30). It has become evident since, that PIBF might be expressed by various reproductive tissues as well as malignant tumors (49–51). A human study illustrated that trophoblast cells in the placenta could express PIBF proteins of 30, 50, and 90 kDa in first trimester (52).

Several human studies suggest an association between PIBF levels and the outcome of pregnancy. In a prospective cohort study attempting to identify early risk factors for miscarriage, PIBF was one of the factors showing a strong association with miscarriage risk (53). In normal human pregnancy, both serum-and urinary PIBF concentrations increase during gestation, while in women, with miscarriage, or preterm labor, urinary PIBF levels fail to increase (54). Preterm birth was predictable by lower than normal pregnancy PIBF values mesaured at 24–28gestational week (55), but not at 11–13 weeks of gestation (56), suggesting, that predictive value of PIBF determination depends on the interval, between sampling and the onset of labor. In line with this, progestogen-treatment of women with threatened miscarriage corrected the initially low PIBF levels, and in parallel, reduced the miscarriage rate to a similar level of healthy controls (57).

While the full length PIBF has been shown to regulate trophoblast and tumor cell invasiveness (58–60), the smaller isoforms are secreted, bind to the PIBF receptor (39, 61) and via their cytokine-like functions, play a role in the materno-fetal relationship, both in animal models and in humans.

Some of the immunological effects of progesterone, e.g., that on NK activity and cytokine balance, are mediated by PIBF.

Earlier studies showed that in mice PIBF protects pregnancy by controlling NK activity (62). Anti-PIBF treatment of pregnant mice results in increased resorption, which are corrected by simultaneously neutralizing NK activity with anti-NK antibodies (62).

Decidual NK cells, are functionally different from their circulating counterparts. Though decidual NK cells selectively overexpress perforin and granzymes A and B (41, 63), their cytotoxic activity is low. In normal pregnancy decidual NK cells contribute to creating a favorable environment for placentation, implantation and embryo development (64), yet they are equipped with cytotoxic molecules, to fight intrauterine infections (65, 66).

In the day 12 mouse decidua, there is an abundance of PIBF positive granulated cells. These cells are missing from the deciduae of alymphoid mice, but when alymphoid mice are reconstituted of with bone marrow from male BALB/c mice, the PIBF positive granulated cells re-appear in the decidua. These data suggest that the PIBF+ cells belong to the lymphoid lineage, and based on their DBA lectin reactivity, to the group of NK cells.

PIBF+ NK cells contain perforin, which co-localizes with PIBF in the cytoplasmic granules. In day 12.5 normal mouse pregnancy only 54% of the PIBF + decidual NK cells contain perforin, whereas in PIBF deficient mice of the same gestational age, not only do most of the PIBF + NK cells disappear, but all of the remaining ones are perforin positive (28).

This implies that in mice PIBF exerts a pregnancy protective effect by keeping NK activity under restraint.

The local mechanism of the protective action of PIBF is less easily studied in humans, than in animal models. Nevertheless, a recent study showed that the otherwise scarcely studied decidual B cells produce PIBF under the effect of IL-33, and that these PIBF + B cells are missing from the choriodecidual area of women with pre-term labor (67) (Nature).

In spite of their high perforin content, spontaneous cytotoxic activity of human decidual NK cells is moderate (68). Progesterone inhibits human NK cytolytic activity *in vitro* (19), and upregulates HLA-G gene expression (4). Because HLA-G is a ligand for NK inhibitory and activating receptors, upregulation of HLA-G by progesterone might be one of the pathways accounting for the low cytotoxic activity of decidual NK cells.

Decidual NK activity appears to be affected by PIBF. PIBF inhibits upregulation of perforin expression in activated human decidual NK cells and prevents degranulation (69, 70).

Though there is no evidence that NK cells directly attack the trophoblast, recurrent miscarriage is often accompanied by increased decidual NK activity (71–75), suggesting that this mechanism might be a factor in the underlying pathology of repeated pregnancy loss.

It is well-established, that while normally progressing pregnancies are characterized by a Th2 dominant cytokine pattern, an excess of Th1-associated cytokines leads to pregnancy termination (76, 77). In humans, recurrent miscarriages are associated with a Thl-dominant peripheral cytokine profile (78–82).

Both progesterone and PIBF play a role in the induction of the Th2 biased cytokine balance. In the presence of progesterone resting human peripheral blood T cells differentiate into Th2-like clones, furthermore, progesterone treatment of Th1-like T cell clones shifts the cytokine production of these cells toward Th2 (2). Neutralization of endogenous PIBF activity in pregnant mice by specific anti-PIBF antibody terminates pregnancy, reduces the synthesis of IL-10, and increases that of IFN-γ (83).

The PIBF receptor is a glycosylphosphatidylinositol (GPI)-anchored protein, which, for signaling, temporarily associates with the alpha chain of the IL-4 receptor (39, 61). Engagement of the PIBF receptor results in immediate STAT6 activation, whereas, a 24 h incubation with progesterone is needed to phosphorylate STAT6, indicating, that the effect of progesterone on Th2 cytokine production is mediated by PIBF (61) (**Figure 3**).

By signaling via this novel form of the IL-4 receptor (39, 61), PIBF induces increased production of IL-3, IL-4, and IL-10 by activated murine lymphocytes (84).

Raghupathy et al. (78, 79) investigated the production of Th1 and Th2 cytokines by progesterone treated peripheral blood lymphocytes isolated from women with recurrent miscarriage. They showed that progestogen induced PIBF production down-regulates the production of Thl-type cytokines and stimulates the production of Th2-type cytokines. Furthermore, progestogen treatment of women with pre-term delivery induces a Th2 dominant cytokine pattern (78, 79).

Taken together, these data suggest, that by up regulating Th2 type cytokine production and by down-regulating NK activity PIBF affects the immune response in a way, which might have an impact on the foeto-maternal relationship.

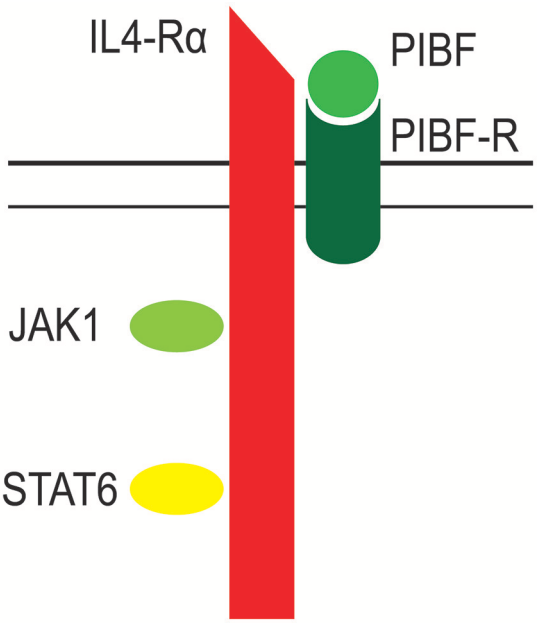


FIGURE 3 | The structure of the PIBF receptor. The receptor for PIBF is a GPI anchored protein. After PIBF binding the receptor associates with the alpha chain of the IL-4 receptor. This initiates intracellular signaling, via the Jak/STAT pathway.

THE PERI-IMPLANTATION EMBRYO COMMUNICATES WITH THE MATERNAL IMMUNE SYSTEM VIA EXTRACELLULAR VESICLES

Earlier studies described a communication between the embryo and the maternal immune system. Embryo culture media were shown to exert an immunosuppressive activity (84). In line with this, incubation of human peripheral lymphocytes with the culture media of fertilized eggs, but not with follicular fluid resulted in increased IL-10 mRNA expression by the lymphocytes (85).

These data suggest that embryo derived signals, can influence the maternal immune response, however, the mechanism of signal transport has not been thoroughly investigated.

In recent years EVs have received much attention. These membrane-coated structures may express phosphatidylserine (PS) in their membrane (86), which reacts with Annexin V. EVs are categorized by their origin and size (87). Exosomes are 30–100 nm, and originate from internalized endocytic vesicles. Microvesicles (100 nm^{-1} μm in diameter), are shed from the plasma membrane by budding, and apoptotic vesicles (1–5 μm in diameter) are released from cells undergoing apoptosis (88).

All types of cells produce EVs which transport various cargos, (including proteins, nucleic acids, and lipids) from one cell to the other. Proteins, e.g., cytokines carried and released by EVs could initiate signaling pathways, and thus alter the biological functions of the target cells (89, 90).

EVs might be considered as candidates for conveying the information from the embryo to the mother. The message carried by EVs has been shown to affect the reproductive process at different points.

EVs have been demonstrated in mouse oocytes (91) as well as in the follicular fluid (92–96) and extra villous trophoblast (97). The tetraspanins CD9 and CD81 expressed by oocyte derived EVs have been suggested to play a role in sperm-oocyte membrane fusion (98–100). Follicular fluid exosomes contain miRNAs, some of them targeting genes that regulate oocyte growth (95) as well as different pathways of reproduction, and endocrine functions (94).

EV—mediated interactions between the endometrium and the blastocyst promote implantation (101). In sheep endometrium, EV production is controlled by progesterone, and endometrium derived EVs were shown to reach the embryo, (102).

EVs from a human uterine epithelial cells express the extracellular matrix metalloprotease inducer (103) which induces the expression of MMPs, thus EVs might also play a role in endometrial remodeling (101, 103, 104).

EVs can be produced by virtually all cell types, however it has been debated, whether a single embryo would be able to produce a detectable amount of EVs. The more so, because the culture medium contains serum or serum albumin, both of which could also be a source of EVs. In a review Tannetta et al. (105) points out the difficulty of measuring EVs in embryo culture medium.

Now there is evidence, that pre-implantation embryos produce EVs both *in vitro* and *in vivo* (106).

Earlier we showed that spent media of *in vitro* cultured human embryos contain a significantly higher number of EVs, than empty media, and the number of nucleic acid containing EVs in day 5 human embryo culture media, might serve as an indicator of embryo competence (106). Other groups have also reported the presence of EVs in embryo culture medium. It is now obvious that embryos release EVs, which are taken up by close by cells (90). Giacomini et al. (107) characterized HLA-G containing EVs isolated from conditioned media from *in vitro* cultured human embryos. EVs were demonstrated in the culture medium of bovine blastocyst and the characteristics of these EVs varied depending on embryo competence (108). Qu et al. (109) showed that the negative effects of culture media replacement during embryo culture are due to the loss of embryo derived EVs, and can be corrected by exosome supplementation. This suggests, that embryo derived EVs do indeed carry molecules that promote normal embryo development.

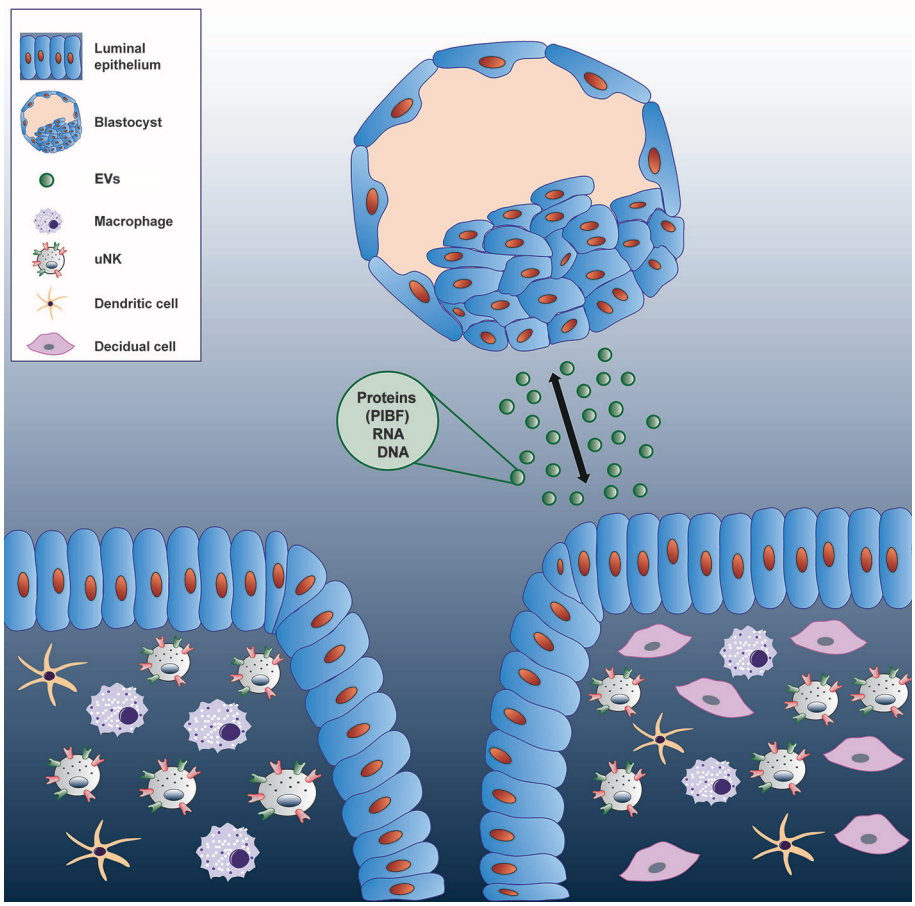


FIGURE 4 | Communication between the embryo and the maternal immune system via extracellular vesicles.

Embryo-derived EVs might also communicate with the maternal immune system by presenting antigens (110, 111), carrying MHC molecules (112–115), or cytokines (116–121). HLA-G-positive EVs isolated from the plasma from healthy term pregnant women have been reported to bind to T lymphocytes (122), and moderately decrease peripheral T lymphocyte STAT3 phosphorylation (122). EVs at the same time can induce pro-inflammatory cytokines and chemokines in primary macrophage cultures (123, 124).

EVs bind to CD8+ and–though to a lesser degree to CD4+ lymphocytes-, via the phosphatidylserine—phosphatidylserine receptor interaction (125). CD4+ and CD8+ cells express similar numbers of phosphatidylserine receptors, therefore, it is likely, that in addition to the phosphatidylserine—phosphatidylserine receptor interaction, other, yet unidentified mechanisms might also be involved in binding of EVs to CD8+ cells. With immuno-electron microscopy we identified PIBF in embryo-derived EVs, and showed that these PIBF containing EVs might affect the immune response (125).

Incubation of murine spleen cells with embryo-derived EVs, increased the number of IL-10+ cells among peripheral CD8+ cells, but not in the CD4+ population. IL-10 producing CD8+ T lymphocytes might moderate antigen-induced inflammatory responses, since these cells have been shown to control influenza virus induced inflammation in the foet (126), and to prevent liver damage during chronic hepatitis C virus infection (127).

Pre-treatment of EVs with an anti-PIBF antibody abrogates the above described effect of the EVs. These data suggest that PIBF transported by the EVs from the embryo to maternal lymphocytes might induce increased IL-10 production by the latter, this way contributing to the Th2 dominant immune responses described during pregnancy. The finding is in line with our earlier data, (83) showing increased IL-10 production of murine spleen cells in the presence of PIBF.

This pathway might have its significance in reproduction. Because embryo derived EVs transport various molecules, - PIBF, among others-, it cannot be ruled out, that these structures act as means of feto-maternal or materno-fetal communication in the peri-implantation period (**Figure 4**).

AUTHOR CONTRIBUTIONS

JS-B wrote the paper. BM-J, SŠ, and JS-B designed and performed the experiments.

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IgG Fc Glycosylation Patterns of Preterm Infants Differ With Gestational Age

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Preterm infants acquire reduced amounts of Immunoglobulin G (IgG) via trans-placental transfer as compared to term infants which might explain their high susceptibility for infections. The reduced amount of IgG antibodies also results in a lower amount of anti-inflammatory Fc N-galactosylated and -sialylated IgG antibodies. This reduction or, even more, a qualitative shift in the type of IgG Fc glycosylation might contribute to the increased risk for sustained inflammatory diseases in preterm infants. It was the aim of our explorative study to investigate the IgG Fc glycosylation patterns in preterm infants of different gestational ages compared to term infants and mothers of preterm infants. In plasma samples of preterm infants ($n = 38$), we investigated IgG concentrations by use of ELISA. Furthermore, we analyzed IgG Fc glycosylation patterns in plasma of preterm infants ($n = 86$, 23–34 weeks of gestation), term infants ($n = 15$) and mothers from preterm infants ($n = 41$) using high performance liquid chromatography. Extremely low gestational age infants (born <28 weeks of gestation during second trimester) had reduced IgG concentrations and decreased proportions of galactosylated (84.5 vs. 88.4%), sialylated (14.5 vs. 17.9%) and bisecting N-acetylglucosamine-containing (8.4 vs. 10.8%) IgG Fc N-linked glycans as compared to preterm infants born ≥28 weeks of gestation (during third trimester) and term infants. Increased non-galactosylated (agalactosylated, 16.9 vs. 10.6%) IgG Fc N-linked glycans were associated with the development of chronic inflammatory bronchopulmonary dysplasia (BPD). However, mothers of preterm infants born during second or third trimester of pregnancy did not show significant differences in IgG Fc glycosylation patterns. Thus, the IgG Fc glycosylation patterns of preterm infants depend on their gestational age. Although lack of bisecting N-acetylglucosamine has been associated with less inflammatory effector functions, the decreased IgG Fc galactosylation and sialylation with lower gestational age suggest a rather pro-inflammatory pattern. The difference in IgG Fc glycosylation patterns between preterm infants and mothers of preterm infants suggests a selective enrichment of IgG glyco forms in preterm infants, which might contribute to or result of the development of sustained inflammatory diseases like BPD.

Keywords: newborn, trans-placental transfer, IgG antibodies, IgG Fc glycosylation, mothers, galactosylation, sialylation, preterm infants

INTRODUCTION

The adaptation of women during pregnancy leads to a feto-maternal immune tolerance, which is disrupted in the settings of preterm birth. Reasons for preterm birth are often associated with rather pro-inflammatory conditions at the feto-maternal interface (e.g., infection, age, stress) (1). With a compromised and immaturely developed immune system, preterm infants are exposed to the extrauterine environment leading to a high susceptibility for infections and sustained lung inflammation, such as bronchopulmonary dysplasia (BPD) of particular very early preterm infants (2–4).

The endogenous Immunoglobulin G (IgG) production in infants starts within 1–3 months after term birth (5). Therefore, specific immune protection of infants against pathogens is provided by the active transport of IgG through the placenta probably exclusively via the neonatal Fc receptor (FcRn) (6–10). However, IgG concentrations of the fetus are largely diminished in the second trimester of pregnancy. Specifically, during week 17–22 of gestation only 5–10% of maternal IgG concentrations are transported via the placenta, which rises to 50% during week 28–32 of gestation (11). Hence, reduced IgG concentrations in preterm infants might contribute to their predisposition for infection (9).

In addition, a resulting reduced total amount of anti-inflammatory galactosylated and sialylated IgG antibodies (12) or, even more, qualitative changes in the type of IgG Fc glycosylation might have an impact on the specific risk profile of preterm infants including inflammation-mediated diseases. IgG Fc glycosylation patterns are characterized as follows: IgG molecules bare an N-glycosylation site on the conserved asparagine at position 297 (N297) of each heavy chain CH2 domain. The glycan is composed of a biantennary core heptasaccharide comprised of four N-acetylglucosamines (GlcNAc) and three mannoses (**Figure 1A**). The core structure can be further modified by addition of fucose, bisecting GlcNAc (bisection), galactose (G), or sialic acid (S) (13, 14).

IgG Fc glycosylation patterns are clinically relevant, as a shift toward more non-galactosylated (agalactosylated; G0) glycans has been linked to inflammation-mediated immune diseases (15–19). Animal models have verified that the absence or presence of galactose and sialic acid themselves can influence the inflammatory properties of IgG, i.e., pro-inflammatory [high proportion of agalactosylated glycan structures; (19, 20)] or anti-inflammatory [high proportion of galactosylated and sialylated glycan structures; (19, 21–27)]. IgG Fc glycosylation patterns without bisecting GlcNAc have been associated with reduced affinity to classical Fcγ receptors and instead less inflammatory conditions (28). The IgG Fc glycosylation pattern thereby influences not only the effector function of antigen-specific IgG in form of immune complexes, but also the immune modulatory effect of total IgG (12, 19, 21–26).

In humans, IgG Fc glycosylation patterns are variable and influenced by several factors, such as genetics, gender, age, and disease state (29–31). During pregnancy, the degree of galactosylation and sialylation of IgG antibodies increases whereas the degree of bisecting GlcNAc slightly decreases (32–36), which may contribute to the tolerance at the feto-maternal interface. Recent data from cord blood samples have confirmed that the proportion of galactosylated and sialylated IgG is higher in infants at term birth compared to older children as expected from the IgG Fc glycosylation pattern of pregnant women (37).

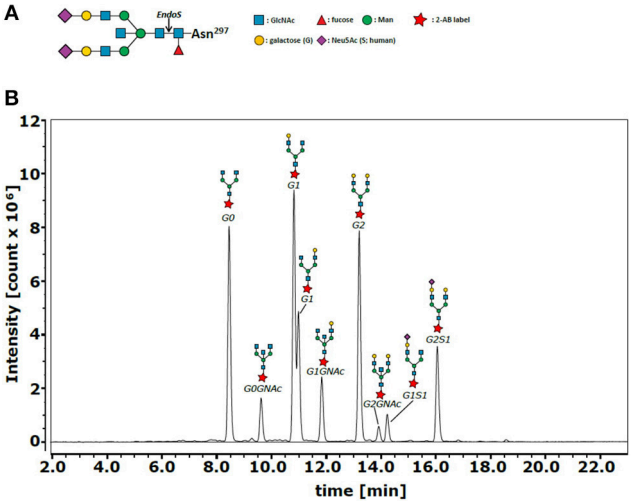


FIGURE 1 | IgG Fc N-glycan structure and representative chromatogram of IgG Fc N-glycans. **(A)** The glycan structure is composed of a biantennary core heptasaccharide comprised of N-acetylglucosamines (GlcNAc or GNAc, blue) and mannoses (Man, green). The core structure can be further modified by addition of fucose (Fuc), bisecting GlcNAc (GNAc, blue), galactose (G, yellow), or sialic acid (Neu5Ac, purple). **(B)** The different Fc N-glycan modifications G0, G0GNAc, G1, G1GNAc, G2, G2GNAc, G1S1, G2S1 and G2S2 (not marked in this sample analysis) were detected using hydrophilic interaction liquid chromatography (representative chromatogram), where glycans with higher hydrophobicity (e.g., more sugar units) were retained longer by the column. Glycan composition of collected fractions containing individual peaks was previously identified by MALDI-TOF analysis (38).

However, the IgG Fc glycosylation patterns of preterm infants, which are highly susceptible to infection and sustained inflammation, have not been studied yet. It was the primary objective of our explorative study to investigate the Fc glycosylation patterns in the context of preterm birth. We hypothesized that IgG Fc glycosylation patterns of preterm infants are comparable to their mother's type of IgG Fc glycosylation (feto-maternal tolerance) or are polarized toward more inflammatory properties, which might contribute to or result of the development of sepsis and sustained lung inflammation.

MATERIALS AND METHODS

Study Cohort

We performed an explorative study in two tertiary care centers for neonates as part of our Immunoregulation of the Newborn (IRoN) study. Samples and clinical data were obtained from infants born between January 1st, 2012 and May 1st, 2015 (center 1; analyzed plasma IgG concentration of infants at mean ± SD 23 ± 2 days of life) and October 1st, 2014 and August

1st, 2017 (center 2; analyzed IgG Fc glycosylation at mean ± SD 32 ± 5 days of life). The inclusion criteria were preterm infants with gestational age ≥23.0 and ≤35.0 weeks without lethal abnormalities, and written informed consent provided by parents or a legal representative. At center 2 (IgG Fc glycosylation), term infants served as controls (blood withdrawal with routine newborn screening at 48–72 h of life). Form mothers of preterm infants born in center 2, we were able to obtain maternal blood from a routine sampling immediately after birth.

Ethics

Written informed consent was obtained from parents on behalf of the infants enrolled into our studies. The study parts were approved by the local committee on research in human subjects at the University of Lübeck (center 2) and by the local committee on research in human subjects at Ärztekammer Bayern (center 1), respectively. All blood samples were obtained within a medically required blood withdrawal procedure. The additional blood volume obtained for research purposes (<1% of whole body blood volume per blood sampling) was in line with current guidelines of the European Medical Agency on the investigation of medicinal products in term and preterm infants; Committee for Medicinal Products for Human Use and Pediatric Committee (39).

Definitions

Gestational age was calculated from the best obstetric estimate based on early prenatal ultrasound and obstetric examination.

Early-onset sepsis (EOS) was defined as sepsis, clinical or culture-proven, occurring within the first 72 h of life.

Late-onset sepsis (LOS) was defined as sepsis, clinical or culture-proven, occurring after the first 72 h of life.

Clinical sepsis was defined as condition when neonatologists decided to treat the infant with antibiotics and continued for at least 5 days due to the following reasons: ≥2 clinical signs of systemic inflammatory response: temperature >38°C or <36.5°C, tachycardia >200/min, new onset or increased frequency of bradycardias or apneas, hyperglycemia >140 mg/dl, base excess <-10 mval/l, changed skin color, increased oxygen need; and at least one laboratory sign: C-reactive protein >10 mg/L, platelet count <100/nl, immature/total neutrophil ratio >0.2, white blood cell count <5/nl (NeoKISS).

Blood culture-confirmed sepsis was defined as clinical sepsis with proof of causative agent in the blood culture.

Necrotizing enterocolitis (NEC) and focal intestinal perforation (FIP) were defined as surgery due to spontaneous intestinal perforation or necrotizing enterocolitis (Bell stage ≥2).

Intraventricular hemorrhage (IVH) was defined according to Papile ultrasound criteria.

Bronchopulmonary dysplasia (BPD) was defined as need for oxygen supplement and/or respiratory support at corrected age of 36 weeks.

Cause of preterm delivery was determined at the discretion of the attending obstetrician, specifically: (1) preterm labor (labor refractory to tocolytic agents) or **amniotic infection syndrome** [AIS; labor ± rupture of membranes, maternal fever (≥39.0°C), and/or one of the following signs: increased maternal inflammatory markers without any other cause (CRP > 10 mg/l or elevation of white blood cell count >15.000/µl), fetal tachycardia, painful uterus and foul-smelling cervical discharge]; (2) pre-eclampsia (pregnancy-induced maternal hypertension, oedema, proteinuria), pathological Doppler (e.g., Arteria umbilicalis Doppler, Ductus venosus flow, Arteria cerebri media Doppler), intrauterine growth restriction as diagnosed by the attending specialist for antenatal ultrasound,

TABLE 1 | Summary of patient demographics from center 1 (IgG quantification).

	<28 weeks	≥28 weeks	Total
n	19	19	38
Gestational age [weeks]	26.4 ± 1.0	30.9 ± 1.9	28.7 ± 2.7
Birth weight [g]	853 ± 160	1261 ± 255	1057 ± 295
Male Gender	11 (58)	8 (42)	19 (50)
Multiple	5 (26)	6 (32)	11 (29)
EOS	0 (0)	0 (0)	0 (0)
LOS	4 (21)	4 (21)	8 (21)
NEC	0 (0)	0 (0)	0 (0)
FIP	1 (5)	0 (0)	1 (3)
IVH	4 (21)	2 (11)	6 (16)
BPD	11 (58)	3 (16)	14 (37)
Cause of delivery			
Preterm labor or AIS	8 (42)	5 (26)	13 (34)
Pathological doppler, placental abruption, pre-eclampsia	8 (42)	8 (42)	16 (42)
Others	3 (16)	6 (32)	9 (24)

EOS, early-onset sepsis; LOS, late-onset sepsis; NEC, necrotizing enterocolitis; FIP, focal intestinal perforation; IVH, intraventricular hemorrhage; BPD, bronchopulmonary dysplasia; AIS, amnion infection syndrome. Data are described as mean ± standard deviation or n (%).

or placental abruption; and (3) others, including cholestasis, etc.

Sample Collection

Peripheral blood (EDTA) samples were stored at room temperature and processed within 24 h after withdrawal. Samples were spun down for 6 min at 1,500 rpm. The clear plasma fraction was transferred and immediately stored at −80°C until further use.

IgG ELISA

A 96 well plate was coated with anti-human IgG-Fc primary antibody (Bethyl, Laboratories, Montgomer, TX). After blocking residual binding sites, plasma dilutions were added. Intravenous immunoglobulin (IVIG, Biotech, Dreieich, Germany) was used as standard. Plasma IgG concentration was detected with horse radish peroxidase-conjugated anti-human-IgG-Fc antibody (Bethyl, Laboratories, Montgomer, TX) and assay developed with TMB substrate (BD Biosciences).

IgG Purification and IgG-Fc Glycan Analysis

Total IgG was purified from human plasma samples using Protein G coupled agarose beads (Genetex, San Antonio, TX) in a 96 well filter plate (Merck, Darmstadt, Germany). In brief, 50 µl settled beads were constituted by washing 3 times with 200 µl

TABLE 2 | Summary of patient demographics from center 2 (IgG Fc glycosylation; infants and mothers of preterm infants).

	<28 weeks	≥28 weeks	Term infants	Total
INFANTS				
n	43	43	15	101
Gestational age [weeks]	26.1 ± 1.2	29.8 ± 1.6	39.6 ± 1.2	29.7 ± 4.7
Birth weight [g]	809 ± 232	1260 ± 229	3220 ± 656	1359 ± 871
Male Gender	26 (60)	18 (42)	8 (53)	52 (51)
Multiple	11 (26)	14 (33)	2 (13)	27 (27)
EOS	13 (30)	6 (14)	1 (7)	20 (20)
LOS	22 (51)	6 (14)	0 (0)	28 (28)
NEC	2 (9)	0 (0)	0 (0)	2 (2)
FIP	4 (9)	0 (0)	0 (0)	4 (4)
IVH	12 (28)	2 (5)	0 (0)	14 (14)
BPD	14 (33)	7 (16)	0 (0)	21 (21)
Cause of delivery				
Preterm labor or AIS	31 (72)	27 (63)	1 (7)	59 (58)
Pathological doppler, placental abruption, pre-eclampsia	6 (14)	13 (30)	1 (7)	20 (20)
Others	6 (14)	3 (7)	13 (87)	22 (22)
MOTHERS				
n	16	25		41
Age of mother at birth [years]	33 ± 6	32 ± 4		32 ± 5
Gestational age [weeks]	25.8 ± 1.6	31.2 ± 2.1		29.1 ± 3.3
Birth weight [g]	756 ± 279	1484 ± 511		1200 ± 562
Male Gender	11 (69)	11 (44)		22 (54)
Multiple	2 (13)	4 (16)		6 (15)
EOS	7 (44)	2 (8)		9 (22)
LOS	9 (56)	1 (4)		10 (24)
NEC	2 (13)	0 (0)		2 (5)
FIP	2 (13)	0 (0)		2 (5)
IVH	4 (25)	0 (0)		4 (10)
BPD	6 (38)	0 (0)		6 (15)
Cause of delivery				
Preterm labor or AIS	9 (56)	14 (56)		23 (56)
Pathological doppler, placental abruption, pre-eclampsia	3 (19)	8 (32)		11 (27)
Others	4 (25)	3 (12)		7 (17)
MOTHER-INFANT-PAIRS				
n	12	8	0	20

EOS, early-onset sepsis; LOS, late-onset sepsis; AIS, amnion infection syndrome; NEC, necrotizing enterocolitis; FIP, focal intestinal perforation; IVH, intraventricular hemorrhage; BPD, bronchopulmonary dysplasia. Data are described as mean ± standard deviation or n (%).

of PBS and applying negative pressure on a vacuum manifold. Protein G was than incubated with 200 µl of a 1:4 plasma dilution (50 µl plasma volume) for 2 h at room temperature with agitation. Unspecific plasma proteins were washed away 5 times with 200 µl of PBS. IgG was eluted three times with 100 µl of 100 mM formic acid (pH 2.5). Elution was neutralized by adding 10 µl of 1 M ammonium bicarbonate into each elution fraction. IVIG (Biotech, Dreieich, Germany) was used as standard.

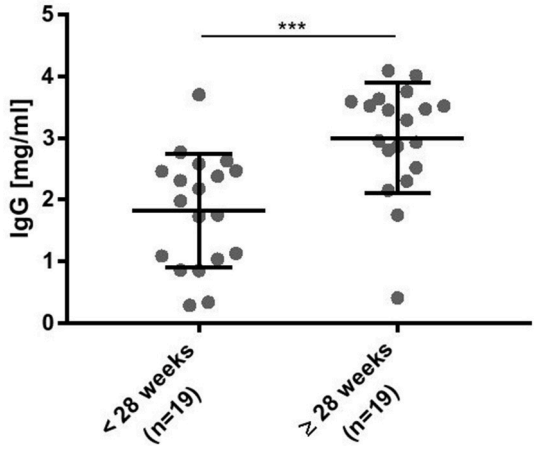


FIGURE 2 | Plasma IgG concentrations were significantly decreased in preterm infants born at lower gestational ages. ELISA data for detection of human IgG-Fc parts. Intravenous immunoglobulin (IVIG) was used as a standard to estimate the plasma IgG concentration (mean ± SD, Mann-Whitney test). ***$p < 0.001$.

From the purified IgG, Fc N-glycan composition was analyzed as previously described (38). Briefly, Fc N-linked glycans were enzymatically released with recombinant endoglycosidase S (EndoS) from *Streptococcus pyogenes*. EndoS hydrolyses specifically the IgG Fc N-glycan of IgG after the first GlcNAc (**Figure 1A**). The N-glycans were purified using self-made graphitized carbon columns (Fisher Scientific, Hampton, NH) and labeled with anthranilamide (Sigma-Aldrich). The labeled glycans were analyzed by hydrophilic interaction liquid chromatography–high performance liquid chromatography (HPLC) on a Dionex Ultimate 3000 (Thermo Fischer Scientific, Waltham, Mass) by using an Xbridge XP BEH Glycan column (1.7 µm, 100 × 2.1 mm i.d.; Waters, Milford, Mass). As previously described (38), glycan composition was identified by MALDI-TOF analysis of collected fractions containing individual peaks. N-glycans with higher hydrophobicity (e.g., more sugar units) were retained longer in the column (**Figure 1B**).

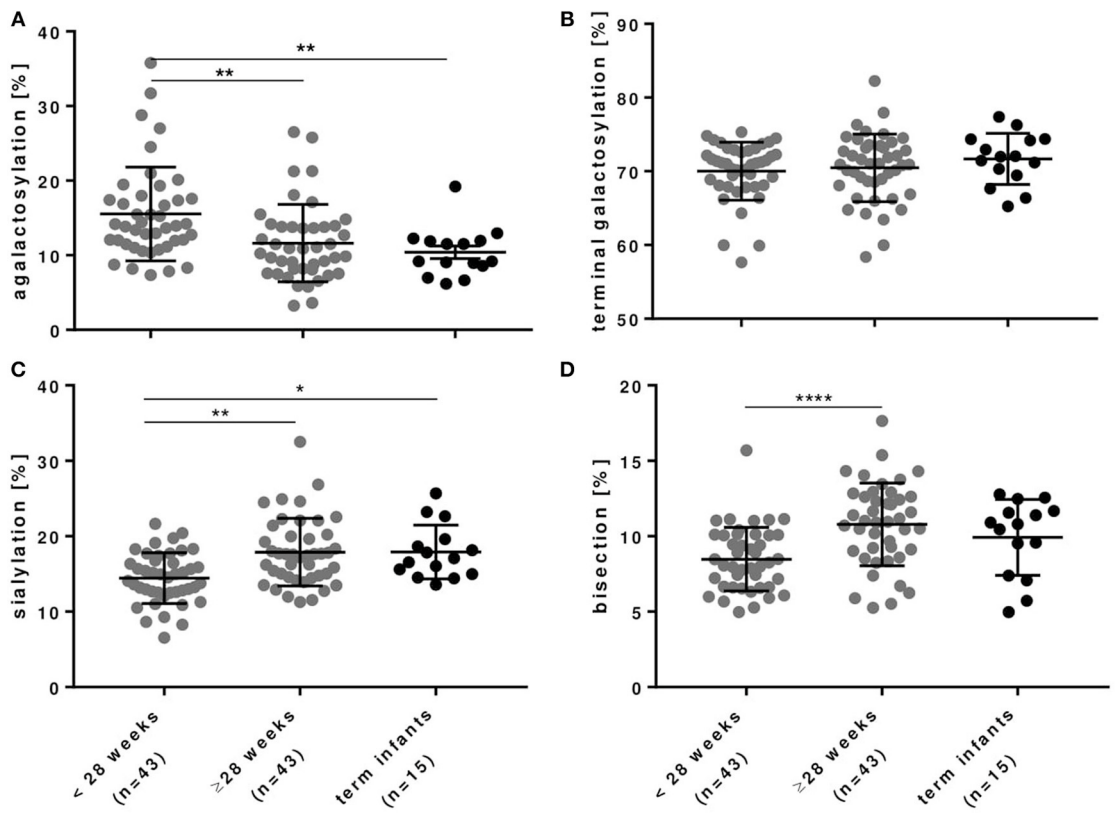


FIGURE 3 | IgG Fc glycosylation in preterm infants is dependent on gestational age. **(A)** IgG with agalactosylated Fc glycans were significantly increased, whereas **(B)** IgG with terminally galactosylated Fc glycans were unchanged and IgG with **(C)** sialylated and **(D)** bisecting Fc glycans were significantly decreased in preterm infants born <28 weeks of gestational age. Term infants served as controls (mean ± SD, Kruskal-Wallis test). *$p < 0.05$, **$p < 0.01$, ***$p < 0.0001$.

The area under the curve (AUC) of the following nine glycan peaks were identified: G0, G0GlcNAc, G1, G1GlcNAc, G2, G2GlcNAc, G1S1, G2S1, and G2S2. Sialylated glycans with bisecting GlcNAc were not detected. The relative proportion of the individual peaks of a sample was calculated by dividing AUC of the individual curve by the AUC of the sum of all nine identified peaks and this multiplied by 100 (for raw data see **Supplementary Tables 1, 2**). For presentation, the four following groups were defined and percentages of the groups calculated as the sum of the percentages of the individual glycan proportions: (1) agalactosylation: G0 ± bisecting GlcNAc; (2) terminal galactosylation: G1 ± bisecting GlcNAc, G2 ± bisecting GlcNAc; (3) sialylation: G1S1, G2S1, G2S2; (4) bisection: G0 + bisecting GlcNAc, G1 + bisecting GlcNAc, G2 + bisecting GlcNAc.

Statistical Analysis

All data were analyzed using GraphPad Prism®version 7. After testing for normal distribution, Kruskal-Wallis test followed by Dunn's multiple comparisons test or the Mann-Whitney test was used for not normally distributed data. Correlations were evaluated using Spearman correlation. Two-way ANOVA followed by Bonferroni's multiple comparison test was done for correlation with clinical parameter. The threshold for

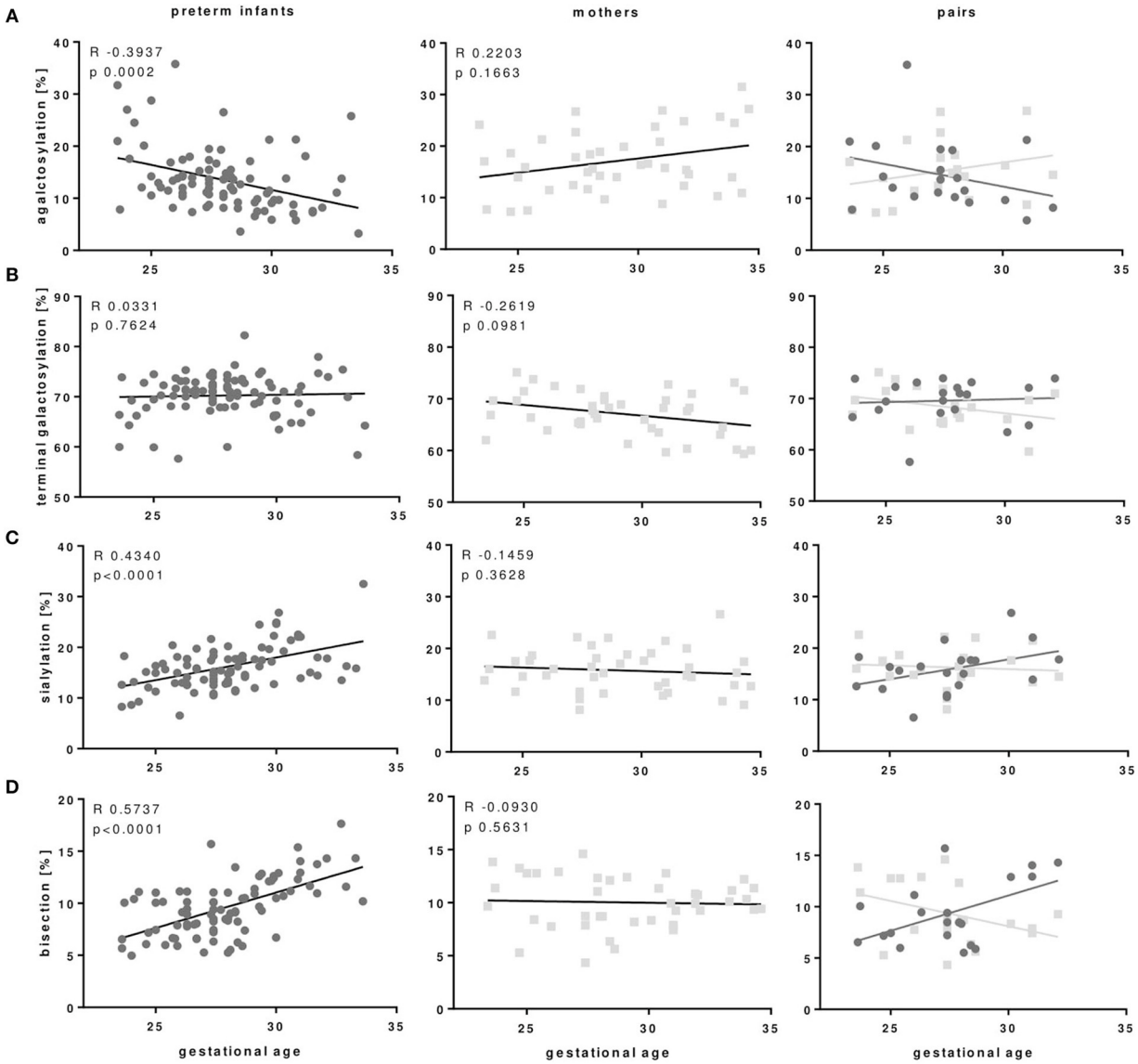


FIGURE 4 | IgG Fc glycosylation patterns of preterm infants but not IgG Fc glycosylation of mothers of preterm infants correlate with gestational age of infants. **(A)** IgG with agalactosylated Fc glycans of preterm infants correlated negatively with gestational age, while Fc agalactosylation of mothers of preterm infants does not correlate with gestational age of their infants. **(C)** Sialylated and **(D)** bisecting Fc glycans correlated positively with gestational age of preterm infants which is not seen in Fc glycosylation of mothers from preterm infants. Terminal galactosylation **(B)** showed no correlation with gestational age in both preterm infants and mothers (Preterm infants: $n = 86$, mothers: $n = 41$, mother-infant-pairs: $n = 20$, Spearman correlation).

significance was a p-value < 0.05 depicted as *, < 0.01 as **, < 0.001 as ***, and <0.0001 as ****.

RESULTS

Clinical Characteristics

We recruited a cohort of preterm infants in two centers and collected plasma from peripheral blood samples and key clinical outcome parameters. In samples of center 1 we analyzed plasma IgG concentrations and in samples of center 2 we investigated IgG Fc glycosylation. To analyze whether plasma IgG concentrations and Fc glycosylation patterns differ with gestational age, we divided the cohort in 2 gestational age groups (center 1/center 2: ≥23(+0 days) and ≤27(+6) weeks, $n = 19/n = 43$; ≥28(+0) and ≤34(+6) weeks, $n = 19/n = 43$; **Tables 1**, **2**).

The cohort of center 1 (plasma IgG concentration) was also selected for a matched pair analysis of BPD diagnosis vs. no BPD. For this matched pair analysis early onset sepsis cases were excluded, and late onset sepsis cases were equal in both groups. In center 2, we also analyzed IgG Fc glycosylation patterns of term infants ($n = 15$) and mothers of preterm infants ($n = 41$) (**Table 2**). For a subgroup of preterm infants and mothers from center 2, we were able to analyze mother-infant-pairs ($n = 20$).

TABLE 3 | IgG Fc glycosylation patterns in infants of different gestational age groups depicted in **Figure 3** (mean ± SD).

	<28 weeks	≥28 weeks	Term infants
Agalactosylation (%)	15.5 ± 6.3	11.6 ± 5.2	10.4 ± 3.3
Galactosylation (%)	84.5 ± 6.3	88.4 ± 5.2	89.6 ± 3.3
Terminal galactosylation (%)	70.0 ± 3.9	70.5 ± 4.6	71.7 ± 3.5
Sialylation (%)	14.5 ± 3.4	17.9 ± 4.5	17.9 ± 3.6
Bisection (%)	8.4 ± 2.1	10.8 ± 2.7	9.9 ± 2.5

Plasma IgG Concentrations Were Decreased in Preterm Infants Born at Lower Gestational Ages

As outlined in **Figure 2**, IgG was detectable in infants born <28 weeks of gestational age and increased significantly with

TABLE 4 | IgG Fc glycosylation patterns of preterm infants associated with (i) gender and clinical parameters defining (ii) cause of birth, (iii) bronchopulmonary dysplasia (BPD) or (iv) sepsis (early and late onset sepsis cases included) (mean ± SD, two-way ANOVA, multiple comparison within group to adjust for gestational age).

	<28 weeks		≥28 weeks	
(i)	**Male ($n = 26$)**	**Female ($n = 17$)**	**Male ($n = 18$)**	**Female ($n = 25$)**
Agalactosylation (%)	15.0 ± 6.5	16.3 ± 6.1	12.4 ± 6.3	11.1 ± 4.2
Galactosylation (%)	85.0 ± 6.5	83.7 ± 6.1	87.6 ± 6.3	88.9 ± 4.2
Terminal galactosylation (%)	70.3 ± 3.7	69.6 ± 4.3	70.9 ± 5.5	70.1 ± 3.8
Sialylation (%)	14.7 ± 3.5	14.1 ± 3.2	16.7 ± 4.0	18.8 ± 4.7
Bisection (%)	8.8 ± 2.3	8.0 ± 1.8	11.1 ± 3.4	10.6 ± 2.2
(ii)	**Preterm labor or AIS ($n = 31$)**	**All other causes ($n = 12$)**	**Preterm labor or AIS ($n = 27$)**	**All other causes ($n = 16$)**
Agalactosylation (%)	15.2 ± 5.6	16.4 ± 8.0	11.3 ± 4.0	12.2 ± 6.9
Galactosylation (%)	84.8 ± 5.6	83.6 ± 8.0	88.7 ± 4.0	87.8 ± 6.9
Terminal galactosylation (%)	70.0 ± 3.4	70.0 ± 5.3	71.2 ± 3.7	69.3 ± 5.8
Sialylation (%)	14.8 ± 3.0	13.6 ± 4.0	17.5 ± 4.1	18.6 ± 5.1
Bisection (%)	8.3 ± 1.8	9.0 ± 2.8	9.8 ± 2.3 ***	12.5 ± 2.7
(iii)	**BPD ($n = 14$)**	**No BPD ($n = 29$)**	**BPD ($n = 7$)**	**No BPD ($n = 36$)**
Agalactosylation (%)	17.0 ± 6.4	14.8 ± 6.2	16.9 ± 8.1*	10.6 ± 3.8
Galactosylation (%)	83.0 ± 6.4	85.2 ± 6.2	83.1 ± 8.1*	89.4 ± 3.8
Terminal galactosylation (%)	69.5 ± 4.2	70.3 ± 3.8	66.6 ± 5.4	71.2 ± 4.1
Sialylation (%)	13.5 ± 2.9	14.9 ± 3.5	16.5 ± 4.6	18.2 ± 4.5
Bisection (%)	8.4 ± 2.2	8.5 ± 2.1	10.9 ± 2.5	10.8 ± 2.8
(iv)	**Sepsis ($n = 27$)**	**No sepsis ($n = 16$)**	**Sepsis ($n = 11$)**	**No sepsis ($n = 32$)**
Agalactosylation (%)	16.6 ± 7.2	13.8 ± 3.9	14.8 ± 7.3	10.5 ± 3.8
Galactosylation (%)	83.4 ± 7.2	86.2 ± 3.9	85.2 ± 7.3	89.5 ± 3.8
Terminal galactosylation (%)	69.5 ± 4.6	70.8 ± 2.4	68.8 ± 5.5	71.1 ± 4.2
Sialylation (%)	13.9 ± 3.5	15.4 ± 3.0	16.4 ± 3.7	18.4 ± 4.7
Bisection (%)	8.7 ± 2.4	8.2 ± 1.6	12.1 ± 2.1	10.4 ± 2.8

*IgG Fc agalactosylation is additionally depicted in **Figure 5**. *$p < 0.05$, ***$p < 0.001$.*

gestational age. Plasma IgG concentrations were not different in a matched pair analysis of preterm infants developing BPD vs. no BPD diagnosis (data not shown).

IgG Fc Glycosylation Patterns Depend on Gestational Age of Preterm Infants

As outlined in **Figures 3**, **4** and **Table 3**, preterm infants had a higher proportion of agalactosylated (accordingly lower proportion of galactosylated) IgG Fc N-glycans with decreasing gestational age ($R = -0.3937$, $p = 0.0002$). No difference in agalactosylated IgG Fc glycans was observed between infants born ≥28 weeks of gestation and term controls. Further, no correlation between terminal galactosylation of IgG and gestational age in preterm infants was found ($R = 0.0331$, $p = 0.76$). However, also IgG Fc sialylation was significantly lower in preterm infants born <28 weeks as compared to infants born ≥28 weeks of gestation and term infants (correlation with gestational age; $R = 0.4340$, $p < 0.0001$). The proportion of IgG glycans with bisecting GlcNAc also correlated with gestational age ($R = 0.5737$, $p < 0.0001$).

IgG Fc Glycosylation Shows Differences Between Preterm Infants and Mothers of Preterm Infants

In the comparison of maternal with neonatal IgG Fc glycosylation patterns we did not find the same correlation between IgG Fc glycosylation patterns in mothers of preterm infants with the gestational age of their infants which we observed between the IgG Fc glycosylation patterns of preterm infants with their gestational age (**Figure 4**).

Clinical Correlation of IgG Fc Glycosylation in Preterm Infants

For the cause of preterm delivery bisecting IgG Fc glycosylation was significantly decreased in preterm infants born ≥28 weeks of gestation with preterm labor or amnion infection syndrome (AIS) as compared to other preterm delivery reasons (**Table 4**).

For the development of BPD, preterm infants born < or ≥28 weeks of gestation had respectively, a tending or significant increased proportion of agalactosylated IgG as compared to preterm infants without BPD (**Figure 5** and **Table 4**). A similar

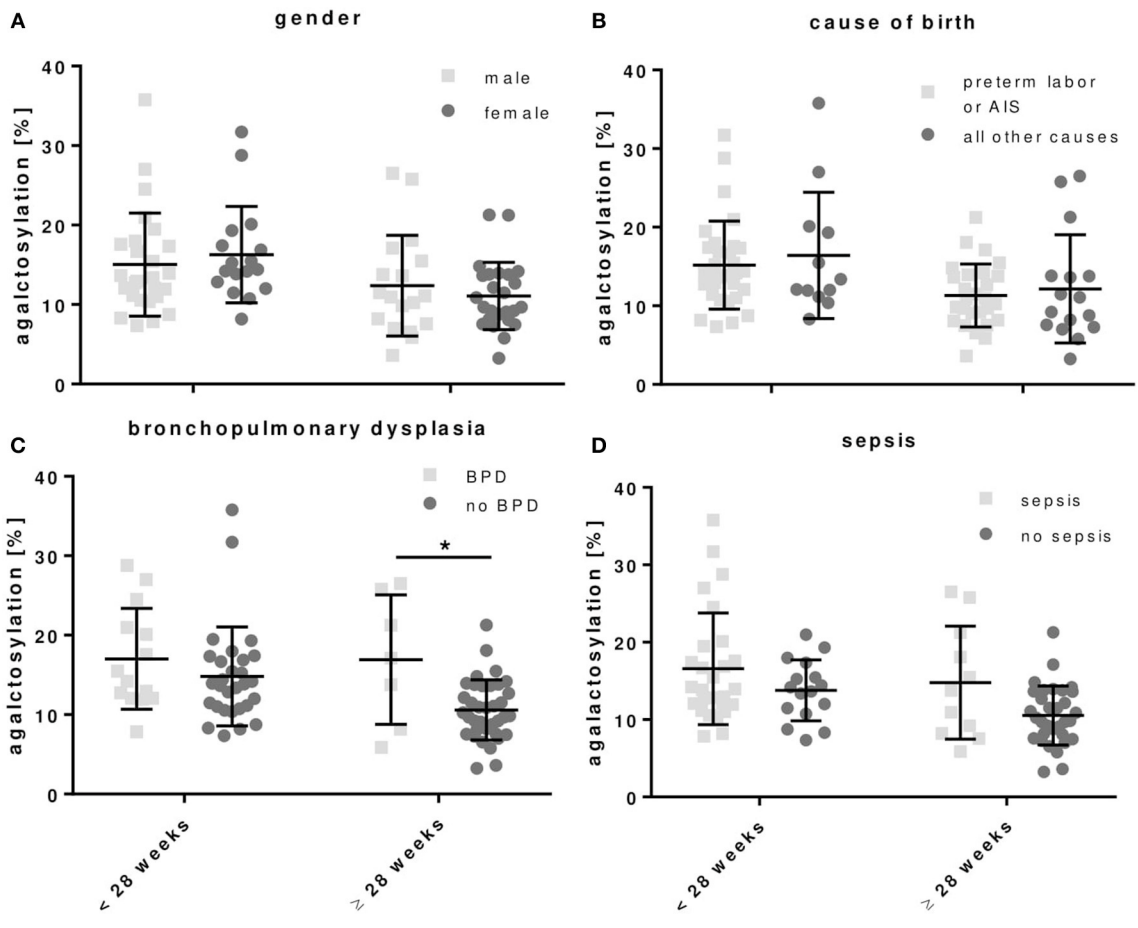


FIGURE 5 | Agalactosylated IgG Fc N-glycans show a tendency to increase in preterm infants developing bronchopulmonary dysplasia (BPD). The correlation was done with (i) gender and clinical parameters defining, (ii) cause of birth, (iii) BPD, and (iv) sepsis (early and late onset sepsis cases included) for all preterm infant groups (mean ± SD, two-way ANOVA, multiple comparison within groups to adjust for gestational age). *$p < 0.05$.

tendency, which was however not significant, was observed in preterm infants with sepsis (**Figure 5** and **Table 4**). No differences in IgG Fc glycosylation were found for the gender (**Figure 5** and **Table 4**).

DISCUSSION

To our knowledge, this is the first explorative study investigating IgG Fc glycosylation patterns in peripheral blood of preterm infants. We noted that IgG Fc glycosylation patterns of preterm infants differ with gestational age whereas the patterns of mothers, as described in the literature (32, 33), did not differ with gestational age of their offspring. Preterm infants born <28 weeks of gestation displayed an IgG Fc glycosylation pattern characterized by a higher proportion of agalactosylated IgG (reduced proportion of galactosylated IgG) and a reduced proportion of sialylated and bisecting IgG. The pattern of Fc glycosylation from mothers, however, did not differ between the second and third trimester of pregnancy suggesting an enrichment of IgG glyco forms in extremely low gestational age infants. This novel finding needs further prospective in-depth-analysis, how these IgG Fc glyco forms are enriched in the smallest, most vulnerable infants, whether they contribute to or are a result of dysregulated immune responses and sustained inflammation like BPD, and how they function.

We were able to confirm that IgG antibodies are transported through the placenta during second trimester of pregnancy, but in a much less amount in infants born <28 weeks of gestation as compared to infants born ≥28 weeks of gestation. In our setting, IgG concentration of preterm infants ≥28 weeks of gestation were still below the levels of term born infants (clinical reference values for infants between 1 and 3 months of age are 2.5–7.5 mg/ml IgG, Immunology laboratory of the medical clinic in Freiburg, Germany, 2007).

Our current understanding of IgG Fc glycosylation patterns and its functional role for the immune system is that different Fc N-linked glycan structures can influence the inflammatory properties of IgG antibodies. Galactosylation and sialylation of total serum IgG but also of antigen-specific IgG in form of immune complexes were shown to mediate more anti-inflammatory effector functions (12, 19, 21–27) whereas a shift toward more agalactosylated and bisecting glycan structures has been associated with pro-inflammatory effects (19, 20, 28). IgG glycosylation of pregnant women in the Fc part was shown to be similar during second and third trimester of pregnancy and directed toward an anti-inflammatory pattern as compared to non-pregnant woman (32, 33). This pattern was also previously found in cord blood samples of term infants (35, 37) and was suggested to contribute to the condition of feto-maternal tolerance and permissive microbiota establishment after birth. In the context of preterm birth, however, the IgG Fc glycosylation patterns are different between infants born during second and third trimester and it is yet unknown whether immune dysregulation derives from a pro-inflammatory setting, i.e., reduced galactosylation and sialylation, or from an anti-inflammatory effect due to reduced bisection leading to lower affinities to activating Fcγ receptors. For this reason, functional studies are needed determining which IgG Fc glycosylation pattern is dominant. We showed that reduced bisection was associated with birth pathology, especially preterm labor and amnion infection syndrome (AIS) suggesting a role of Fc glycosylation patterns for preterm birth. Additionally, we showed a trend of increased IgG Fc agalactosylation in inflammatory-mediated diseases of preterm infants (i.e., chronic lung disease) supporting the hypothesis of a pro-inflammatory effect.

Since IgG of infants in the first month of life are mainly of maternal origin (5), several studies investigated the role of trans-placental transfer through FcRn to be selective for certain Fc glycosylation. *In vitro*, FcRn has a higher affinity to galactosylated IgG (40). A few studies showed a transfer of more galactosylated IgG to infants born at term by comparing IgG glycosylation in healthy pregnant women and umbilical cord blood of term infants (35, 41, 42). In another study, a similar IgG Fc glycosylation pattern between maternal sample and cord blood was reported (43). In our setting, IgG Fc glycosylation patterns in peripheral blood revealed a difference between preterm infants born during second and third trimester compared to IgG Fc glycosylation of mothers from preterm infants which is not changing over the second and third trimester. The reduced galactosylation, sialylation and bisection in extremely preterm infants might reflect the enrichment of certain IgG glyco forms at lower gestation. Since endogenous IgG production slowly starts at 1–3 months (5), we assumed that detected IgG in preterm infants is still of maternal origin. Several mechanisms for the enrichment of the described IgG glyco forms in preterm infants born <28 weeks of gestation might be possible. First, a selective IgG transport via the placenta might play a role in early stages of pregnancy. Second, trimming (44) or extracellular modification (45) of the sugars from IgG Fc glycans in the circulation of early preterm infants might result in a dominance of the detected IgG Fc glycosylation pattern. Third, IgG in breast milk might modify the IgG Fc glycosylation pattern also it only contains about 0.01–0.06 mg/ml in women from western countries (46).

Taken together, our findings suggest an enrichment of certain IgG glyco forms in extremely low gestational age infants and that a high proportion of "pro-inflammatory" agalactosylated IgG Fc N-glycans in those infants might contribute to or result from inflammation-mediated diseases. However, IgG Fc agalactosylation was not significantly associated with the diagnosis of BPD or sepsis in extremely preterm infants, only with the diagnosis of BPD in preterm infants born ≥28 weeks of gestation. Future studies need to evaluate larger cohorts using multivariable linear regression accounting for possible confounding factors. Furthermore, functional *in vitro* and *in vivo* studies are needed to reveal consequences for effector functions of IgG glycosylation pattern in term and preterm infants.

The use of peripheral blood from infants in contrast to umbilical cord blood has the advantage not to be contaminated with maternal blood in the context of clinical sampling. Despite the limited sample volumes, we obtained peripheral blood samples of highly vulnerable infants. Our data are hypothesis-generating and add another component to the discussion of intravenous substitution of pooled immunoglobulins

(IVIg) to preterm infants. Large trials have shown no benefit (INIS trial) with regard to sepsis risk or neurodevelopmental outcome (47), but to e.g., reduction of the inflammatory cytokine IL-6 (48). However, the role of IgG Fc glycosylation has never been reflected in the context of IVIg treatment in preterm infants and also not in the context of BPD. The effect might be beneficial in sustained inflammation of preterm infants, if the intravenous immunoglobulins would be immunomodulatory with higher proportions of galactosylation and sialylation, since this would simulate more closely the measured IgG glycosylation patterns in term infants and might mediate anti-inflammatory effects in preterm infants.

In summary, our data suggest that IgG Fc glycosylation patterns differ in preterm infants and their mothers suggesting an enrichment of certain IgG glyco forms in early stages of gestation. Further investigations with a larger cohort are needed to determine the enrichment and functional role of the observed IgG Fc glycosylation pattern in early preterm infants and to verify its correlation with sustained inflammation of preterm infants.

AUTHOR CONTRIBUTIONS

NT, YB, JP, AH, ME, and CH contributed conception and design of the study. CH and CW provided clinical samples. YB performed experiments and HPLC analysis. NT correlated HPLC analysis with clinical data and performed statistical analysis. NT and YB wrote first draft of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.03166/full#supplementary-material

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HIF-1α-Deficiency in Myeloid Cells Leads to a Disturbed Accumulation of Myeloid Derived Suppressor Cells (MDSC) During Pregnancy and to an Increased Abortion Rate in Mice

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Abortions are the most important reason for unintentional childlessness. During pregnancy, maternal immune cells are in close contact to cells of the semi-allogeneic fetus. Dysregulation of the maternal immune system leading to defective adaptation to pregnancy often plays a role in pathogenesis of abortions. Myeloid-derived suppressor cells (MDSC) are myeloid cells that suppress functions of other immune cells, especially T-cells, thereby negatively affecting diseases such as cancer, sepsis or trauma. They seem, however, also necessary for maintenance of maternal-fetal tolerance. Mechanisms regulating MDSC expansion and function during pregnancy are only incompletely understood. In tumor environment, hypoxia is crucial for MDSC accumulation and activation. Hypoxia is also important for early placenta and embryo development. Effects of hypoxia are mediated through hypoxia-inducible factor 1α (HIF-1α). In the present study we aimed to examine the role of HIF-1α in myeloid cells for MDSC accumulation and MDSC function during pregnancy and for pregnancy outcome. We therefore used a mouse model with targeted deletion of HIF-1α in myeloid cells (myeloid HIF-KO) and analyzed blood, spleens and uteri of pregnant mice at gestational day E 10.5 in comparison to non-pregnant animals and wildtype (WT) animals. Further we analyzed pregnancy success by determining rates of failed implantation and abortion in WT and myeloid HIF-KO animals. We found that myeloid HIF-KO in mice led to an abrogated MDSC accumulation in the pregnant uterus and to impaired suppressive activity of MDSC. While expression of chemokine receptors and integrins on MDSC was not affected by HIF-1α, myeloid HIF-KO led to increased apoptosis rates of MDSC in the uterus. Myeloid-HIF-KO resulted in increased proportions of non-pregnant animals after positive vaginal plug and increased abortion rates, suggesting that activation of HIF-1α dependent pathways in MDSC are important for maintenance of pregnancy.

Keywords: HIF, pregnancy, MDSC, abortion, apoptosis

INTRODUCTION

Abortions are one of the most important pregnancy complication; at least 25%, probably up to 50% of women are affected (1). Recurrent Abortions (RA) are defined as three or more consecutive abortions and affect about 1-3% of couples of childbearing age (2). Besides chromosomal abnormalities, anatomic anomalies and infections, a dysregulation of the immune system seems to play an important role (1, 2). During pregnancy, maternal immune cells and cells of the semi-allogeneic fetus are in close contact. To avoid rejection of the fetus, the maternal immune system has tightly to be regulated (3). Mechanisms inducing maternal-fetal tolerance during pregnancy and those leading to complications like recurrent abortions are incompletely understood.

Myeloid derived suppressor cells (MDSC) are myeloid cells with suppressive activity on other immune cells, especially on T-cells (4). Depending on their phenotype, they can be sub-grouped in two populations – monocytic MDSC (MO-MDSC) and granulocytic MDSC (GR-MDSC). In mice, MDSC can be identified by co-expression of the myeloid lineage differentiation antigen Gr-1 and CD11b. MO-MDSC and GR-MDSC can further be differentiated by the expression of the two epitopes of Gr-1, i.e., Ly6G and Ly6C; MO-MDSC are defined as $CD11b^{+}/Ly6G^{-}/Ly6C^{high}$ and GR-MDSC as $CD11b^{+}/Ly6G^{+}/Ly6C^{low}$ (5, 6). Primarily, accumulation of MDSC has been described in cancer patients (7, 8) and tumor bearing mice (5), where they inhibit T-cell response against the tumor leading to disease progression. Later, we and others could show that MDSC and especially GR-MDSC also accumulate during pregnancy in the fetal and maternal organism and that their immune-modulatory properties may be crucial for maternal-fetal tolerance (9–13) and immune adaptation of the newborn (14–17). However, only little is known about mechanisms leading to MDSC-accumulation during pregnancy.

Hypoxia plays an important role in normal placental development as well as in pathogenesis of placental pathologies (18, 19). Hypoxia-inducible factor 1 (HIF-1) is a key regulator of the response to hypoxia, initiating transcription of various genes. HIF-1 is a heterodimer consisting of the subunits HIF-1α and HIF-1β; HIF-1β is constitutively expressed, while expression of HIF-1α is induced under hypoxic conditions (20). During early gestation, HIF-1α is highly expressed in the placenta, which is characterized by low oxygen pressure (21). In the context of malignancies HIF-1α may be critical for MDSC-activation in the hypoxic environment of tumors (22–24). Until now, nothing is known about the role of HIF-1α for MDSC accumulation and activation during pregnancy.

In the present study, we evaluated the impact of HIF-1α on MDSC accumulation and activation during pregnancy in a mouse model of HIF-1α deficiency in myeloid cells. We found that HIF-1α deficiency in myeloid cells (myeloid HIF-KO) (1) led to a diminished accumulation of MDSC during pregnancy, especially in the uterus of pregnant mice, (2) MDSC from myeloid HIF-KO mice had lower suppressive activity than wildtype (WT) MDSC, (3) MDSC from myeloid HIF-KO mice exhibited higher apoptosis rates and (4) myeloid HIF-KO mice had increased abortion rates compared to WT animals.

Taken together, we describe a role of HIF-1α for MDSC accumulation and function during pregnancy and for pregnancy maintenance. Disturbed activation of HIF-1α and resulting alterations in MDSC homeostasis during pregnancy may be a yet unknown mechanism for immunological pregnancy complications.

Abbreviations: BM, bone marrow; GR-MDSC, granulocytic MDSC; HIF-1α, hypoxia inducible factor 1 α; HIF-KO, HIF-1α knockout; MDSC, myeloid derived suppressor cells; MO-MDSC, monocytic MDSC; NK, natural killer; WT, wildtype.

METHODS

Mice

$HIF\text{-}1\alpha^{flox}$ (B6.129-$Hif1a^{tm3Rsjo}$/J) mice and LysMcre (B6.129P2-$Lyzs^{tm1(cre)Ifo}$/J) mice were obtained from The Jackson Laboratory (Bar Harbor, Maine, USA). $HIF\text{-}1\alpha^{flox}$ mice and LysMcre mice were crossed to get animals double homozygous for $HIF\text{-}1\alpha^{flox}$ and LysMcre, carrying a deletion of HIF-1α in myeloid cells ($HIF\text{-}1\alpha^{flox}$/LysMre, myeloid HIF-KO). C57BL/6J (WT) mice were also obtained from The Jackson Laboratory. All animals were maintained under pathogen-free conditions in the research animal facility of Tuebingen University, Tuebingen, Germany. All experimental animal procedures were conducted according to German federal and state regulations.

Syngeneic matings of myeloid HIF-KO and WT mice were set up at 8-12 weeks of age. Gestational ages were determined by visualizing the presence of a vaginal plug (E0.5 = vaginal plug day).

Abortion rates were determined by visual inspection of fetal-placental units and defined as ratio between resorbing units and total implantation sites. Resorbing units were either dark, small and necrotic or pale, small and with no visible fetus inside the amniotic cavity.

Tissue Collection and Single Cell Preparations

Non-pregnant and pregnant mice at gestational age E10.5 were euthanized by CO_2 inhalation. Blood (0.5–1 ml) was collected by intracardial puncture immediately after death and placed into EDTA-tubes. Red blood cells were removed from whole blood by Ammonium chloride lysis. Spleens were removed and tissue was pushed through a 100 μm filter (Greiner bio-one, Frickenhausen, Germany) using a syringe plunger. Red blood cells were removed by Ammonium chloride lysis and the resulting cell suspension was then passed again through a 40 μm filter (Greiner bio-one, Frickenhausen, Germany) Uterine horns were removed in toto. The fetuses and the fetal part of placenta were dissected from the uteri and blood vessels were removed. Uteri were then placed into PBS, minced into ∼1 mm^3 pieces and pushed through a 40 μm filter. All cell suspensions were then adjusted to 1–4 × 10^6 cells/ml in PBS.

In-vitro Generation of MDSC

in-vitro generation of MDSC was performed according to previously established protocols (25, 26). For *in-vitro* generation

of MDSC non-pregnant WT and myeloid HIF-KO mice were euthanized and femora removed. Bone marrow was collected by rinsing the bones with PBS with a syringe and a 25G needle. Bone marrow cells were then washed, adjusted to $3x10^5$ cells/ml and cultured for 72 h at 37°C in culture medium [Dulbecco's modified eagle medium, DMEM (Thermo Fisher Scientific, Darmstadt, Germany), supplemented with 10% fetal calf serum (FCS, Biochrom, Berlin, Germany) and 1% Penicilline/Streptomycin (Biochrom, Berlin, Germany)] supplemented with 100 ng/ml recombinant murine G-CSF (Peprotech, Hamburg, Germany) and 12.5 ng/ml recombinant murine GM-CSF (Peprotech, Hamburg, Germany). After 72 h of culture non-adherent cells were removed and adherent MDSC detached with trypsin (Biochrom GmbH, Berlin, Germany). >90% of cells were Gr-1^+/CD11b^+ as determined by flow cytometry, thereby exhibiting surface characteristics of MDSC.

Cell Isolation and Flow Cytometry

For isolation of $CD4^+$ from splenocytes, cells were labeled with T-cell Biotin-Antibody Cocktail followed by two sequential Anti-Biotin magnetic bead separation steps (Miltenyi Biotec, Bergisch-Gladbach, Germany) according to the manufacturer's instructions. Purity of $CD4^+$ T-cells after separation was >90%, as assessed by flow cytometry.

For extracellular staining, freshly isolated cells were washed in washing buffer [PBS with 0.1% bovine serum albumin (BSA)] and FcRs were blocked with purified anti-CD16/32 (clone 2.4G2) for 10 min. Then, fluorescent-conjugated extracellular antibodies were added. Antibodies were purchased from BD biosciences [CD3 (145-2C11), CD4 (RM4-5), CD8a (53-6.7), CD11b (M1/79), CD19 (1D3), CD45 (30-F11), NK1.1 (PK136), Gr-1 (RB6-8C5), Ly-6C (AL-21), Ly-6G (1A8), CXCR5 (2G8), annexin V] and R&D systems [CXCR1 (FAB8628P), CXCR2 (FAB2164C), CXCR4 (FAB21651C), CXC3CR1 (FAB5825P), IL4-Rα (FAB530P), Integrin-α4 (FAB2450P) Integrin-β2 (FAB2618P), L-Selectin (FAB5761P)]. For immune cell quantification, cells were pre-gated to CD45. Among $CD45^+$ cells, cell types were identified as follows: T-cells $CD3^+$, T-Helper cells $CD3^+$/CD4+, cytotoxic T-cells $CD3^+$/$CD8^+$ B-cells $CD3^-$/$CD19^+$, NK-cells $CD3^-$/$NK1.1^+$, MDSC CD11b^+/Gr-1^+, MO-MDSC CD11b^+/Ly6C^+/Ly6G^-, GR-MDSC CD11b^+/Ly6C^{low}/Ly6G^+, monocytes CD11b^+/Gr-1^-.

Data acquisition was performed with a FACScalibur flow cytometer (BD Bioscience) and analyzed via CellQuest (BD Biosciences).

T-Cell Suppression Assay

Freshly isolated $CD4^+$ splenocytes were stained with carboxyfluorescein-succinimidyl ester (CFSE, Invitrogen, Heidelberg, Germany) according to the manufacturer's instructions. Cells were suspended in RPMI 1640 media containing 1% penicillin/streptomycin and 10% FCS. CFSE-labeled $CD4^+$ T-cells (2×10^5) suspended in 100 µl media were stimulated with 2×10^5 mouse T-Activator CD3/CD28 Dynabeads (Thermo Fisher Scientific, Dreieich, Germany) and 50 ng recombinant murine Interleukin-2 (rmIL-2, R&D Systems, Wiesbaden-Nordenstadt, Germany). *In-vitro* generated MDSC also suspended in RPMI 1640 containing 1% penicillin/streptomycin and 10% FCS were added in different ratios (1:1, 1:2 and 1:4). After 5 days of culture, $CD4^+$ T-cell proliferation was determined by CFSE dye dilution by flow cytometry. Proliferation index, defined as the ratio of $CD4^+$ T-cell proliferation after addition of MDSC and $CD4^+$ T-cell proliferation without MDSC, was determined. $CD4^+$ T-cell proliferation without MDSC was set to a fixed value of 1.

Statistical Analysis

Statistical analysis was done using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA). Data were analyzed for Gaussian distribution using D'Agostino and Pearson omnibus normality test. Immune cell quantification experiments were analyzed using the Kruskal Wallis test and Dunn's multiple comparison test. T-cell suppression was analyzed using the Wilcoxon matched pairs signed rank test. Expression of surface receptors and apoptosis rates were analyzed using the Mann-Whitney test. Data analyzing pregnancy success (rate of pregnant animals after positive vaginal plug and rate of animals with at least one abortion) were analyzed by Fishers exact test. Abortion rate was analyzed by Mann-Whitney test. Normally distributed data were analyzed using paired *t*-test, not normally distributed data were evaluated using the Wilcoxon matched pairs signed rank test. A p-value < 0.05 was considered as statistically significant.

RESULTS

HIF-1α Deficiency in Myeloid Cells Leads to a Diminished Accumulation of MDSC in the Pregnant Uterus

HIF-1α has been shown to be essential for MDSC function in the tumor environment (22). Therefore, we first asked whether HIF-1α plays a role for MDSC accumulation also during pregnancy. We analyzed expression of MDSC in blood, spleens and uteri of non-pregnant and pregnant WT mice at embryonic day 10.5 (E10.5) in comparison to myeloid HIF-KO mice. Pregnant WT mice had significantly higher proportions of CD11b^+/Gr-1^+ MDSC in blood, spleen and uteri than non-pregnant WT mice (**Figures 1A–C**). In myeloid HIF-KO mice, MDSC counts in non-pregnant animals tended to be higher in blood, spleen and uterus compared to WT mice. Pregnancy induced further MDSC accumulation only in blood, not in spleens or uteri. In the latter, we even found a tendency toward lower MDSC numbers in pregnant than in non-pregnant myeloid HIF-KO mice (**Figures 1A–I**). MDSC-accumulation in WT animals was mainly attributed to the CD11b^+/Ly6G^+/Ly6C^{low} GR-MDSC subpopulation (**Figures 1D–F**), not the CD11b^+/Ly6G^-/Ly6C^{high} MO-MDSC subpopulation (**Figures 1G–I**). Gating strategy for MDSC, GR-MDSC and MO-MDSC in spleens and uteri are depicted in **Figures 1J–K**.

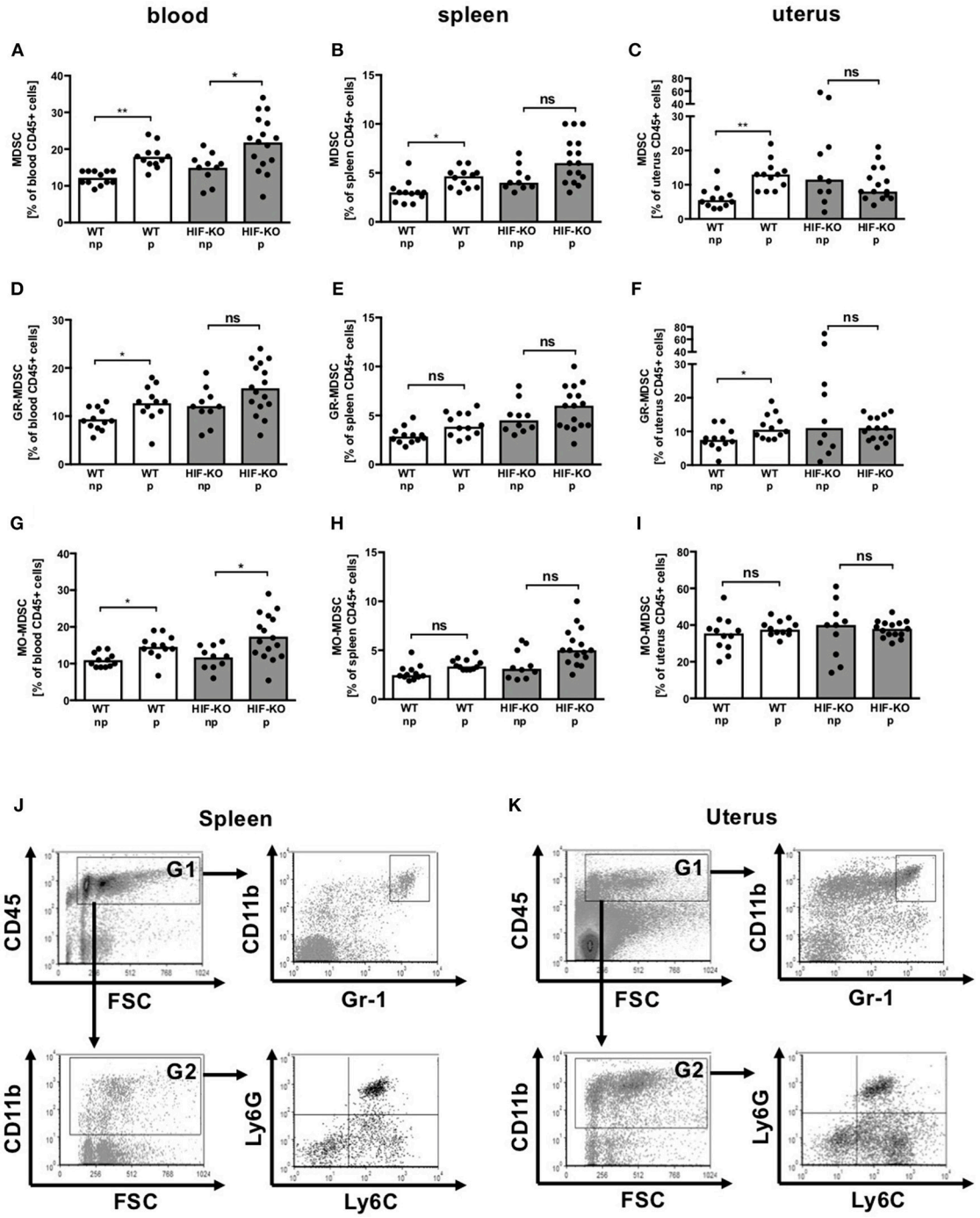


FIGURE 1 | Quantification of MDSC in blood, spleens and uteri of wildtype and myeloid HIF-KO mice. Non-pregnant (np) and E10.5 pregnant (p) wildtype (WT) and myeloid HIF-KO (HIF-KO) mice were euthanized and blood, spleens and uteri were collected. Tissues were homogenized and filtered to obtain single cell suspensions. Cells were then analyzed by flow cytometry. **(A–I)** Scatter diagrams with bars showing percentages of total MDSC **(A–C)** GR-MDSC **(D–F)**, and MO-MDSC **(G-I)** from total $CD45^+$ leucocytes in peripheral blood **(A,D,G)**, spleens **(B,E,H)** and uteri **(C,E,I)**. Each symbol represents an individual sample and the median is indicated. $n = 10–16$, $^*p < 0.05$; $^{**}p < 0.01$; ns not significant; Kruskal-Wallis test and Dunn's multiple comparison test. **(J,K)** Representative density plots showing the gating strategy for total MDSC ($CD11b^+/Gr\text{-}1^+$), GR-MDSC ($CD11b^+/Ly6C^{low}/Ly6G^+$) and MO-MDSC ($CD11b^+/Ly6C^{high}/Ly6G^-$) in spleens **(J)** and uteri **(K)**.

Regarding other leucocyte subpopulations, we found a decrease in percentages of B-cells in blood and NK-cells in spleens of both WT-mice and myeloid HIF-KO-mice during pregnancy (**Supplementary Figures 1D,H**). Percentages of T-cells decreased in uteri of myeloid HIF-KO mice during pregnancy, but remained unchanged in WT-mice

(**Supplementary Figure 1C**). Percentages of monocytes increased in blood and spleens of pregnant myeloid HIF-KO but not WT-mice (**Supplementary Figures 1J,K**), while they decreased in uteri of pregnant WT-mice and remained unchanged in uteri of myeloid HIF-KO mice (**Supplementary Figure 1L**).

HIF-1α Deficiency Leads to Diminished Suppressive Activity of MDSC

Next, we asked whether HIF-1α deficiency also has effects on suppressive activity of MDSC. To test this hypothesis, we generated MDSC from bone marrow (BM) of WT mice and myeloid HIF-KO mice and tested their suppressive activity on $CD4^+$ T-cell proliferation. MDSC generated from BM of WT mice efficiently suppressed $CD4^+$ T-cell proliferation in a concentration dependent manner to 48.0 ± 14.2 % (1:4), 24.6% ± 12.5% (1:2) and 14.3% ± 3.4% (1:1). In contrast, MDSC generated from BM of myeloid HIF-KO mice displayed substantially lower suppressive activity on $CD4^+$ T-cell (103.2% ± 25.2% (4:1) $p < 0.01$ vs. WT mice, 71.6% ± 26.5% (2:1) $p < 0.01$ vs. WT mice and 47.5% ± 15.4% (1:1) $p < 0.05$ vs. WT mice, $n = 5$, **Figures 2A,B**).

Diminished Accumulation of MDSC in the Pregnant Uterus Is Not Due to an Altered Expression of Chemokine Receptors or Integrins

As the most impressive difference between WT mice and myeloid HIF-KO mice was a diminished influx of MDSC into the pregnant uterus in myeloid HIF-KO mice, we asked whether deficiency for HIF-1α might lead to a diminished expression of chemokine receptors or integrins on MDSC leading to defects in their migratory capacity. Therefore, we analyzed expression of chemokine receptors CXCR1 (CD181), CXCR2 (CD182), CXCR4 (CD84), CXCR5 (CD185), CX3CR1 and IL4Rα (CD124), as well as ITGA4 (CD49d), ITGB2 (CD18) and L-selectin (CD62L) in MDSC from spleens and uteri of WT and myeloid HIF-KO mice. We found no differences in the expression of CXCR1, CXCR2, CXCR4, CXCR5, and CX3CR1 between WT and myeloid HIF-KO mice, neither in splenic MDSC nor in uterine MDSC. Interestingly, we even found higher expression of IL4Rα, ITGA4, ITGB2, and L-Selectin on myeloid HIF-KO MDSC (**Supplementary Figure 2** and **Figure 3**), not explaining the diminished accumulation in pregnant uteri.

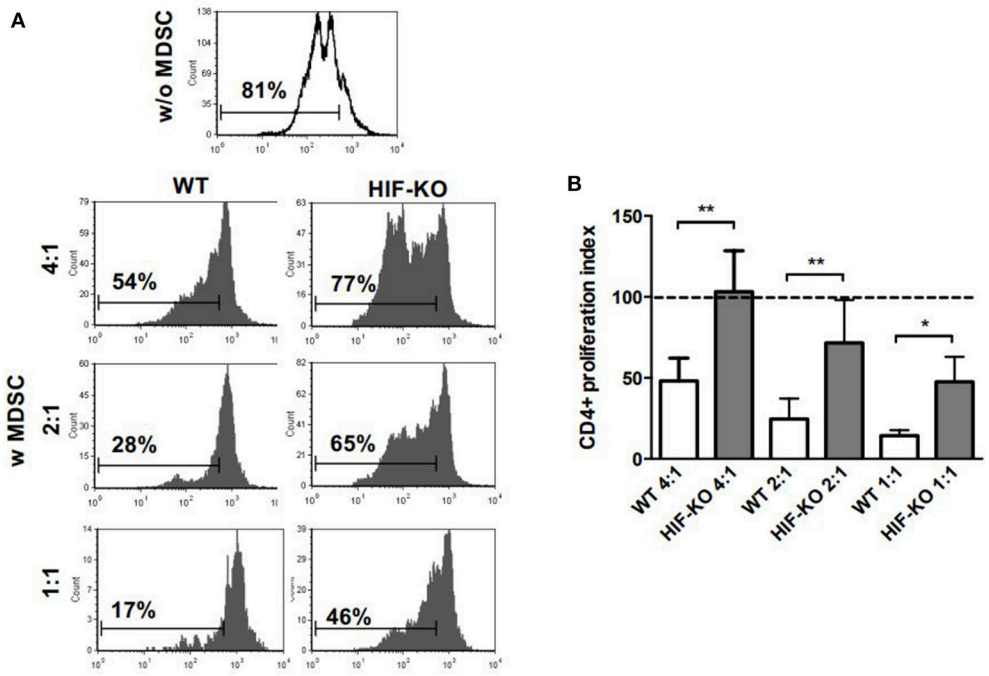


FIGURE 2 | Inhibition of $CD4^+$ T-cell proliferation by MDSC generated from wildtype and myeloid HIF-KO mice. Non-pregnant wildtype (WT) mice and myeloid HIF-KO mice (HIF-KO) were euthanized and bone marrow cells were collected. Cells were cultured for 4 days with G-CSF and GM-CSF. After 4 days non-adherent cells were removed and adherent MDSC were detached with Trypsin/EDTA. MDSC were then added to $CD4^+$ T-cells, freshly isolated from spleens of non-pregnant wildtype mice by MACS, stained with CFSE and stimulated with anti-CD3/CD28 microbeads. After 4 days, proliferation of $CD4^+$ T-cells was assessed by CFSE dye dilution. Proliferation index was determined as ratio of T-cell proliferation with and without addition of MDSC. **(A)** Representative histogram plots showing proliferation of $CD4^+$ T-cells without (white histogram, w/o MDSC) addition of MDSC and with (gray histograms, w MDSC) addition of MDSC generated from WT mice (left side) and from HIF-KO mice (right side) in T-cell:MDSC ratios of 4:1, 2:1 and 1:1. **(B)** Inhibitory effect of MDSC from WT mice (white bars) and HIF-KO mice (gray bars) on proliferation of $CD4^+$ T-cells. Dashed line shows proliferation of target $CD4^+$ T-cells without addition of MDSC. Inhibition of T-cell proliferation by MDSC was measured at the indicated ratios by CFSE dye dilution. Bars show mean and standard deviation of 5 samples pooled from 5 independent experiments. $^*p < 0.05$; $^{**}p < 0.01$ compared with target cells alone; Wilcoxon matched-pairs signed-rank test.

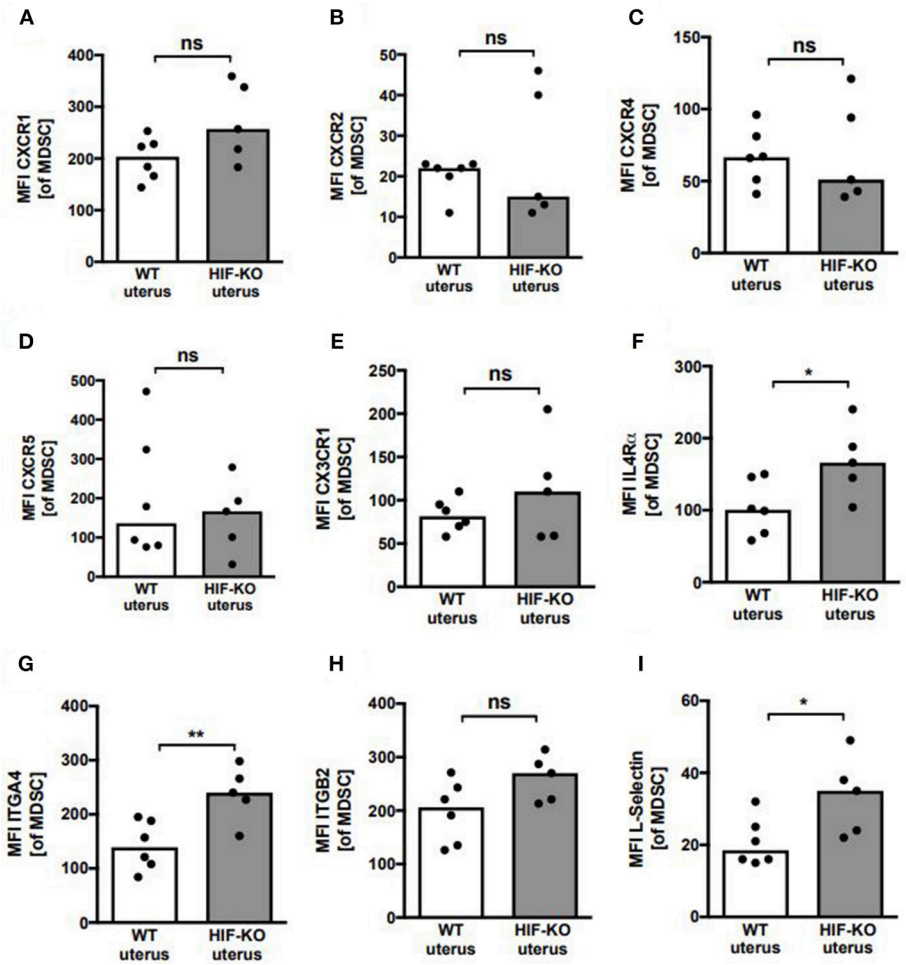


FIGURE 3 | Expression of chemokine receptors and integrins on uterine MDSC from wildtype and myeloid HIF-KO mice. **(A–I)** E10.5 pregnant wildtype (WT) and myeloid HIF-KO (HIF-KO) mice were euthanized and uteri were collected. Tissues were homogenized and filtered to obtain single cell suspensions. Cells were then analyzed by flow cytometry. Scatter diagrams with bars showing MFI for indicated chemokine receptor and integrin expression on $CD11b^+/Gr\text{-}1^+$ MDSC from wildtype (white bars) and myeloid HIF-KO (HIF-KO) mice. Each symbol represents an individual sample and the median is indicated. $n = 5–6$, $^*p < 0.05$; $^{**}p < 0.01$; ns, not significant; Mann-Whitney test.

HIF-1α Deficiency in Myeloid Cells Leads to Increased Apoptosis Rates of MDSC

To further figure out the mechanism(s) underlying the diminished accumulation of MDSC in uteri of pregnant myeloid HIF-KO mice, we analyzed apoptosis rates of MDSC in spleens and uteri of pregnant WT and myeloid HIF-KO mice by annexin V staining. We found that apoptosis rates of MDSC from spleens and uteri of myeloid HIF-KO mice were about twice as high as those of WT-MDSC (median 20.6 vs. 8.1% for spleens and 39.5 vs. 25.6% for uteri, $n = 5–6$, $p < 0.05$, **Figures 4A–C**).

HIF-1α Deficiency in Myeloid Cells Leads to an Increased Abortion Rate in Mice

To evaluate the clinical significance of myeloid HIF-KO during pregnancy, we analyzed pregnancy outcomes in myeloid HIF-KO mice and WT mice at E10.5. Placentae were prepared and numbers of resorbing units quantified. Resorbing units were either dark, small and necrotic, pale or small and with no visible fetus inside the amniotic cavity. We found that the rate of non-pregnant animals after positive vaginal plug was significantly higher in myeloid HIF-KO animals (16 vs. 7%, $n = 13–20$, $p < 0.05$, Fishers exact test, **Figure 5A**). Furthermore, we found a higher rate of animals with at least one abortion in myeloid HIF-KO animals than in WT mice (87 vs. 58%, $n = 12–16$, $p < 0.0001$, Fishers exact test, **Figure 5B**). At day E10.5, we found no differences in the number of implantation sites between myeloid HIF-KO mice and WT mice (**Supplementary Figure 3**), however, myeloid HIF-KO mice had significant higher abortion rates than WT mice (median 19.5% vs. 9.5%, $n = 12–16$, $p < 0.05$, **Figures 5C,D**).

Discussion

The role of MDSC in maintaining pregnancy is increasingly recognized. Molecular mechanisms leading to MDSC-accumulation during pregnancy, however, are still incompletely understood. In the present study, we investigated

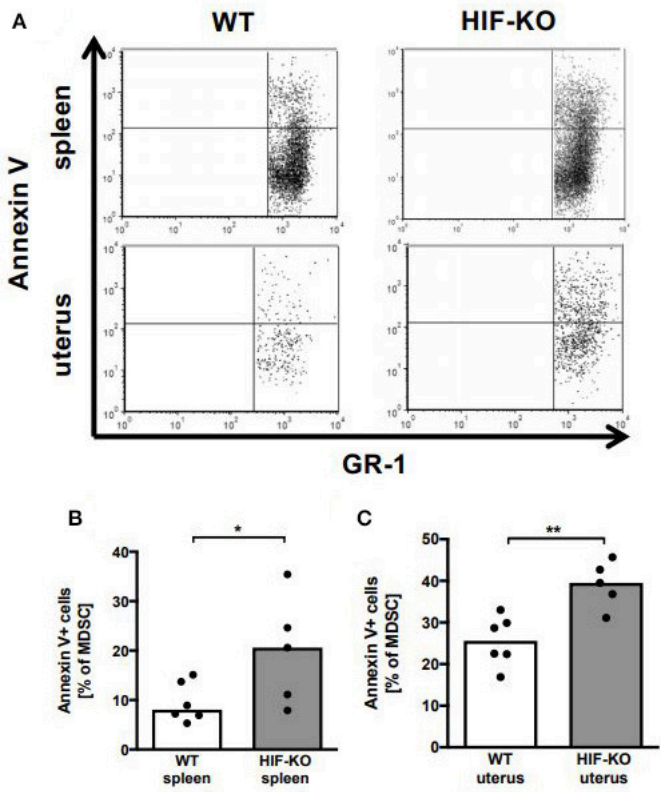


FIGURE 4 | Apoptosis of MDSC from spleens and uteri from wildtype and myeloid HIF-KO mice. E10.5 pregnant wildtype (WT) and myeloid HIF-KO (HIF-KO) mice were euthanized and spleens and uteri were collected. Tissues were homogenized and filtered to obtain single cell suspensions. Cells were then analyzed for apoptosis annexin V staining and flow cytometry. **(A)** Representative density plots from spleens (upper plots) and uteri (lower plots) of Wildtype (WT) and myeloid HIF-KO (HIF-KO) mice for GR-1 vs. annexin V. **(B,C)** Scatter diagrams with bars showing percentages of annexin V^+ MDSC from total MDSC in spleens **(B)** and uteri **(C)** from wildtype (white bars) and myeloid HIF-KO (HIF-KO) mice. Each symbol represents an individual sample and the median is indicated. $n = 5–6$, $^*p < 0.05$; $^{**}p < 0.01$; Mann-Whitney test.

the role of the hypoxia-regulated transcription factor HIF-1α for MDSC-accumulation during pregnancy and for pregnancy outcome.

First, we found that knockout of HIF-1α in myeloid cells led to a diminished accumulation of MDSC in the pregnant uterus, illustrating a relationship between myeloid HIF-1α expression and MDSC accumulation during pregnancy. One other study recently described a HIF-1α dependent accumulation of MDSC under tumor conditions; in a mouse model of hepatocellular carcinoma, HIF-1α activation in tumor cells led to an overexpression of the ectonucleotidase CD39 mediating a differentiation arrest of MDSC thereby leading to their accumulation (27). Other studies investigating the interplay between HIF-1α-activation and MDSC focused on MDSC function and not on MDSC accumulation (22–24). Interestingly, besides the uterine milieu, where hypoxia is known to play a role during different stages of pregnancy (18, 19), we also found a diminished MDSC-accumulation in spleens of myeloid HIF-KO mice. This suggests that, in addition to hypoxia, other factors may activate HIF-1α during pregnancy. Besides inflammatory pathways such as NF-κB that has been described to activate HIF-1α (28), one study reported an activation of HIF-1α by the sex hormone estrogen (29). Furthermore it has been shown that *in vitro* estrogen activates MDSC during pregnancy (30). The impact of estrogen on HIF-1α activation in MDSC and their functional activation during pregnancy is content of ongoing studies.

Interestingly, MDSC-accumulation in the pregnant uterus of wildtype mice was accompanied by a reduction of uterine monocytes. This reduction was not observed in uteri of myeloid HIF-KO mice. Since MDSC can differentiate to mature myeloid cells and hypoxia has been described to prevent MDSC differentiation via HIF-1α (27), the decreased monocyte numbers in pregnant uteri of WT mice might be a result of a HIF-1α-driven maturational arrest.

Second, we found that MDSC generated from myeloid HIF-KO mice had substantially reduced suppressive activity compared to MDSC generated from WT mice. This is in line with other studies describing an increased suppressive activity of MDSC under hypoxic tumor conditions mediated by HIF-1α (22). We now show that lack of HIF-1α leads to impaired suppressive activity of MDSC also in normoxia. The underlying mechanism for reduced suppressive activity of HIF-KO MDSC remains unclear. One of the main effector mechanisms used

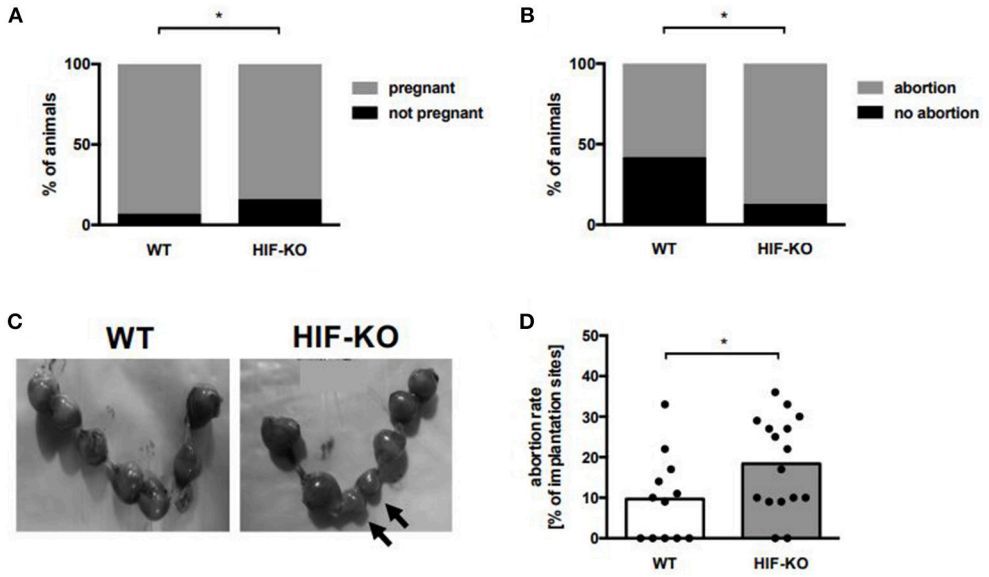


FIGURE 5 | Pregnancy success in wildtype and myeloid HIF-KO mice. Wildtype and myeloid HIF-KO mice were term-bred and the day when a vaginal plug was detected was defined as day E0.5. At day E10.5 mice were euthanized and the abdominal cavity was opened. Uteri containing foeto-placental units were removed and inspected. Total implantation sides and resorbing units were counted. **(A)** Bar graph showing the percentages of non-pregnant (black part) and pregnant (gray part) animals among wildtype (WT) and myeloid HIF-KO (HIF-KO) mice after positive evaluation for vaginal plug. $n = 13–20$, $^*p < 0.05$, Fishers exact test. **(B)** Bar graph shows the percentages of individuals without (black part) and with (gray part) at least one abortion among wildtype (WT) and myeloid HIF-KO (HIF-KO) mice. $n = 12–16$, $^*p < 0.05$, Fishers exact test. **(C)** Representative pictures of uteri from a pregnant wildtype (WT) and a pregnant myeloid HIF-KO mouse (HIF-KO). Arrows indicate resorbing units. **(D)** Scatter diagram with bars showing abortion rates of pregnant wildtype (white bar) and pregnant myeloid HIF-KO mice (gray bar). Abortion rates were defined as ratio between resorbing units and total implantation sites. Each symbol represents an individual animal and the median is indicated. $n = 12–16$, $^*p < 0.05$; Mann-Whitney test.

by MDSC to suppress T-cells is the production of iNOS (6). It has been shown that the Th1-cytokine IFN-γ activates HIF-1α leading to increased iNOS expression (31) and that myeloid cells acquire suppressive activity under hypoxic tumor conditions that can be abrogated by inhibiton of iNOS (23) so that it could be speculated that decreased generation of NO in HIF-KO MDSC leads to their decreased suppressive activity. Another mechanism linking HIF-1α activation and MDSC-function is the expression of the immune checkpoint molecule PD-L1. Noman et al. showed that expression of PD-L1 on myeloid cells can be stimulated by Hypoxia via HIF-1α and that blockade of PD-L1 decreases MDSC-mediated T-cell suppression by down-regulating MDSC IL-6 and IL-10 (24). Our group showed that expression of PD-L1 on human MDSC can be stimulated by E.coli (32). Thus, stimulation of PD-L1 expression via HIF-1α could also be a mechanism for MDSC activation during pregnancy.

To figure out mechanisms leading to the disturbed accumulation of MDSC in the pregnant uterus of myeloid HIF-KO mice we analyzed expression of chemokine receptors and integrins on MDSC. Although upregulation via HIF-1α signaling pathways has been described for many surface receptors including CXCR1 and CXCR2 (33), CXCR4 (34), and IL-4Rα (35), we found no differences in their expression on MDSC from WT and myeloid HIF-KO mice. ITGB2 and ITGA4 were found to be upregulated in HIF-KO MDSC confirming other studies that showed negative regulation of ITGA4 by HIF-1α (36) but not explaining the reduced MDSC-accumulation in the uterus. Taken together, our results suggest that HIF-1α knockout in myeloid cells does not alter MDSC migration to the uterus.

We found increased apoptosis rates of MDSC isolated from spleens and uteri of myeloid HIF-KO mice compared to WT mice. This is in line with most previous studies indicating that HIF-1α promotes survival in cancer and endothelial cells (37–41). In contrast, there are also studies describing a proapoptotic role for HIF-1α (42, 43). During pregnancy, data on the role of HIF-1α for apoptosis are conflicting (44, 45). However, previous studies focused on placental cells and not immune cells in the uterus. Regarding our results, the increased apoptosis rates in HIF-KO MDSC may explain their reduced accumulation in the pregnant uterus. Correspondingly, in peripheral organs, myeloid HIF-KO MDSC still expanded, albeit to a lesser extent than WT-MDSC.

Last, we show that myeloid HIF-KO mice had higher rates of non-pregnant animals after positive vaginal plug and higher abortion rates than WT-mice pointing to the potential clinical relevance of reduced MDSC accumulation in the uterus. General loss of HIF-1α causes severe failure in placental formation, resulting in embryo lethality by E10.5. In these animals, placental defects are mainly caused by disturbed development of vascularization and disruption of trophoblast differentiation (46–48). In the embryo, HIF-1α is required for heart development, chondrogenesis and bone

formation (49–51). Clinical studies showed upregulation of HIF-1α in trophoblasts of patients with missed abortions (52, 53), and increased HIF-1α activity in placental tissues has been associated with preeclampsia (54–56). However, reports on HIF-1α-regulation in myeloid cells during pregnancy are lacking. The role of hypoxia and HIF-1α in myeloid cells during other inflammatory processes is inconclusive. In a mouse model of LPS-induced Sepsis, HIF-1α induced a proinflammatory phenotype in monocytes and deletion of HIF-1α led to improved survival (57). In dendritic cells, hypoxia and HIFs are described to mediate both, proinflammatory and anti-inflammatory properties (58, 59). Under tumor conditions however, hypoxia and HIF-1α seem to drive the development and function of immunosuppressive myeloid cells like TAMs and MDSCs (23, 60, 61). Correspondingly, a recent study showed that overexpression of HIF-1α in myeloid cells leads to diminished transplant rejection and induction of a regulatory phenotype in myeloid cells in a model of heart transplantation (62). To our knowledge, our study is the first describing an impact of myeloid HIF-1α-expression on pregnancy outcome. Several previous studies in humans (11, 63, 64) and mice (10, 64–66) have shown that reduced MDSC accumulation is associated with abortions. Adoptive transfer experiments with abortion-prone mice furthermore showed that abortion rates were reduced by MDSC-transfer (65, 66). Absence of MDSC also led to failed implantation (13). These results together with our finding of a reduced MDSC-accumulation in myeloid HIF-KO mice make it tempting to speculate that diminished MDSC accumulation is responsible for the disturbed course of pregnancy in these animals.

In conclusion, we show here that HIF-1α expression in myeloid cells is required for a successful pregnancy and that loss of HIF-1α in myeloid cells leads to diminished accumulation, increased apoptosis and impaired MDSC function in the pregnant uterus. These results not only enlarge our knowledge about regulation of MDSC-accumulation and function during pregnancy, but may also help to better understand MDSC biology in other hypoxic conditions such as in solide tumors and inflamed tissues. Targeting HIF-1α may be a promising strategy to modulate MDSC-function.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Einrichtung für Tierschutz, Tierärztlichen Dienst und Versuchtierkunde Tübingen. The protocol was approved by the Einrichtung für Tierschutz, Tierärztlichen Dienst und Versuchtierkunde Tübingen and the Regierungspräsidium Tübingen.

AUTHOR CONTRIBUTIONS

NK and CG conceptualized and designed the study. NK, SD, JS, and BS performed experiments. NK, SD, and CG analyzed data. NK drafted the initial manuscript. NK, JP-F, CP, and CG reviewed and revised the manuscript. All authors approved the final manuscript as submitted and agreed to be accountable for all aspects of the work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00161/full#supplementary-material

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Elevated Soluble PD-L1 in Pregnant Women's Serum Suppresses the Immune Reaction

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Background: Programmed death-ligand 1 (PD-L1) is expressed not only on some cancer cells, but also on the outer surface of placental syncytiotrophoblasts, which is assumed to induce maternal immune tolerance to fetal tissue via programmed death-1 (PD-1) receptors on T cells. Recently, levels of soluble forms of PD-L1 (sPD-L1) were reported to be higher in the serum of pregnant women (PW) than in non-pregnant women (non-PW). However, there have been no reports of the functional significance of PW's serum containing high sPD-L1 levels. Therefore, the aim of the present study was to clarify the role of sPD-L1 in the sera of PW as an immunosuppressive molecule by *in vitro* assays.

Methods: As a *post-hoc* analysis of our previous cohort study, 330 pairs of serum from PW during the third trimester and cord blood (CB) from paired offspring without major complications were examined. Serum levels of sPD-L1 and sPD-1 were measured by ELISA. On mixed lymphocyte culture (MLC), ^{3}H-thymidine uptakes in the presence of PW's, offspring's, or non-PW's serum were compared. Peripheral blood mononuclear cells (PBMCs) were cultured in the presence of PW's serum stimulated with PHA, and then cytokine levels were measured in supernatants by multiple cytokine analysis with or without anti-PD-L1blocking antibody.

Results: The median sPD-L1 level was 8.3- and 6.9-fold higher in PW than in offspring and non-PW, respectively, whereas sPD-1 levels were lower in PW and offspring than in non-PW. On MLC, ^{3}H-thymidine uptake in the presence of autoantigen was strongly reduced by co-culture with serum of both PW and offspring, compared with serum of non-PW. In contrast, uptake in the presence of alloantigen was moderately inhibited by PW's serum, whereas it was less suppressed by offspring's serum, compared with non-PW's serum. In the culture of PBMCs, tumor necrosis factor-α, interferon gamma, interleukin (IL)-2, and IL-4 levels were significantly higher in the presence of anti-PD-L1 blocking antibody than in culture not treated with antibody (all $P < 0.05$) or culture treated with isotype control antibody (all $P < 0.05$).

Conclusion: The levels of sPD-L1 are elevated in PW's serum, which may, at least in part, suppress maternal immunity.

Keywords: PD-1, PD-L1, cord blood, pregnant woman, serum, MLC, IFN-γ

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INTRODUCTION

Programmed death-ligand 1 (PD-L1) is expressed on some cancer cells to suppress anti-tumor immunity by interacting with the programmed death-1 (PD-1) receptor expressed on T cells (1). Indeed, blocking this interaction by administering monoclonal antibodies targeting either the PD-1 or PD-L1 molecule improves the prognosis of cancer patients (2, 3). By immunohistochemical staining with anti-PD-L1 antibody, PD-L1 was proven to be highly expressed on the outer surface of syncytiotrophoblasts on chorionic villi in placenta facing the maternal blood stream in the uterus, but not on the inner aspect of the syncytiotrophoblasts facing chorionic villous stroma, as well as not on cytotrophoblasts facing fetal blood vessels (4–6). It has long been the immunological paradox of pregnancy that, even though tissues of the fetus including the placenta express allogeneic paternal antigen in addition to autogenic maternal antigen, they are not rejected by maternal immune systems. Thus, this paradox may be explained at least in part by PD-L1 being highly expressed on placenta; like cancer cells, PD-L1 on the placenta suppresses T cells attached to the placenta and induces immune tolerance to fetal tissues via PD-1 receptors on T cells. In fact, blockade of the PD-1 pathway by anti-PD-L1 monoclonal antibody during pregnancy increased abortions in mice (7).

Both PD-L1 and PD-1 exist in membrane-bound form and induce local or peripheral immune tolerance by maintaining the quiescence of autoreactive T cells. Recently, soluble forms of PD-L1 and PD-1 (sPD-L1 and sPD-1), which are considered to be generated by proteolytic cleavage of the membrane-bound forms, have been detected in the serum of cancer patients (8, 9). ELISA has confirmed that sPD-L1 binds to PD-1 (10). In addition, a recent meta-analysis showed that a higher level of sPD-L1 is associated with worse overall survival of cancer patients (11), indicating that sPD-L1 may work as a systemic suppressor of anti-tumor immunity, in addition to local suppression of anti-tumor immunity by membrane-bound PD-L1 on cancer cells. In 2018, sPD-L1 levels were reported to be higher in the serum of pregnant women (PW) than in non-pregnant women (non-PW), and postpartum women (12). In contrast to sPD-L1, sPD-1 may block the interaction between PD-L1 and PD-1 on T cells by competitive inhibition (13), resulting in enhanced activity of autoreactive T cells and contributing to anti-cancer effects. These experimental results were further supported by both an animal model in which sPD-1-producing virotherapy successfully improved the prognosis of tumor-bearing mice (14) and clinical evidence in which non-small cell lung cancer patients with increased serum levels of sPD-1 showed prolonged survival (15). However, there have been no reports regarding serum sPD-1 levels in PW or the functional significance of elevated sPD-L1 levels in PW's serum. Therefore, the aim of the present study was to clarify the role of sPD-L1 in sera of PW as an immunosuppressive molecule by *in vitro* assays.

METHODS

Study Design

As a *post-hoc* analysis, 330 pairs of PW and their offspring were randomly selected from our previous cohort study (16) conducted at Shiomidai Hospital, a general hospital in Kanagawa Prefecture, Japan. The inclusion criteria were: PW $\geq$ 20 years old at enrollment; lack of major complications, such as gestational diabetes mellitus, pregnancy-induced hypertension, pre-eclampsia, preterm labor, or the need for emergent cesarean section; and lack of high-risk fetal conditions, such as twins, intrauterine growth retardation, and congenital malformations. PW were enrolled from June 2011 to September 2012. Because sPD-L1 levels vary with age, 20 commercial serum samples from non-pregnant healthy women in their twenties and thirties were initially purchased for use as age-matched controls. To compare serum sPD-L1 levels among non-PW with known smoking status, 21 commercial serum samples from non-pregnant healthy women were also purchased: non-smokers, $n = 7$; past smokers, $n = 7$; and current smokers, $n = 7$.

Ethics

The study protocol was approved by the ethics committee at the Jikei University School of Medicine, the clinical study committee at Jikei Hospital, and the institutional review board at Shiomidai Hospital. Clinical data and samples were anonymized immediately after their collection at birth in a non-linkable fashion. Data monitoring was performed in the Division of Epidemiology, the Jikei University School of Medicine, with all data monitored and fixed by HM, who did not participate in ELISA measurements or statistical analyses. All women provided their written, informed consent. The serum samples used for controls were purchased from Tokyo Future Style, Inc. (Tsukuba, Ibaraki, Japan).

Measurement of sPD-L1 and sPD-1 Levels

Serum samples were collected from PW at 34 weeks of gestation. The offspring's serum (5–10 mL) was sampled from the placental side after placental delivery at birth. The serum samples were stored at −80°C prior to use. Serum levels of sPD-L1 and sPD-1 were measured by MO, using ELISA kits from Abcam (Cambridge, MA, USA) and RayBiotech (Norcross, GA, USA), respectively, according to the manufacturers' protocols. Each sample was tested in triplicate for sPD-L1 and in duplicate for sPD-1, with the medians used for analysis. The lower detection limits for ELISA were 3.9 pg/mL for sPD-L1 and 20 pg/mL for sPD-1. The upper detection limits for ELISA were 1,300 pg/mL for sPD-L1 and 6,000 pg/mL for sPD-1.

Mixed Lymphocyte Culture

Reactions of lymphocytes in the presence of either autoantigen or alloantigen were measured by ^{3}H-thymidine uptake using a mixed lymphocyte culture (MLC) assay system at SRL Inc (Hachioji, Tokyo, Japan). Briefly, peripheral blood mononuclear cells (PBMCs) were obtained from three healthy male volunteers, named A, B, and C. For the MLC assay with autoantigen, fresh PBMCs were co-cultured with 13-Gy-irradiated PBMCs from the same donor in three patterns, i.e., fresh A—irradiated A, fresh B—irradiated B, and fresh C—irradiated C. For the MLC assay with alloantigen, fresh PBMCs were co-cultured with 13-Gy-irradiated PBMCs from different donors in four patterns, i.e., fresh A—irradiated B, fresh A—irradiated C, fresh B—irradiated A, and fresh C—irradiated A. These cells were

cultured for 5 days with RPMI1640 and 20% of either a mixture of serum samples from PW, offspring, or non-PW, randomly selected from the cohort of this study. Each kind of serum was a mixture of at least 10 samples. Cells were pulsed with ^{3}H-thymidine during the last 17.5 h of incubation and counted in a liquid scintillation counter (Microplate Scintillation and Luminescence Counter; Perkin Elmer, Inc, Waltham, MA, USA). This MLC experiment was repeated for three sets using the same serum samples.

Cytokine Assay

PBMCs were obtained from a healthy female volunteer. Then, 2.0×10^4 PBMCs per well in 96-well U-bottom plates were co-cultured with RPMI1640 and a mixture of serum samples derived from at least 10 PW randomly selected from the cohort of this study. The serum concentration was brought to 5%, and treated with 5 μg/ml of phytohemagglutinin (PHA) (J-CHEMICAL, Inc., Chuo-ku, Tokyo, Japan) for stimulation. Samples treated with anti-PD-L1 blocking antibody (5 μg/ml) (Monoclonal Antibody MIH1, Functional Grade, eBioscience, San Diego, CA, USA), isotype control (5 μg/ml) (mouse IgG1 kappa Isotype Control, Functional Grade, eBioscience), and samples without antibody were cultured according to the method of Andorsky et al. (17). After 72 h of culture, supernatants were harvested from culture, and tumor necrosis factor-α (TNF-α), interleukin 6 (IL-6), interferon gamma (IFN-γ), IL-2, and IL-4, as markers of broad immune responses, were measured by quantitative multiplex detection using the Human Magnetic Luminex Screening Assay (R&D System, Minneapolis, MN, USA), since these cytokines were reported to be secreted from PBMCs stimulated by PHA (18).

Statistical Analyses

Because the skewness test showed that sPD-L1 levels and sPD-1 levels were not normally distributed, their levels were compared among control non-PW, PW, and CB using the Kruskal-Wallis rank test (KW test). If the result of the KW test was significant, then the Mann-Whitney test was used to compare between two groups. Spearman's rank correlation, represented as rho, with linear regression was used to quantify the strengths of associations between two continuous variables: rho ≥0.4, strong; 0.4> rho ≥0.2, moderate; and rho <0.2, weak. The test for trends across ordered groups developed by Cuzick (19) was used to examine the relationships between anthropometric measurements at birth and quartiles of sPD-L1. Multivariate analysis with linear regression was performed to adjust for potential confounders of the associations between anthropometric data and sPD-L1 quartiles, gestational weeks, and sex of offspring. For ^{3}H-thymidine uptake on MLC, as well as production levels in cytokine assays, the KW test and the Mann-Whitney test were used to compare among three groups and between two groups, respectively. Stata version 14.0 software (StataCorp, College Station, TX, USA) was used for all analyses. Values of $P < 0.05$ were considered significant. These analyses were not corrected for multiple comparisons.

RESULTS

Participants' Characteristics

In a *post-hoc* manner, 330 pairs of PW and offspring were analyzed in this study; the demographic and clinical data are shown in **Table 1**. Moreover, 20 serum samples from non-PW were used as controls.

Serum Levels of sPD-L1 and sPD-1 Among PW, Offspring, and Non-PW

Serum levels of sPD-L1 were first compared among PW, offspring, and non-PW to find significant differences (KW test: $P = 0.0001$) (**Figure 1A**). The median level of sPD-L1 was 8.3-fold higher in the serum of PW than in offspring ($P < 0.0001$) and 6.9-fold higher in PW than in non-PW ($P < 0.0001$). The median level of sPD-L1 was lower in offspring than in non-PW ($P = 0.003$). Then, serum sPD-1 levels were compared among the three groups to find significant differences (KW test: $P = 0.0006$) (**Figure 1B**). In the serum of non-PW, the median level of sPD-1 was 4.8-fold higher than in serum of PW ($P = 0.0003$), and 4.6-fold higher than in serum of offspring ($P = 0.0001$). However, the sPD-1 level was not significantly different between PW and offspring ($P = 0.72$). The median sPD-1/sPD-L1 ratio of non-PW was 41.6-fold higher than in PW ($P < 0.0001$) and 4.6-fold higher than in offspring ($P = 0.001$), and the ratio of offspring was 9.1-fold higher than in PW ($P < 0.0001$) (**Figure 1C**).

Collinearity of sPD-L1 and sPD-1 Serum Levels Between PW and Paired Offspring

The sPD-L1 levels in PW had a strong positive association with those in offspring (Spearman's rho = 0.40; $P < 0.0001$) (**Figure 2A**). The sPD-1 levels in PW also had a strong positive association with those in offspring (Spearman's rho = 0.54; $P < 0.0001$) (**Figure 2B**). In contrast, there were no associations

TABLE 1 | Participants' characteristics.

	Pregnant women $n = 330$	Offspring $n = 330$	Non-pregnant women $n = 20$
Age (y) mean (*SD*)	32 (5)	–	29 (7)
Female ratio (%)	100%	48%	100%
Body height (cm) mean (*SD*)	158.6 (5.2)	48.7 (1.9)	
Body weight (kg) mean (*SD*)	52.9 (8.1)	3.057 (0.410)	
Weight change (kg) mean (*SD*)	8.6 (3.6)		
Gestational weeks mean (*SD*)	38.7 (1.3)		
Apgar score, median (25–75%)			
1 min		9 (8–9)	
5 min		9 (9–10)	

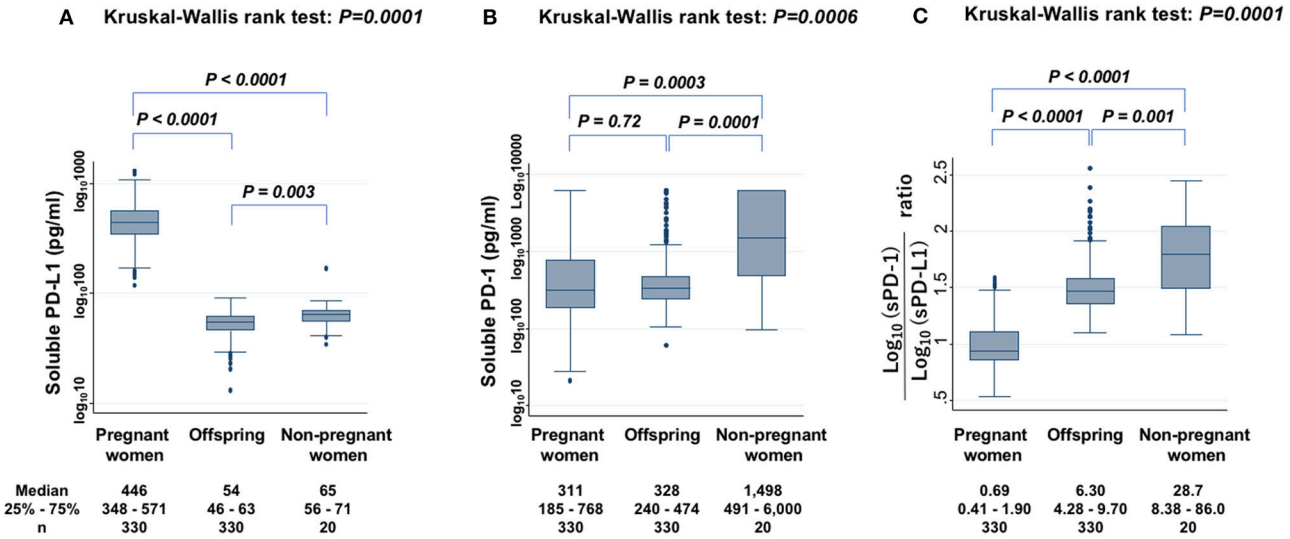


FIGURE 1 | Levels of sPD-L1 **(A)**, sPD-1 **(B)**, and the ratio of sPD-1 to sPD-L1 **(C)** in serum from PW, offspring, and non-PW controls. The Kruskal-Wallis rank test (KW test) was used for comparisons among three groups. The Mann-Whitney test was used to compare two groups. Values were transformed by the common logarithm (log_{10}) prior to analysis and are shown in the graph, although the median and 25−75th percentiles are presented as absolute values.

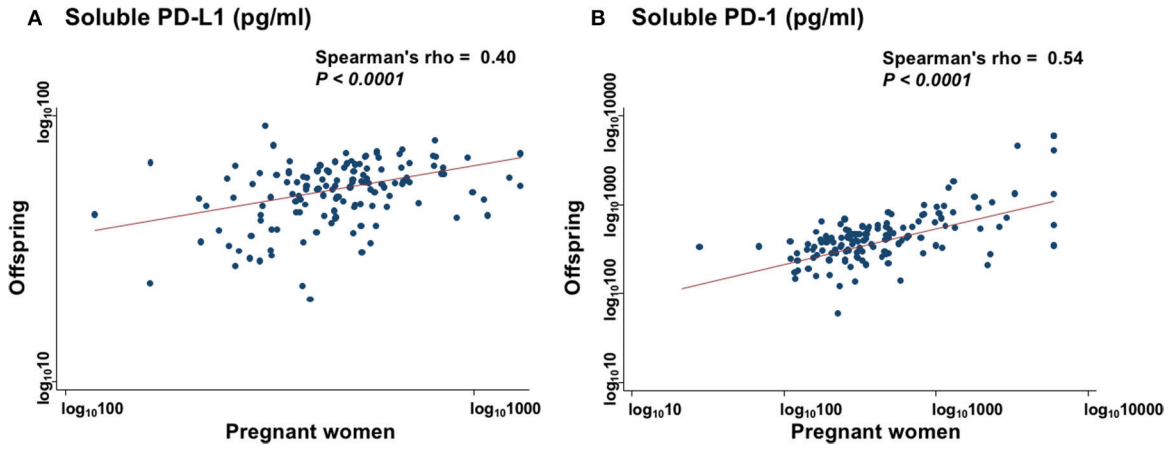


FIGURE 2 | Collinearity levels of sPD-L1 **(A)** and sPD-1 **(B)** in serum between PW and matched offspring. Spearman's rank correlation with linear regression was used to quantify the strength of association; sPD-L1 values were transformed by common logarithm (log_{10}) prior to analysis.

between sPD-L1 and sPD-1 levels in serum of PW and of offspring.

Mixed Lymphocyte Culture

Level of ^{3}H-thymidine uptake in the presence of autoantigen (**Figure 3A**) and alloantigen (**Figure 3B**) were compared among three groups: PW, offspring, and non-PW. In the presence of autoantigen, median ^{3}H-thymidine uptake was strongly reduced by co-culture with serum from both PW (57% reduction; $P = 0.047$) and offspring (78% reduction; $P = 0.02$), compared with that of non-PW. On the other hand, there was no difference in the uptake between sera from PW and offspring. In the presence of alloantigen, ^{3}H-thymidine uptake was moderately reduced by co-culture with serum from PW (23% reduction; $P = 0.005$), and not significantly reduced by co-culture with offspring's sera ($P = 0.33$), compared with that of non-PW.

Cytokine Assay

In the presence of PW's sera, anti-PD-L1 blocking antibody increased the secretion of TNF-α (**Figure 4A**), IFN-γ (**Figure 4B**), IL-2 (**Figure 4C**), and IL-4 (**Figure 4D**), significantly more than samples without antibody or those treated with isotype antibody (all $P < 0.05$). On the other hand, the levels of these cytokines did not show significant difference between samples without antibody and those treated with isotype antibody. There was a similar tendency for IL-6, but it was not significant (data not shown).

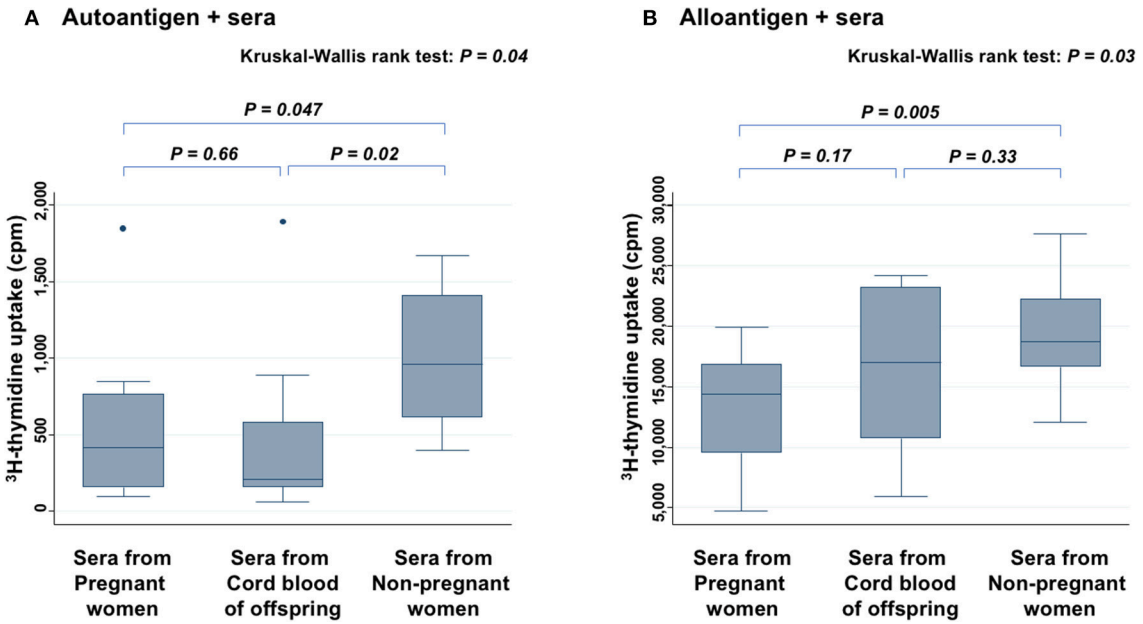


FIGURE 3 | Mixed lymphocyte culture assays in the presence of autoantigen **(A)** and alloantigen **(B)**. Levels of ^{3}H-thymidine uptake of peripheral blood mononuclear cells (PBMCs) are compared among sera from PW, offspring, and non-PW. The Kruskal-Wallis rank test (KW test) was used for comparisons among three groups. The Mann-Whitney test was used to compare two groups.

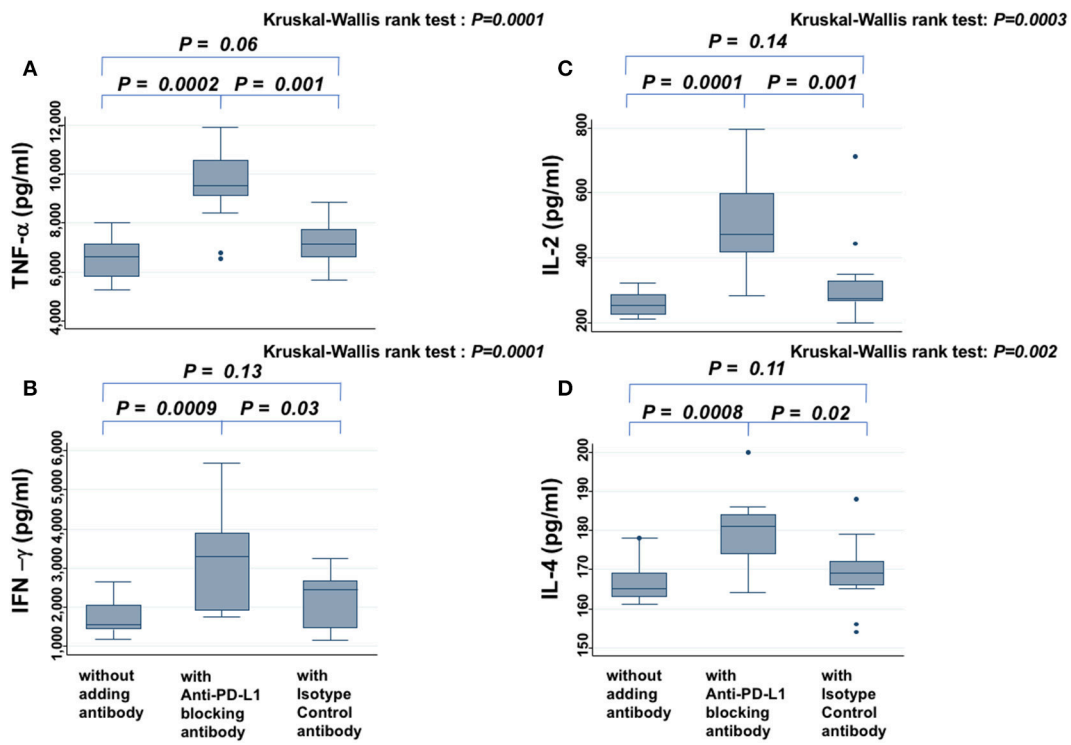


FIGURE 4 | Cytokine assay. Levels of TNF-α **(A)**, IFN-γ **(B)**, IL-2 **(C)**, and IL-4 **(D)** secreted from PBMCs in the presence of PHA and PW's serum are compared among samples without antibody, those treated with anti-PD-L1 blocking antibody, and those treated with isotype control antibody. The Kruskal-Wallis rank test (KW test) was used for comparisons among the three groups. If there was a significant difference, the Mann-Whitney test was used to compare two groups.

Levels of sPD-L1 and Smoking Status in PW and Non-PW

The levels of sPD-L1 were compared among non-smokers, past smokers, and current smokers among PW to find significant differences (KW test: $P = 0.0006$) (**Figure 5A**). The sPD-L1 levels were 13 and 31% lower in past and in current smokers, respectively, than in non-smokers. Serum levels of sPD-L1 were also measured among non-PW (KW test: $P = 0.03$) (**Figure 5B**). In particular, the sPD-L1 levels were 27% lower in current smokers than in non-smokers plus past smokers (Mann-Whitney test: $P = 0.03$), whereas there was no significant difference in sPD-L1 levels between non-smokers and past smokers.

Offspring Anthropometric Measurements and sPD-L1 Levels in PW

Offspring anthropometric measurements at birth were compared with serum sPD-L1 levels in PW as both quartiles and continuous variables.

The median body weight at birth in the highest sPD-L1 quartile (Q4) was 270 g heavier than in the lowest quartile (Q1). Thus, there was a significant trend for increased body weight with higher levels of sPD-L1 (trend test, $P < 0.0001$) and a moderate Spearman's rho (0.24, $P < 0.0001$) (**Supplementary Figure 1**). On multivariate adjustment with gestational weeks and offspring's sex, body weight remained a significant factor.

The median body height of the highest quartile (Q4) of sPD-L1 was 0.6 cm taller than that of the lowest quartile (Q1). There was thus a significant trend for increased body height with higher levels of sPD-L1 (trend test, $P < 0.0001$) and a weak Spearman's rho (0.13, $P = 0.02$) (**Supplementary Figure 2**). On multivariate adjustment with gestational weeks and offspring sex, body height remained a significant factor.

The median head circumference of the highest quartile (Q4) of sPD-L1 was 0.4 cm longer than that of the lowest quartile (Q1). There was thus a significant trend for increased head circumference with higher levels of sPD-L1 (trend test, $P = 0.009$) and a weak Spearman's rho (0.15, $P = 0.007$) (**Supplementary Figure 3**). On multivariate adjustment with gestational weeks and offspring sex, head circumference remained a significant factor.

The median chest circumference of the highest quartile (Q4) of sPD-L1 was 0.5 cm longer than that of the lowest quartile (Q1). There was thus a significant trend for increased chest circumference with higher levels of sPD-L1 (trend test, $P < 0.0001$) and a moderate Spearman's rho (0.21, $P = 0.0001$) (**Supplementary Figure 4**). On multivariate adjustment with gestational weeks and offspring sex, chest circumference remained a significant factor.

DISCUSSION

In the present study, the median serum sPD-L1 level in PW during the third trimester was high, 8.3-fold higher than in offspring and 6.9-fold higher than in healthy age-matched non-PW controls. Although serum levels of sPD-L1 were reported to increase throughout gestation (12) when blood samples from 30 PW were collected, this increase of sPD-L1 in PW was reconfirmed by expanding the sample size to 330 PW, and their offspring's CB was found to have lower levels of

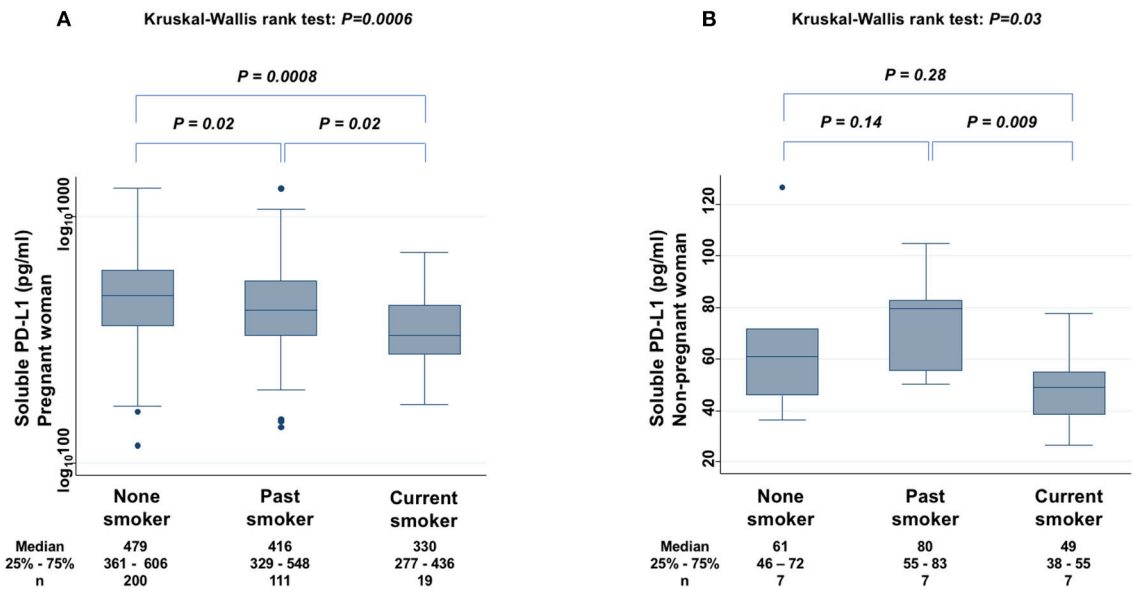


FIGURE 5 | Relationship between sPD-L1 levels and smoking status in PW **(A)** and in non-PW **(B)**. The Kruskal-Wallis rank test (KW test) was used for comparisons among three groups: non-smoker, past smoker, and current smoker. The Mann-Whitney test was used to compare two groups; sPD-L1 values were transformed by the common logarithm ($\log_{10}$) prior to analysis and are shown in the graph **(A)**, although the median and 25−75th percentiles are presented as absolute values.

sPD-L1 than the serum of non-PW. In contrast to sPD-L1, sPD-1 levels were lower in PW and offspring than in non-PW. Opposite to sPD-L1, sPD-1 was reported to competitively inhibit the interaction between PD-L1 and PD-1 on T cells (13). Therefore, it was assumed that when both increased sPD-L1 and decreased sPD-1 are present in the serum of PW, the immune reaction may be more suppressed than in offspring with decreased sPD-L1 and decreased sPD-1 in the serum. Thus, in serum from PW, the sPD-1/sPD-L1 ratio was very low and could inhibit lymphocyte proliferation in response to both autoantigen and alloantigen. In serum from offspring's CB, the sPD-1/sPD-L1 ratio was moderately low and could inhibit lymphocyte proliferation in response to autoantigen, but not significantly to alloantigen. To clarify whether the elevated sPD-L1 protein in PW's serum contributes to immune suppression, *in vitro* experiments of cytokine production assays with PHA stimulation, where an attempt was made to block specific functions of sPD-L1 in PW's serum with anti-PD-L1 antibody, were added. These experiments showed that various cytokines were increased in the presence of anti-sPD-L1 blocking antibody, suggesting that the elevated sPD-L1 protein in PW's serum may be functional, able to suppress broad immune reactions, and may thus be considered to protect the placenta and fetus from maternal immunosurveillance, at least in part.

With respect to clinical data, levels of sPD-L1 in PW showed a negative association with smoking. It has been well reported that maternal smoking impairs placental structure and function (20, 21). In addition, expression of PD-L1 mRNA in trophoblasts was reported to be increased with rising oxygen concentrations and decreased rapidly by a low oxygen concentration (5). On the other hand, administration of PD-L1 protein was demonstrated to protect against pre-eclampsia in the rat (22). Judging from these lines of evidence, it was then assumed that smoking may decrease oxygen supply to the placenta and secretion of sPD-L1 to the maternal blood stream, and the decreased sPD-L1 may further impair placental function and reduce sPD-L1 secretion to form a vicious circle. Of interest, even among non-PW, serum levels of sPD-L1 were suppressed in current smokers. PD-L1 is usually expressed on the macrophage lineage (23). Smoking impairs alveolar macrophage activation (24), which can be normalized by smoking cessation (25). In this case, smoking may be assumed to suppress sPD-L1 levels through impaired macrophage function.

There are several limitations of the present study. First, PW with major complications, such as gestational diabetes mellitus, pregnancy-induced hypertension, pre-eclampsia, preterm labor, or the need for emergent cesarean section, and who lacked high-risk fetal conditions, such as twins, intrauterine growth retardation, and congenital malformation, were excluded. Thus, associations between sPD-L1 levels of PW and these complications could not be examined. Instead, associations with smoking status and with fetal growth were examined to show that serum sPD-L1 levels can be a biomarker of placental function. Second, functional assays were added to support the hypothesis that sPD-L1 in addition to PD-L1 expressed on the surface of the placenta suppresses the maternal immune reaction to reject the placenta and fetus. It was demonstrated that the serum of PW showed stronger suppressive effects on autogenic and allogeneic immune reactions than serum of non-PW. Moreover, this suppressive effect of PW's sera was blocked using anti-PD-L1 antibody, as demonstrated by cytokine production assays. Third, MLC, which is used to evaluate the possibility of graft vs. host or host vs. graft disease in the field of bone marrow transplantation or organ transplantation, was used to examine immune reactions in the presence of PW's serum, considering the immunological paradox of pregnancy. However, there are many other types of functional assays. Fourth, since placental tissue was not collected in this cohort, for example, direct interactions between syncytiotrophoblasts and T cells could not be examined. Instead, the focus was not on membrane-bound PD-L1 molecules expressed on syncytiotrophoblasts, but on the soluble form of PD-L1 in the serum of PW. Fifth, sPD-L1 was measured at only one point in the third trimester during pregnancy. Sixth, placental PD-L1 protein expression was not measured by immunohistochemistry.

In conclusion, this is the first study to measure both sPD-L1 and sPD-1 levels in the serum of PW, as well as paired offspring, and it showed that: (1) sPD-L1 levels were very high in the serum of PW in the third trimester, but low in paired offspring; (2) sPD-1 levels were lower in both PW and offspring than in non-PW; (3) there was a strong correlation of sPD-1 levels, as well as sPD-L1 levels, between PW and offspring; (4) on MLC, ^{3}H-thymidine uptake in the presence of autoantigen was strongly reduced by co-culture with sera from both PW and offspring, compared with non-PW's serum, while uptake in the presence of alloantigen was moderately inhibited by sera from PW, but it was not significantly suppressed by offspring's serum, compared with non-PW's serum; (5) adding the anti-PD-L1 blocking antibody to sera from PW raised cytokine secretion (6); and, finally, sPD-L1 levels in PW and in non-PW were suppressed by smoking.

The novel finding of this study was that PW's serum may suppress both autogenic and allogeneic immune reactions, whereas offspring's serum may suppress mainly the autogenic immune reaction and only partly allogeneic immune reactions. It was further shown that anti-PD-L1 antibody impairs the immunosuppressive effects of PW's serum, and it was suggested that elevated sPD-L1 levels in PW's sera may be functional and play, at least in part, a role in suppressing the maternal immune reaction to alloantigen, i.e., placenta and fetus. Further research is needed to confirm this.

AUTHOR CONTRIBUTIONS

MO and MU conceptualized and designed the study, drafted the initial manuscript, and reviewed and revised the manuscript. TK and HM collected and fixed clinical data and reviewed and revised the manuscript. MU performed experiments and analyzed data statistically. All authors approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00086/full#supplementary-material

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REVIEW
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Therapeutic Potential of Regulatory T Cells in Preeclampsia—Opportunities and Challenges

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Inflammation is a central feature and is implicated as a causal factor in preeclampsia and other hypertensive disorders of pregnancy. Inflammatory mediators and leukocytes, which are elevated in peripheral blood and gestational tissues, contribute to the uterine vascular anomalies and compromised placental function that characterize particularly the severe, early onset form of disease. Regulatory T (Treg) cells are central mediators of pregnancy tolerance and direct other immune cells to counteract inflammation and promote robust placentation. Treg cells are commonly perturbed in preeclampsia, and there is evidence Treg cell insufficiency predates onset of symptoms. A causal role is implied by mouse studies showing sufficient numbers of functionally competent Treg cells must be present in the uterus from conception, to support maternal vascular adaptation and prevent later placental inflammatory pathology. Treg cells may therefore provide a tractable target for both preventative strategies and treatment interventions in preeclampsia. Steps to boost Treg cell activity require investigation and could be incorporated into pregnancy planning and preconception care. Pharmacological interventions developed to target Treg cells in autoimmune conditions warrant consideration for evaluation, utilizing rigorous clinical trial methodology, and ensuring safety is paramount. Emerging cell therapy tools involving *in vitro* Treg cell generation and/or expansion may in time become relevant. The success of preventative and therapeutic approaches will depend on resolving several challenges including developing informative diagnostic tests for Treg cell activity applicable before conception or during early pregnancy, selection of relevant patient subgroups, and identification of appropriate windows of gestation for intervention.

Keywords: pregnancy, preeclampsia, placenta, embryo implantation, maternal vascular adaptation, inflammation, Treg cells, immune tolerance

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INTRODUCTION

Preeclampsia and related hypertensive disorders complicate 3–5% of pregnancies. They are a leading cause of maternal deaths and perinatal morbidity and mortality (1) and are enormously expensive to health care systems, with an estimated cost in the US of $2.18 billion for the first 12 months of life alone (2). Preterm birth and fetal intrauterine

growth restriction (IUGR) are common sequalae, causing developmental challenges for the neonate that adversely impact cardiovascular, metabolic, and neurodevelopmental health (3). Preeclampsia also has long-term consequences for maternal cardiovascular health (4). Despite extensive research, the pathophysiological origins of preeclampsia remain unclear and effective preventative interventions are lacking. Current clinical management is aimed at alleviating symptoms and delaying delivery, rather than preventing occurrence by modifying the underlying cause (5, 6).

An emerging view is that critical initiating events before pregnancy and in the conception and implantation phase determine preeclampsia susceptibility, eliciting changes in placental development much earlier in gestation than when symptoms appear (7–9). This is particularly the case for the severe, early onset form of preeclampsia where failed maternal vascular adaptation to pregnancy is implicated—but also likely contributes to later onset disease (10, 11). There is strong evidence that failure of the maternal immune response to adapt correctly in early pregnancy underpins the placental and cardiovascular anomalies that become evident in later gestation. Disturbance in the immune response appears to be central and causal of later placental and hypertensive symptoms (8, 12, 13).

The adaptive immune response, with its typical features of immunological priming and memory, appears integral to the pathophysiological origin of the condition. Preeclampsia is more common in first pregnancies, particularly after limited sexual contact with the conceiving partner due to short sexual cohabitation, use of barrier contraceptive methods or assisted reproduction (14–16). Prior pregnancy with the same partner offers protection, but this is partner-specific and is lost with a new partner, implying alloantigen specificity (17). Assisted reproduction with donor oocytes, where there is no prior contact with the donor's alloantigens, is associated with a 4.3-fold increase in preeclampsia compared to natural conception (18). The risk is also increased with donor sperm but this is reduced with multiple exposures to the same donor (19). Pregnancy-induced memory in T cells (20) and in uterine NK cells (21) likely contributes to the protective benefit of prior pregnancy, and mechanisms by which seminal fluid may also induce memory are emerging (22). Recognizing this protective role for the adaptive immune response offers scope for new approaches to tackle this prevalent condition.

All women show evidence of altered immunity and elevated inflammatory activation in pregnancy. Immune adaptation for pregnancy commences during the pre-implantation phase when conception and implantation evoke a controlled inflammatory response in the female reproductive tract, which must be rapidly resolved by specific cytokines and pro-tolerogenic mechanisms into an anti-inflammatory milieu, in order for pregnancy to progress (23). In preeclampsia there are excessive pro-inflammatory mediators and inappropriate activation of effector immune cells, detectable in peripheral blood and gestational tissues from the first trimester (24, 25), implying incomplete or insufficient establishment of anti-inflammatory mechanisms (**Figure 1**).

In healthy pregnancy, inflammation associated with conception and implantation is rapidly resolved, and then remains suppressed by anti-inflammatory protective mechanisms, amongst which the specialized subset of $CD4^+$ T lymphocytes called regulatory T cells (Treg cells), are pivotal (26–28). Through their critical roles in constraining inflammation, suppressing effector immunity, and modulating vascular function, Treg cells are emerging along with uNK cells and macrophages as key coordinators of implantation and early placental development (23, 29–31).

In preeclampsia, Tregs in the maternal peripheral blood and decidua are fewer in number (26, 28, 32) and their suppressive function is impaired (33, 34), while pro-inflammatory Th17 cells (27), $CD8^+$ effector T cells and trophoblast apoptosis (28) are increased. The underlying reasons are unclear, but number and functional capacity of Treg cells are known to vary between individuals and are influenced by agents and exposures identified as pre-pregnancy antecedents of preeclampsia and other adverse pregnancy outcomes. Risk factors for impaired Treg activity include elevated inflammatory load associated with obesity and metabolic dysfunction (35), autoimmune conditions and systemic inflammatory exposures (36, 37), nutritional deficiencies (38, 39), and age (40). Their abundance and phenotype in the uterus are furthermore regulated by relevant clinical factors including prior pregnancy, disparity between male and female partner alloantigens, and seminal fluid contact (23). Other obstetric disorders including fetal growth restriction, gestational diabetes, and spontaneous preterm birth also have an inflammatory etiology, but amongst these conditions, the causal link between Treg cell dysregulation and preeclampsia is most clear.

In this review, we make the case that interventions to boost the number, functional competence and stability of Treg cells may offer realistic preventative and therapeutic strategies to protect against preeclampsia in at-risk women. Several pharmacological agents and cell therapy approaches to target Treg cells are in clinical trials or under development for auto-immune disorders and organ transplantation (37, 41, 42). We argue that as Treg therapies move closer to reality in other clinical settings, these interventions warrant evaluation for their potential utility in preeclampsia and related obstetric disorders with an immune etiology.

TREG CELLS—ESSENTIAL FOR MATERNAL ADAPTATION TO PREGNANCY

Several mechanisms of active immune tolerance arise in early pregnancy to dampen inflammation and suppress allo-reactive immune responses that otherwise threaten conceptus survival. These include attenuated expression of polymorphic MHC molecules on placental tissues, trophoblast production of anti-inflammatory and pro-tolerogenic cytokines and hormones, and epigenetic modulation of decidual cell chemokine expression to

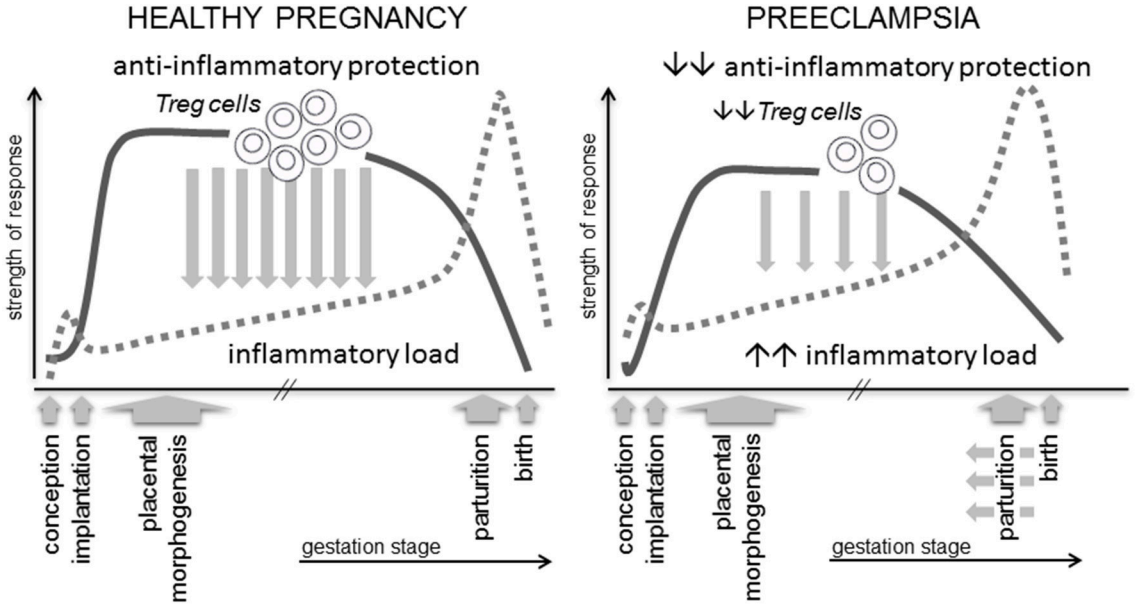


FIGURE 1 | A robust immune response is essential for healthy pregnancy. Regulatory T cells (Treg cells) are a critical and rate-limiting element of the anti-inflammatory protection required to suppress inflammation and prevent adverse effects of anti-fetal effector immune responses. Treg cells arise as a consequence of an inflammation-like response during the peri-conception phase, and their abundance, suppressive function and stability are influenced by events prior to and during early pregnancy. Insufficient numbers, reduced suppressive function or instability are linked with preeclampsia and IUGR, potentially mediated by insufficient Treg cell capacity to support normal placental development and to suppress the elevated inflammatory load typical of this condition.

prevent effector T cells (Teff) cells accumulating at the maternal-placental interface (43–45).

Amongst the various mechanisms of maternal tolerance, $CD4^+$ Treg cells are essential for embryo implantation and early placental development (46, 47). Their capacity to constrain and resolve the inflammation elicited during embryo implantation, and suppress generation of immune effector cells in local lymph nodes, is pivotal to controlling inflammation and promoting immune tolerance over the course of gestation, until an inflammatory shift emerges again at parturition (**Figure 1**). This function is consistent with essential roles for Treg cells in immune homeostasis throughout the body, where they prevent autoimmunity to self-antigens, suppress Teff cells reacting to non-dangerous foreign antigens, regulate and limit excessive inflammation (48–50), and have important roles in tissue repair and homeostasis (51).

Different subsets of T cells with regulatory functions exist. $CD4^+$ Treg cells, $CD8^+$ Treg cells, gamma/delta T cells, Tr1 cells, and NKT cells can all exert suppressive functions and appear to operate collaboratively to control immune responses. $CD4^+$ Treg cells are of particular interest because of their strong association with preeclampsia, and their potential for therapeutic manipulation (41). $CD4^+$ Treg cells comprise about 1–3% of total T helper cells in humans and 3–10% in mice, and are defined by their expression of the master transcription factor Forkhead Box P3 (FOXP3). As well as FOXP3, $CD4^+$ Treg cells constitutively express surface molecules including the IL2 receptor α-chain (CD25), the immune checkpoint receptor cytotoxic T-lymphocyte protein 4 (CTLA4), and glucocorticoid-induced tumor necrosis factor receptor (GITR), and in humans are $CD127^-$ or $CD127^{low}$ (49, 52).

There are two types of $CD4^+$ Treg cells (referred to hereon as "Treg cells"). Thymus-derived Treg cells (tTreg cells) emerge from the thymus after self antigen-driven selection as functional suppressor T cells. Peripheral Treg cells (pTreg cells) differentiate from naïve $CD4^+$ precursors after contact with antigens in peripheral lymph nodes or tissues (52). Differentiation of naïve $CD4^+$ T cells into pTreg cells requires cognate antigen to be presented by antigen-presenting cells (APCs) such as pro-tolerogenic dendritic cells (tDCs) in the presence of IL2 and TGFB. The CD4 cells are thereby induced to express FOXP3 and become committed to suppressive function (53). These cells then promote a cycle of *de novo* Treg cell generation and drive the development of long-lasting immunologic memory, which is reinforced by persistent antigen exposure (54). Like pTreg, tTregs can also be induced to proliferate and acquire greater suppressive function by antigen contact in the periphery (51, 55, 56). In humans, tTregs and pTregs are not readily distinguishable but in mice, tTregs express neuropilin 1 (Nrp1) while pTregs are generally Nrp1 low or negative (52).

pTreg cells and tTreg cells exert anti-inflammatory and immune suppressive activity by secreting a range of soluble factors including IL10 and TGFB, as well as through cell contact-dependent mechanisms. Importantly, Treg cell suppressive function inhibits proliferation and cytokine release from pro-inflammatory $CD4^+$ Teff cells, T helper 1 (Th1) and T helper

17 (Th17) cells, which typically produce pro-inflammatory IFNG and IL17, respectively. Activated Treg cells interact with DCs through CTLA4, to cause down-regulation of DC co-stimulatory molecules CD80 and CD86, which drive Teff cell activation (49).

ALTERED TREG CELLS ACCOMPANY AND MAY PRECEDE PREECLAMPSIA ONSET IN WOMEN

In women, T cells comprise 10–20% of decidual immune cells in the first trimester (57). Many decidual T cells are $CD8^+$, including regulatory subsets (58, 59). Amongst the $CD4^+$ T cells, around 10–30% express FOXP3, which is a substantial enrichment compared to peripheral blood (60–62). The Tregs comprise of both tTregs and pTregs and exhibit heterogeneous phenotypes that vary across the menstrual cycle and phase of pregnancy (32, 63, 64).

There is substantial evidence that many pregnant women with preeclampsia have fewer and less functionally competent Treg cells, accompanied by increased Teff cell activity, particularly Th1 and Th17 cells in decidual tissue and peripheral blood (26–28, 34, 65, 66). In a recent meta-analysis, a total of 17 independent primary studies were evaluated, and all but 2 showed consistent evidence of association between both severe, early-onset and late onset preeclampsia with fewer Treg cells in the third trimester (67). As well as reduced numbers, the suppressive function of Treg cells is often compromised in preeclampsia (33, 34, 68). The decrease in Treg cells may be proportional to the severity of disease (26), although relationship with time of disease onset and co-incidence of fetal growth restriction have not been consistently documented. There is evidence of an altered balance in Treg cell subsets in preeclampsia, with reports of fewer peripheral blood naïve $HLADR^{neg}$ $CD45RA^+$ Treg cells (68, 69) and fewer $CD45RA^+CD31^+$ recent thymic emigrant Tregs (64) in peripheral blood. Decidual Treg populations may be differentially affected, given decidual tDCs exhibit a reduced capacity to induce pTreg in preeclampsia (32).

Treg cell changes become evident in peripheral blood and gestational tissues shortly after conception and accumulate in decidua reaching their highest levels in early to mid-gestation, before decreasing as term approaches (28, 61, 70). A recent study utilizing chorionic villous sampling (CVS) at week 10–12 of gestation, showed that women who progress to preeclampsia demonstrate dysregulated expression of decidual and immune cell genes from this early time (71). In another study, elevated expression of IL6 which counteracts Treg stability and promotes Th17 generation (72), as well as reduced numbers of alternatively activated M2 macrophage and T cell markers, were detected in CVS tissues of women who later develop preeclampsia associated with fetal growth restriction (IUGR) (73). Although longitudinal studies to track Treg cells over the course of gestation are not yet reported in women with preeclampsia, there is good evidence that low abundance of circulating Treg cells in the first trimester is predictive of miscarriage before 12 weeks (74). Collectively, these observations underpin a working hypothesis that disturbed immune adaptation in early pregnancy precedes impaired placental development, setting the scene for later emergence of preeclampsia and related complications of pregnancy (8, 10, 29, 75).

This fits an emerging paradigm which positions early pregnancy as the origin of disorders of deep placentation that underpin early onset, severe preeclampsia, and also contribute to IUGR, preterm labor, premature rupture of membranes, and late spontaneous abortion (11, 76, 77). So-called shallow placentation arises from insufficient trophoblast invasion and failure to adequately remodel spiral arteries and to achieve high capacity maternal blood flow, which further compromises placental development and function, and leads to IUGR (1, 7, 8).

Treg cells are emerging as key regulators in the decidual leukocyte network which controls implantation and placental development. Through interactive cross-regulation, growth factor secretion and extracellular matrix remodeling, this network controls the decidual immune environment which facilitates trophoblast invasion and cytotrophoblast shell development, and enables remodeling of the decidual vasculature to support placental development (10, 78).

Inappropriate function or insufficient numbers of Treg cells in the decidua are linked with inadequate extravillous trophoblast invasion, and poor spiral artery remodeling, in turn destabilizing placental development and resulting in "shallow" placentation (12, 79). There is also a clear link between Treg deficiency and both recurrent implantation failure and recurrent pregnancy loss, where more severe forms of impaired uterine receptivity arrest trophoblast invasion and early placental development (80, 81). Thus, it is not difficult to envisage how insufficient Treg cells in the preconception and peri-conception phase could be a key upstream trigger for the sequence of events leading to impaired vessel remodeling and shallow placentation, which ultimately cause the overt symptoms of preeclampsia in later gestation (**Figure 2**).

TREG CELL REGULATION OF THE DECIDUAL IMMUNE ENVIRONMENT

Mouse models have been instrumental for defining mechanisms through which Treg cells exert anti-inflammatory activity to influence the decidual environment and early placental development. Kinetic studies show that Treg cells accumulate in the uterine decidua from very early in pregnancy, and that these originate after naïve T cell activation and proliferation in local lymph nodes, causing numbers to expand through the first half of gestation (20, 46). After recruitment into the implantation site, Treg cells comprise around 30% of decidual T cells in the mouse (46).

Extensive experiments wherein Treg cells are selectively depleted, or overwhelmed by exacerbated Teff cell responses, show an essential role for Treg cells in preventing generation of destructive immunity to fetal alloantigens (82–85). Without sufficient Treg cells, an aggressive Th1 and Th17 mediated-response causes fetal loss in allogeneic but not syngeneic pregnancy (46).

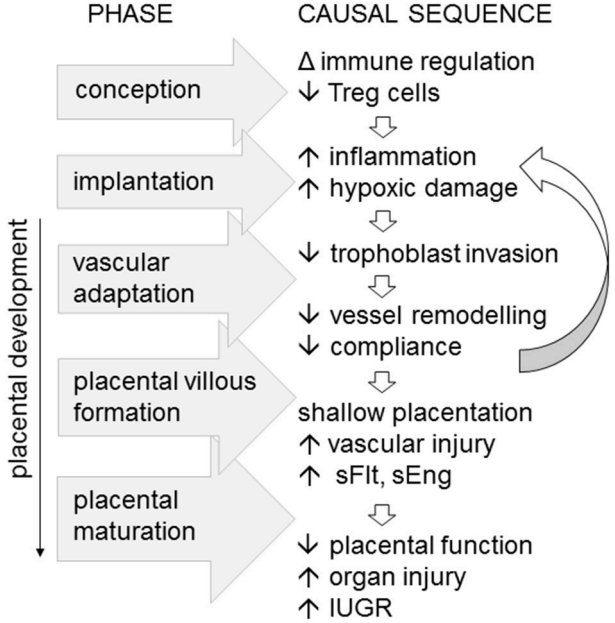


FIGURE 2 | Immune imbalance associated with insufficient or incompetent Treg cells are implicated as an upstream cause of preeclampsia, particularly in severe, early onset disease. In a working model of the sequence of pathophysiological events, fewer Treg cells generated at conception impact the decidual environment at implantation, to limit trophoblast invasion, constrain maternal vascular adaptation, and vessel compliance. The resulting "shallow" placentation causes vascular inflammatory injury accompanied by elevated soluble Fms-like tyrosine kinase (sFlt) and soluble endoglin (eEng), reducing placental function, and causing maternal organ injury and *in utero* growth restriction (IUGR) of the fetus.

Depending on the severity and timing of manipulation, Treg depletion can manifest as implantation failure, miscarriage or fetal growth restriction. Several studies show the pre- and peri-implantation phase is highly vulnerable. Administration of anti-CD25 Ab before or shortly after mating causes complete implantation failure (86–88). Depleting $FOXP3^+$ cells from *FOXP3-Dtr* mice during early placentation increases later fetal resorption (85, 89), but depletion in mid-gestation only moderately reduces fetal viability (20), unless mice receive a second hit inflammatory challenge such that Treg depletion exacerbates the adverse impact (90–92). Mice deficient in T cells due to *Rag1*-null mutation are highly vulnerable to inflammation-induced fetal loss, but this is reversed by administering $CD4^+$ T cells that differentiate to Tregs after transfer (90). Midgestation depletion of $CD25^+$ cells using anti-CD25 mAb has a less severe impact than in early pregnancy, but this may be because Teff cells are also removed (86, 93). Additionally, other tolerogenic mechanisms including IL10 secretion by uNK cells (94) may compensate for Treg deficiency once placental development is complete.

Mouse models with a high rate of spontaneous fetal loss also demonstrate a critical role for Tregs in embryo implantation. CBA/J females mated with DBA/2J males have fewer decidual Tregs and elevated Th1 cells (87, 95). Adoptive transfer of Tregs from donor CBA/J females mated with Balb/c males boosts decidual Tregs and corrects fetal loss (87), but only if Treg transfer occurs before embryo implantation (87). These findings confirm that Tregs are most essential in the uterus during the peri-implantation period, consistent with a central role in orchestrating the transition to an anti-inflammatory mileu required for placental development (**Figure 1**).

Treg cells co-localize in clusters with uNK cells and other leukocytes in the human decidua basalis (78), where they exhibit activity expected to potently influence the local immune environment by enforcing an anti-inflammatory phenotype in other leukocyte lineages. In particular Tregs regulate uNK phenotype, through releasing TGFB and IL10 to control DC release of uNK viability factor IL15 (96), and suppress uNK cytolytic activity (91, 97). This may be particularly important in first pregnancy, given that uNK cells acquire memory and assume a more differentiated "trained" phenotype in subsequent pregnancies (21). Whether there is an interaction between antigen-experienced Tregs and trained uNK cells, remains to be investigated.

Treg cells also regulate M2 macrophages (98), mast cells (99), and tDCs, releasing heme oxygenase-1 which maintains immature DCs (100) and promotes indoleamine 2,3-dioxygenase (IDO) production to impair Th1 cell survival (101, 102). M2 macrophages and tDCs promote further Treg generation (98, 100) and produce an array of cytokines that reinforce a pro-tolerogenic decidual environment, including TGFB, CSF2 (GMCSF), IL4, IL10, CSF3 (GCSF), and prostaglandin E (103). Decidual Tregs also express other hallmark mediators of Teff suppression CD25, CTLA4, and PD-L1 (61, 91, 104–106).

Uterine NK cells and DCs are implicated as key regulators of decidual transformation (107–109) so through regulating uterine DC and uNK phenotype, Tregs would indirectly influence the extent and quality of the decidual response. Furthermore, trophoblasts engage with Tregs in a reciprocal interaction to modulate the secretory profile of both lineages (110). Together, these coordinated interactions allow Tregs to constrain inflammation and limit oxidative stress caused by trophoblast invasion during early placental development (13, 25, 111).

TREG CELLS INFLUENCE MATERNAL VASCULAR REMODELING AND EARLY PLACENTAL DEVELOPMENT

Treg cells are emerging as critical participants in the process of maternal vascular remodeling, through their modulating effects on the decidual leukocyte network (**Figure 3**). There is extensive evidence to demonstrate key roles for uNK cells (30, 112), macrophages (31), and mast cells (99) in decidual vessel transformation, and in collaborating with invading trophoblasts to restructure the endothelial surface and smooth muscle wall (7, 13, 104). As detailed above, Treg cells exert potent anti-inflammatory actions on uNK cells (91, 97), M2 macrophages (98), mast cells (99), and tDCs (100), thereby influencing the vascular remodeling process.

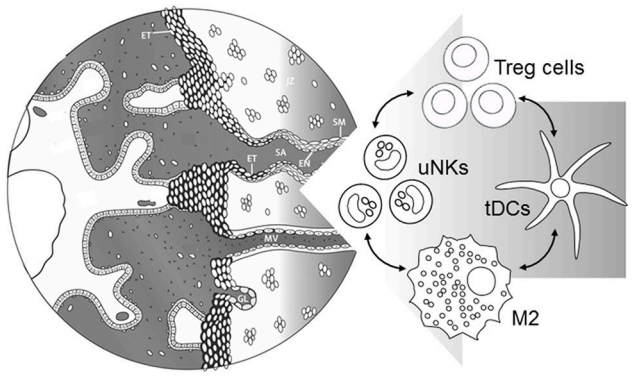


FIGURE 3 | Events during early placental development require immune cells in the decidua to support maternal blood vessel (MV) adaptation and spiral artery (SA) transformation. Treg cells interact with uNK cells, M2 macrophages, and tDCs to suppress inflammation and provide secretory factors regulating extravillous trophoblast (ET) invasion, and functional changes in endothelial cells (EN) and surrounding smooth muscle (SM). Severe early-onset preeclampsia is accompanied by insufficient Treg cells, which in turn would be expected to contribute directly and indirectly through immune cell networks to impair vascular adaptations required for robust placental development. Left hand part of Figure is adapted from Steegers et al. (1).

This is unsurprising given growing evidence that Treg cells play important roles in modulating cardiovascular function, and vascular homeostasis throughout the body (113). In hypertensive mouse models, Treg cell infusion reduces blood pressure and vascular damage, and reverses hypertensive sequelae (114, 115). Treg cell-derived cytokines, particularly IL10 and TGFB, suppress inflammatory endothelial cell activation and inhibit development of atherosclerosis (113).

Rodent models of preeclampsia support a critical function for Treg cells in the pathophysiological events underlying abnormal placental development, through coordinated interactions with uNK cells, DCs, and macrophages (**Figure 3**). Experiments in mice deficient in T cells and/or NK cells show that T cells interact with uNK cells to influence the maternal hemodynamic response to pregnancy (116, 117). When cause-and-effect relationships are explored by antibody-mediated or genetic modulation of T cell subsets, Treg cells are implicated as having causal roles in the maternal and fetal symptoms of preeclampsia models.

Treg-deficient mice consistently show impaired uterine spiral arterial modification, reduced placental blood flow, and fetal growth restriction (85, 89, 118). Depletion of $FOXP3^+$ Tregs in early pregnancy causes later dysfunction in uterine arteries accompanied by increased endothelin-1 production (89). Peripheral Treg cells are particularly implicated, as indicated by experiments in mice with a null mutation in the CNS1 gene which is a *FOXP3* enhancer element essential for pTreg cell but dispensable for tTreg generation. CNS1 is only present in eutherian mammals, suggesting its introduction into the FOXP3 locus to enable pTreg generation, in turn facilitated evolution of placentation (85). In CNS1-null mice, impaired remodeling of material spiral arteries underpins defective placental development (85). Compromised trophoblast invasion and failed transformation of spiral arteries is also seen in mice where neutrophil depletion causes insufficiency of pro-angiogenic, neutrophil-induced Treg cells (118).

In the reduced uterine perfusion pressure (RUPP) model of preeclampsia in rats, reduced uterine artery flow is induced by clip placement on the abdominal aorta and right and left uterine artery arcades at day 14 of gestation, resulting in placental ischemia and oxidative stress. The model replicates human preeclampsia symptoms with hypertension accompanied by increased circulating VEGF, sEng, Flt1, and placental growth factor (PlGF), plus elevated inflammatory cytokines and IUGR. A substantial (∼50%) reduction in decidual and placental Treg cells, and elevation in total $CD4^+$ T cells and Th17 cells, is a consequence of the RUPP intervention. Remarkably, the preeclampsia symptoms induced in this model are T cell dependent since the RUPP intervention does not cause hypertension and IUGR in T cell deficient athymic rats, and disease can be induced by passive transfer of Th17 effector $CD4^+$ T cells (119). Treg cell deficiency is a key driver of hypertension and IUGR and these symptoms are mitigated when Treg cells from pregnant control donor rats are administered shortly after the RUPP procedure (120). Treatment strategies applied to boost endogenous Treg cells, including IL10 administration (121) or low dose CD28 superagonist (120), also reduce hypertension and IUGR.

Rodent models show that the protective effects of Treg cells are crucial from the early implantation phase, when vascular adaptation and early placental development begin. Several studies using different approaches to deplete Treg cells at various time points show the peri-implantation phase is most severely affected, with extensive Treg cell depletion at embryo implantation causing complete implantation failure (46, 86, 87). Experiments in the abortion-prone CBA/J x DBA/2 mouse model indicate transferred Treg cells can rescue the underlying placental defect, but only if Treg cells are transferred from healthy pregnant mice at or before the time of embryo implantation (87). Treg cell replacement influences other immune cells in the decidua, including mast cells, to repair placental and vascular defects and prevent sFlt elevation and fetal loss (99). Consistent with a critical role for peri-implantation Treg cells, a genetic model of preeclampsia involving overexpression of human angiotensinogen and renin in rats showed greater responsiveness to Treg cell therapy when it was applied in early gestation (122).

Even subtle disturbances to the T cell response in early pregnancy may impact later pregnancy progression. This may be the consequence of an altered environment during T cell activation, as we have recently demonstrated for $CD8^+$ T cells in pregnancy (123). Other studies in mice show that reduced numbers or altered function of Treg cells at conception can disrupt fetal-placental development without immediate adverse effects, but with a legacy that becomes apparent in mid- or late gestation, particularly when a "second-hit" inflammatory challenge is applied (106, 124).

THE TREG CELL RESPONSE IS DETERMINED IN THE PRE- AND PERI-CONCEPTION PHASE

The conditions under which the Treg cell populations of pregnancy originate are likely to be critical to the reduced quantity and impaired quality of the Treg response in preeclampsia. Uterine recruitment of Tregs in readiness for possible embryo implantation commences in the proliferative phase of each cycle, with an estrogen-driven increase peaking around ovulation (125). $CD4^+$ $FOXP3^+$ cells, thought to be pTregs based on expression of the Helios marker, are a major subset amongst the expanding Treg populations in blood and decidua in early human pregnancy (32). $Helios^+$ Treg cells appear to be preferentially recruited into the decidua in the first trimester (63). Amongst peripheral blood tTregs the population of $CD45RA^+CD31^+$ cells, which have recently emigrated from the thymus, expand prominently in the first trimester and differentiate into $CD45RA^-CD31^-$ memory Tregs (64).

The majority of decidual T cells in women have a memory phenotype ($CD45RA^-$ or $CD45RO^+$) (59, 126) and show evidence of fetal antigen specificity (62), which indicates antigen exposure must occur to elicit the full Treg cell response. HLA-C is the only polymorphic HLA expressed by human placental trophoblasts, and fetal-maternal HLA-C mismatch is associated with a greater expansion in decidual Tregs (127). Many decidual Tregs show fetal HLA-C antigen specificity (62, 128), but whether other reproductive or tissue antigens are involved has not been investigated.

In preeclampsia, Treg cell deficiency is most pronounced in pTreg cells (32), as well as $CD45RA^+CD31^+$ recent thymic emigrant tTregs less able to acquire a memory phenotype (64). This implies there may be an underlying problem with antigen priming. Consistent with this, dysfunctional DCs with reduced HLA-G and ILT4 (32), and/or insufficent PD-L1 (129), have been reported in preeclampsia.

Contact with fetal alloantigens must occur under conditions that favor antigen presentation and stable Treg cell (not Teff cell) development. These conditions occur in two waves in the reproductive process. Paternally-derived transplantation antigens shared by the fetus are first and most frequently contacted during transmission of seminal fluid at coitus, at conception and in pre-conception cycles (22). Seminal fluid primes the activation of pTregs that are specific for paternal transplantation antigens which will later be expressed by fetal and placental cells. Additionally, once pregnancy is established and maternal blood comes into contact with the syncytiotrophoblast surface, placental exosomes are released into maternal blood, providing a second wave of alloantigen exposure (130, 131).

Again, mouse models have been informative in tracing the origins and regulation of Treg cells and point to specific events as critical for generation of the Treg cell pool in early pregnancy (132). The two stages of T cell activation can be tracked through the first half of gestation using T cell transgenic mice (93). The strength of seminal fluid as the initial priming event is first seen as a burst of T cell proliferation in the peri-conception phase, evident in cells recovered from the uterus-draining para-aortic lymph nodes (dLN) on day 3.5 post-coitus (pc), followed by a steady progressive increase during the post-implantation phase once placental morphogenesis is complete (93).

The first wave of proliferation of Treg cells can be detected within days of insemination in the lymph nodes draining the reproductive tract, in the peripheral blood, and spleen (46, 133). Seminal fluid contains paternal alloantigens and high levels of TGFB, and elicits an inflammation-like response in female reproductive tract tissues. DCs and macrophages recruited into female tissues take up seminal fluid alloantigens, traffic to the dLN and present antigen to naive T cells (93). Treg expansion is maximized in allogeneic compared with syngeneic matings, demonstrating a contribution of male alloantigens (134), but endogenous antigens might also contribute to the activation and expansion of Tregs in early pregnancy (135). Amongst the responding pTregs, paternal antigen-reactive pTreg cells are selectively enriched (133, 136). Data from mice with a mutation in the CNS1 gene show elevated fetal loss when pTreg alone are deficient, suggesting that pTreg cells have non-redundant functions important for viviparous pregnancy (85).

A population of tTreg cells of thymic origin also expand systemically prior to conception. These cells are recruited into the uterus after proliferation in the dLN during the estrous stage of the reproductive cycle in mice, in response to rising estrogen at ovulation (125, 137). After mating, factors in seminal fluid induce tTreg to proliferate and express elevated FOXP3 and CTLA4, both markers of suppressive competence, accompanied by demethylation of the Treg-specific demethylation region (TSDR) in the *FOXP3* locus (138). This expansion of tTreg cells occurs in parallel with the seminal fluid antigen-driven expansion of pTreg cells. This population may well have different functional qualities to pTreg, although these are still to be defined.

After recirculation via peripheral blood, Treg cells are recruited into the fetal-maternal interface in response to chemokines secreted by uterine epithelial cells including CCL19 (133), and may be stimulated to undergo further rounds of proliferation locally in the uterine tissue (46). The resulting expansion of the Treg cell pool induces a state of hypo-responsiveness to paternal alloantigens, concurrent with embryo implantation when the conceptus first contacts maternal tissues (136, 139). The kinetics of Treg cell induction in the peri-conception phase ensures sufficient abundance of Treg cells in the endometrium at embryo implantation, when their function is most critical (86, 87, 140). Continued release of paternally-inherited alloantigen from trophoblasts over the course of pregnancy sustains the T cell response until post-partum (20, 93). After birth, a population of paternal alloantigen-reactive Tregs are sustained, and in the event of a subsequent mating with a male expressing the same alloantigens, there is accelerated expansion of Treg cells driven by proliferation of fetal-specific Treg cells retained from the prior pregnancy (20).

Mouse studies imply that the immune response initiated at seminal fluid priming is a crucial initiating step and highly vulnerable phase for Treg cell tolerance to be established. In particular, responding pTreg cells require appropriate environmental signals including the cytokines IL2 and TGFB,

to ensure naïve T cells differentiate into Treg cells and not Th1 or Th17 effector T cells (93, 133). Both the size of the Treg cell pool and the suppressive competence of pTreg cells will be determined by the strength of the antigenic challenge, and the nature of the cytokine context in which antigen contact occurs—parameters which are determined by seminal fluid composition as well as female tract factors. Since newly generated pTreg cells that have only recently commenced FOXP3 expression appear more vulnerable to phenotype switching and lineage instability (141), the extent to which pTreg cells primed at coitus will commit to a secure Treg fate will be substantially influenced by the conception environment. Relevant factors impacting this environment would include MHC disparity between male and female partners, the abundance and phenotype of DCs involved in antigen presentation, and bioavailability of local cytokines, hormones and other positive and negative regulators including microRNAs and the local microbiome (23).

Similar events occur in women, where the cervical immune response to seminal fluid mirrors the mouse response, causing elevated cytokine production, recruitment of leukocytes and T cell activation (22, 142), consistent with prior seminal fluid contact contributing to priming the paternal antigen-specific Treg cell response of pregnancy (62). It is yet to be proven that seminal fluid induces pTreg cells in women, and other factors must contribute to uterine Treg accumulation since IVF pregnancy can be established without seminal plasma contact. Expansion of uterine Tregs after conception may be further facilitated by human chorionic gonadotropin (hCG) secreted by invading placental trophoblasts (143). This builds on the hormone-driven expansion of Treg cells in the follicular phase of the menstrual cycle, correlating with progressively elevating serum E2 levels (125). However, the *in vivo* cervical response and an array of *in vitro* studies demonstrating that seminal fluid skews DC cells to an tDC phenotype and induces Treg cells *in vitro*, is consistent with a key role for seminal fluid in women (22, 144, 145). A priming effect of seminal fluid contact in women also explains the benefit of cumulative seminal fluid contact with the conceiving partner in protecting from preeclampsia (17, 146).

IS INSUFFICIENT PRIMING A CAUSE OF TREG CELL DEFICIENCY IN PREECLAMPSIA?

An important question is why some women have fewer Treg cells and/or impaired Treg function at the outset of pregnancy. The nature and significance of factors that cause variation in the uterine Treg cell response are unclear and require investigation. As detailed above, antigen priming in the appropriate environmental context is a critical factor in the strength and quality of any peripheral tissue Treg cell response. In the reproductive tissues, the strength and quality of antigen and immune-regulatory signals in the female reproductive mucosa during priming would be paramount, as well as the number and timing of prior exposures to the conceiving partner's seminal fluid and any previous pregnancies with that partner (25).

This raises the possibility that some women develop preeclampsia after conceiving without adequate prior priming to male partner alloantigens. It seems likely that pTregs reacting with paternal alloantigens would be more vulnerable than tTregs to variations in population size, antigen experience and memory, functional competence, and stability. Because newly generated pTregs are particularly susceptible to phenotype-switching and lineage instability (141), the priming environment would be a key determinant of a secure fate amongst pTreg with male partner alloantigen specificity. Recent evidence in mice that seminal fluid contact regulates tTreg cells, inducing proliferation and reinforcing a suppressive phenotype through epigenetic modulation, suggests tTreg as well as pTreg are impacted (138).

Priming may be dysregulated due to seminal fluid composition or female responsiveness to seminal fluid signals (22, 25). It has been shown that recurrent miscarriage patients produce more $CD4^+IL17^+$ and $CD4^+IFNG^+$ cells and fewer $CD4^+CD25^+FOXP3^+$ Tregs, compared to fertile controls, when $CD4^+$ T cells are cultured with DCs and partner's seminal fluid antigens (147). The balance of immune-regulatory agents in seminal fluid, particularly pro-tolerogenic TGFB, varies between men, and within men over time (148). The anti-tolerogenic cytokine IFNG, which drives generation of Th1 immunity, fluctuates substantially and can become elevated in seminal fluid in the event of infection or other inflammatory conditions (149). IFNG interferes with synthesis of CSF2 required to drive the T cell proliferative response at conception (150, 151), skews Th0 differentiation toward Th17 cells (48, 152), and increases Treg susceptibility to transdifferentiate into Th17 cells (153).

CLINICAL AND LIFESTYLE FACTORS IMPACTING THE TREG CELL RESPONSE

A suboptimal Treg cell adaptation for pregnancy could also occur in women due to intrinsic Treg deficiency. The specific factors determining between individual variation in Treg numbers and functional capacity are yet to be fully defined. An interaction between genetic, epigenetic, and environmental factors seems likely, based on data from animal models and limited studies in population cohorts (154). The thymic output of tTreg, and peripheral tissue induction of pTreg cells, are independently regulated and can be affected by a range of metabolic and nutritional parameters, inflammatory exposures, autoimmune conditions, and age (35–40). Common health conditions that affect the immune system including intestinal microbial dysbiosis and dietary deficiencies, particularly vitamin A and vitamin D, have been associated with poor adaptive immunity and may be a common cause of compromised Treg activity (38). Exposure to sunlight (55) and exercise (56) are also recently identified to support Treg homeostasis, but how these are linked to variation in human Treg parameters are yet to be defined.

In hyper-inflammatory conditions caused by autoimmune, infectious or metabolic disorders some pTreg cells exhibit phenotypic plasticity and instability, with increased disposition to shift phenotype, or lose *FOXP3* expression and become reprogrammed into a Teff fate (154). Studies in mice and

humans demonstrate that FOXP3$^+$ T cells can be induced by inflammatory stimuli to express IL17 and IFNG characteristic of Teff cells (155, 156), and may then transdifferentiate into effector Th17-like cells, known as "exTregs," which can amplify inflammatory pathology (157).

Epigenetic regulation of *FOXP3* through demethylation of the TSDR region is a key factor in the resilience of Tregs to inflammatory stress, that controls whether T cells can express sufficient *FOXP3* to overrule Teff functions and maintain a Treg suppressive phenotype (158). Increased TSDR methylation and associated underexpression of *FOXP3* is a feature of some autoimmune conditions (159).

There is little information on whether Treg cells exhibit signaling defects, lineage instability or methylation changes in preeclampsia. Given the evidence of elevated Th1 and Th17 cells counteracting the decrease in Treg number and function in preeclampsia, defects in both Treg cell induction and/or stability seem plausible, and would explain the concurrent reduction in Treg cell suppressive function (34, 68). Although large populations have not been examined, exploratory studies in preeclampsia suggest an elevated incidence of gene variants within the promoter region of *FOXP3* that may affect expression levels and hence Treg stability (160, 161). Elevated IL6 trans-signaling, which is known to promote Treg instability and transdifferentiation, has been described in women with recurrent miscarriage (162). IL6 is associated with reduced TGFB output and IL2-mediated STAT5 signaling (163), and is a possible candidate contributing to impaired Treg capacity in preeclampsia, given that elevated expression of IL6 is seen in gestational tissues of women who later develop the condition (73).

POTENTIAL INTERVENTIONS TO TARGET TREG CELLS IN PREGNANCY

Recognition that excessive inflammation secondary to insufficient anti-inflammatory protection is a key driver of preeclampsia gives rise to the prospect of targeting the immune response to prevent or suppress progression of the disease. In particular, Treg cells provide an attractive target, because of (1) the clear link between compromised Treg cells and preeclampsia; (2) a logical mechanistic pathway placing insufficient Treg cells as an upstream event in the placental and systemic pathophysiological sequalae; (3) compelling evidence from preclinical rodent models showing that insufficient Treg cells can elicit preeclampsia-like symptoms, while boosting Treg cells mitigates symptoms, and (4) encouraging progress in development of Treg cell therapies for other autoimmune and inflammatory conditions.

Interventions to boost Treg cell populations and their suppressive competence are under development and show promise in autoimmunity and tissue transplantation (41, 42), and more recently have been considered for cardiovascular disease (113). Treg cell therapies relevant to preeclampsia could take one of three alternative approaches: (1) lifestyle and health advice during preconception planning to assist immune adaptation to pregnancy; (2) neutraceutical, pharmacological, or other strategies to increase Treg cell numbers and/or function in an antigen non-specific, systemic manner, or (3) cell therapy treatments that involve *ex vivo* generation and/or expanding Treg cells in a highly-individualized process. These clearly represent different degrees of technical challenge, invasiveness, cost and risk. While lifestyle adjustments or dietary supplements are generally safe and tractable, cell therapies are labor-intensive, expensive, and higher risk.

The evidence base for approaches to target Tregs in other clinical settings is building (41, 42), but to date little consideration has been given to applications in reproductive conditions. To advance new treatments targeting Treg cells for preeclampsia prevention and mitigation, research on several fronts is required. Most immediate goals should be to develop appropriate diagnostics, and to investigate and validate pre-pregnancy planning interventions to boost Treg cells. There should also be careful consideration of the rationale for initiating clinical studies, using robust clinical trial methodology, to evaluate pharmacological and/or cell therapy treatments for application when Treg deficiencies are not responsive to lower intervention approaches.

Diagnosis of Treg Cell Deficiency

To progress understanding of Treg cell insufficiency in preeclampsia, and to develop therapeutic options targeting these, it is essential that effective diagnostic tools are developed and validated. These should detect common and informative defects in Treg cell parameters that define competency for healthy pregnancy, and ideally be applied to peripheral blood if preference to endometrial biopsies. Treg tests should be appropriate for routine use during pregnancy planning or early after conception, to provide a therapeutic window for early treatment interventions to prevent progression to miscarriage or later obstetric conditions. To date little work has been done to investigate Treg cell deficiency before conception, or in early pregnancy, in the blood or endometrium of women who go on to develop preeclampsia. Ongoing studies to address the pre-pregnancy origins of preeclampsia may begin to address this (9).

A recent meta-analysis of Treg cell parameters quantified in preeclampsia highlights the considerable variability in markers that different groups have measured to date (67). A useful step will be to develop a consensus definition of minimum essential Treg markers to facilitate harmonization across future studies, and to determine the best stage in preconception cycles or early pregnancy for analysis (164). Given the significance of tTreg cells vs. pTreg cells, and of naïve vs. memory cells in preeclampsia (68, 69), extensive marker panels to discriminate these subsets in flow cytometry-based tests will be most informative. Along with standard markers CD4, CD25, CD127, and FOXP3, markers that reflect memory, suppressive capacity, and activation status amongst Treg cells should be measured. These may include GITR which is emerging as a superior marker of active, functional Tregs (165), plus CTLA4, CD45RO, HLADR, and potentially intracellular cytokines or transcription factors which appear particularly informative in the preeclampsia setting (27, 34).

Ideally, Treg cell assays should also inform on suppressive competence in a paternal antigen-specific manner. Assessment of suppressive function by *in vitro*-based assays and/or analysis of *FOXP3* methylation status has been the gold standard for assessment of suppressive potential, but new markers such GITR, CD154, and PI16 may supersede these tests and be more amenable to a clinical diagnostic setting (165–167). With increasing availability of tetramer-based diagnostic tools for identifying TCR specificity, identification of partner alloantigen-reactive Treg cells may in time become feasible.

Optimal Timing for Interventions

A major challenge for developing strategies to target Treg cells in pregnancy is timing—how and when would Treg cell deficiency be diagnosed, and how would this relate to the window of opportunity for intervention? With evidence that Treg cells are most critical during the implantation and early placentation phase of pregnancy, interventions in cycles prior to conception, or as soon as possible after conception would likely be most advantageous. The timing would need to align with hormone-driven regulation across the menstrual cycle, and might leverage events controlling estrogen and progesterone-regulated expansion of the Treg pool (125). Interventions would need to be coupled with Treg screening of high risk women during pre-pregnancy planning or early after conception to allow the best chance to identify patient subgroups that could be amenable to therapy.

Treg Cells and Preconception Care

A tractable approach worthy of further investigation is advice on immune system health and boosting immune priming during preconception planning. In nulliparous women, the available evidence suggests on average, 3–6 months of sexual cohabitation without using barrier contraceptives is required for sufficient seminal fluid priming to minimize the chance of preeclampsia (15). Consistent with this, in a recent study of 340 women, women in the highest 10th percentile of exposure to partner's seminal fluid had a 70% reduced odds of preeclampsia relative to women in the lowest 25th percentile (168). Thus, advising nulliparous women to avoid use of barrier contraceptive methods and to consider increasing vaginal coitus prior to conceiving may reduce preeclampsia risk. A key question is the impact of different non-barrier approaches to facilitating immune priming, such as oral contraceptive pill or intrauterine device, which both deliver immune-modulating hormones. Further studies are required to evaluate the impact on preeclampsia rates of preconception advice on seminal fluid contact and contraceptive choice, and to investigate whether a partner-specific Treg cell response is involved.

Detecting and correcting any immune imbalance due to clinical, nutritional and/or, lifestyle factors is also likely to be effective for pregnancy planning and reducing susceptibility to preeclampsia. Elevated inflammatory load due to chronic infection, smoking, diabetic and pre-diabetic conditions, obesity and/or microbiome dysbiosis in women would be expected to adversely affect intrinsic Treg cell parameters and responsiveness to priming (35, 169), while in men these conditions may increase seminal fluid IFNG and reduce capacity to elicit a healthy female response (149). A range of autoimmune conditions known to impact reproductive function likely have a shared underlying etiology and correcting the immune disorder with validated approaches would reasonably yield dividends for pregnancy health (170). Microbiome disorders, and vitamin and micronutrient deficiencies also affect Treg cells, and treating these might have utility in boosting Treg cell activity in the reproductive setting, as has been shown for some other immune disorders (38). It will be important for future studies to trial the efficacy of alternative approaches to pre-pregnancy care, in order to determine the most effective interventions.

Pharmaceutical Interventions to Expand Treg Cells for Pregnancy

High-risk women with a previous pre-eclamptic pregnancy are an obvious target for preconception care to boost immune tolerance. However, duration of sexual cohabitation is unlikely to be limiting in this patient group, and couple-intrinsic issues such as insufficient HLA disparity between partners, or HLA incompatibility resulting in low immunogenicity of male alloantigens, could theoretically interfere with priming and expansion of the Treg cell pool. In selected women with a demonstrated intrinsic Treg deficiency, approaches that target Treg cells might warrant consideration.

Agents of potential utility to induce Treg cell-mediated tolerance in women include cytokines and other biological agents. Two cytokines that been used clinically to attempt to enhance embryo implantation and placentation, CSF3 (171) and CSF2 (172), act on myeloid immune cells and promote recruitment and function of tDCs in the reproductive tract mucosa. Mouse studies are consistent with their fertility-promoting effects being mediated via tDC-mediated induction of Treg cells (151, 173), but their effect on T cells has not been studied in women. IL10 and several microRNAs that act to expand the Treg cell pool and increase functional competence, and are known to be induced naturally in the female tract response to seminal fluid, are also worthy of investigation (106, 174).

Several existing drugs deployed in pregnancy may act at least partly through Treg cells. Studies in mice suggest that progesterone mediates suppression of the Teff cell response, affecting $CD4^+$ T cell and Treg cell phenotype (175, 176). Progesterone effectively suppresses the generation of Th1 cells and Th17 cells and induces Treg cell differentiation (177–179). Treg cells induced by progesterone have increased capacity to suppress the activation and expansion of Teff cells (177, 178). This fits with evidence of progesterone-regulated increases in uterine Treg cell populations in mice and in women (125, 137). Physiological levels of progesterone increase the functional population of $CD4^+FOXP3^+$ cells in pseudopregnant mice and increase the splenic $CD4^+FOXP3^+$ cell proportions in mid gestation (180). Progesterone also acts to selectively repress *IFNG* gene expression in $CD4^+$ T cells (181), allowing enhanced induction of Treg cells and suppression of Th1 and Th17 differentiation (84, 182). A Cochrane meta-analysis

demonstrated a benefit of progesterone for reducing recurrent miscarriage in women (183), but whether this impacts Treg cells is unknown. Furthermore, the outcomes in this setting are confounded by the large proportion of losses related to embryo chromosomal abnormalities, rather than immune dysregulation in the endometrium (184). There is no proven clinical benefit of progesterone in preeclampsia, and a Cochrane review did not find sufficient evidence to support its clinical use to prevent preeclampsia when administration was commenced between 16 and 28 weeks gestation in 4 clinical trials (185). Administration in early pregnancy would likely be required to improve Treg cells at the relevant developmental phase, but the effect of early administration of progesterone on susceptibility to preeclampsia has not been assessed.

Although immune suppressive glucocorticoid drugs conventionally used in autoimmune conditions are sometimes administered in assisted reproduction settings, these suppress both Treg cells and Teff cells, and carry risks when used in pregnancy (186). Intravenous immunoglobulins (IVIg) and Intralipid have also been empirically used in artificial reproductive technology settings to enhance implantation and in recurrent miscarriage clinics to reduce miscarriage rates (187). IVIg did not demonstrate an improvement in livebirth outcomes in 8 small studies in 303 women who suffered recurrent miscarriage (188). Although there is some evidence to suggest that intralipid infusions are associated with immune suppression and alter NK cell activity (189), their effects on Treg cell parameters has not been measured and their clinical benefit for implantation disorders or miscarriage is unproven in clinical trials. The impact of administering intralipid and IVIg infusion in early pregnancy on the development of early or late onset preeclampsia has not been assessed.

There are several drugs under development for autoimmune diseases, including immune checkpoint regulators and other immune-active biologics, that may afford greater selectivity for Treg cells than the immune modulating treatments described above (42). These approaches may be worthy of cautious evaluation in reproductive conditions. Drugs targeting immune checkpoint regulators CTLA4 and PD-1 offer enormous potential, and studies in preclinical models offer encouragement. A recent study in rats where preeclampsia-like symptoms are induced by L-NAME administration showed treatment with PD-L1-Fc protein was effective in reversing Treg/Th17 imbalance and mitigating placental damage (129). Substantial promise for a CD28 superagonist treatment was demonstrated in a rat model of preeclampsia induced by overexpression of human angiotensinogen. Administration of CD28 superagonist was highly effective in increasing Treg cells and alleviating maternal hypertension, proteinuria and IUGR, particularly when treatment was applied from the pre-implantation phase (122). Low dose IL2 has been used to expand Tregs in several conditions, including in abortion-prone mice where protection against fetal loss was achieved (135).

Other relevant agents include humanized antibodies against T cell markers such as anti-CD3, anti-CD52, and anti-CD45 RO/RA which reestablish immune tolerance by selectively depleting Teff cells and retaining Treg cells (37). Other approaches utilize cytokine specific monoclonal antibodies to promote Treg cells—these include anti-TNFA which is approved for use in rheumatoid arthritis and Crohn's Disease, or protolerogenic cytokines such as TGFB and IL10 (37).

Epigenetic regulation of *FOXP3* to impart elevated suppressive function and stability in Tregs is another candidate approach. Inhibitors of DNA methyltransferases such as 5-aza-2′-deoxycytidine (Aza), or factors involved in DNA methylation such as Ten-eleven translocation (TET) protein, have been utilized *in vitro* to drive hypomethylation of the *FOXP3* locus, resulting in strong, stable *FOXP3* expression in Treg cells (190–192). In mouse models, administration of DNMT inhibitors enhances Treg number, *FOXP3* expression and suppressive capacity which assists in reducing inflammation associated with LPS-induced lung injury (193), and prolongs cardiac allograft survival (194). Histone deacetylase (HDACs) inhibitors have also been shown to boost Treg cells and improve suppressive function, resulting in decreased inflammatory bowel disease and increase tissue graft survival in mice (195). These agents carry risks as well as potential, so any application in a reproductive setting would need to be carefully evaluated, initially in preclinical studies.

Cell Therapy Interventions to Boost Treg Cells for Pregnancy

Cell therapy provides a challenging but highly personalized and thus potentially more effective approach to tackling Treg-mediated conditions (37). Cell therapy involves either (i) isolating *in vivo* differentiated Treg cells and expanding them *ex vivo* or (ii) generating and expanding pTreg cells *in vitro*, before subsequent reinfusion. These approaches are in development for transplantation and severe autoimmune disease, but would currently be difficult to justify for a non-life-threatening pregnancy condition. However, given that in preeclampsia the Teff response is not overwhelming, once Treg cell therapy becomes a reality it may prove to be more amenable than other conditions where an extreme immune deviation is beyond rescue (37, 41).

A substantial benefit of cell therapy is that antigen-specific Treg cells can be manipulated without systemic effects on the immune response, with lower risk of off target effects in the mother and fetus than with pharmacological approaches. Studies in type 1 diabetes and other diseases show that T cell receptor (TCR) reactivity with relevant antigens in the target tissue improve Treg cell recruitment and capacity to persist and execute effective suppression, with a low chance of non-specific immune suppression (41, 196). This is a challenge for many disease conditions that might be considered for Treg cell therapy, when Treg cells reactive to tissue-specific antigens are rare. However, the relevant antigens in pregnancy are paternal alloantigens where the starting frequency is much higher. A large proportion of naturally-occurring naïve $CD4^+$ T cells, pTreg cells and tTreg cells react with allo-antigens and could readily be expanded amongst polyclonal populations. Furthermore, because of their capacity to suppress immune responses in an antigen non-specific manner (bystander suppression) and their capacity to

skew T cell responses to other tissue antigens toward tolerance (infectious tolerance), it is possible to regulate the immune response in a whole organ using Treg cells reactive with a single antigen (37, 41). In pregnancy, this means that Treg cells reactive with just one or a subset of paternally-inherited fetal alloantigens, or perhaps even a male minor histocompatibility antigen such as H-Y, could reasonably be effective in suppressing immune responses to a wide range of placental and fetal antigens.

Enormous potential is offered by new gene modification developments in generating alloantigen-specific Treg cells using chimeric antigen receptor technology. This approach overcomes the challenge of the low frequency of antigen-reactive T cells occurring naturally in peripheral blood, by genetically manipulating Treg cells with self-specificity to express either a TCR complex, or a chimeric antigen receptor (CAR) reactive to specific antigens. Use of CAR technology can reliably generate potent, functionally competent, and stable alloantigen-specific human Treg cells that have utility in a wide range of human autoimmune diseases (197). Ongoing clinical trials are showing exciting results in Crohn's disease and are likely to soon be applied in the tissue transplant setting (198). There is a prospect that in time, obstetric disorders may be amongst the range of diseases to benefit from CAR T cell therapy—but again, this must wait until relevant reproductive antigens are identified, and can be targeted in a patient-specific manner.

CONCLUSIONS

Immune imbalance or "maladaptation" has been implicated as central and causal in disease development in preeclampsia, and Treg cells are identified as a pivotal immune cell lineage. Their unique combination of anti-inflammatory, and immune modulatory properties affords Treg cells a potent capacity to support maternal vascular adaptation and placental development, suppress inflammation and sustain maternal tolerance of the fetus. The effects of Treg cells appear most critical at the time of pregnancy establishment and during early placental morphogenesis. Insufficient or dysfunctional Tregs provides a mechanism through which environmental, metabolic, and genetic factors can converge to increase disease risk (154), likely interacting with clinical factors such as prior pregnancy and immune compatibility between partners, which are known to be important pre-pregnancy antecedents of preeclampsia (10).

Given the rapid advances in Treg cell immunology including informative diagnostics based on flow cytometry of peripheral blood, and development of a range of low and high intervention treatments, the prospect of targeting Treg cells in at-risk women to treat early placental disturbances and effectively mitigate preeclampsia onset, warrants evaluation. It will be important to focus on developing diagnostics and interventions for application before or during early pregnancy, to divert the course of disease development before placental or fetal injury occurs. Proof-of-concept experiments in rodent models of preeclampsia already demonstrate the utility of biological agents PF-L1 Fc (129), CD28 superligand (122), and low dose IL2 (135) to boost Treg cell numbers and stability.

Experimental evaluation of any strategy to increase Tregs in a human reproductive setting must take a highly cautious approach and be founded in robust clinical trial design principles. It is critical that safety for mothers and infants is paramount, and the different risk-benefit ratio of reproductive and obstetric conditions, compared to life-threatening immune diseases, is recognized. Possible adverse consequences of artificially boosting maternal Treg cells, including reduced pathogen defense (199) or even reduced immune surveillance against malignancy (200) would need to be considered. Notwithstanding the substantial work to be done to evaluate alternative approaches and identify responsive patient groups, there is an imperative to invest in developing immune therapy options with the goal to reduce the morbidity and mortality associated with preeclampsia.

AUTHOR CONTRIBUTIONS

SR, EG, AC, and LM assembled and interpreted the relevant literature and prepared manuscript drafts and Figures. JP, MH, SB, and GD provided expert knowledge on content and revised manuscript drafts.

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REVIEW
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New Paradigm in the Role of Regulatory T Cells During Pregnancy

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Semi-allogenic fetuses are not rejected by the maternal immune system because feto-maternal tolerance induced by $CD4^+CD25^+FoxP3^+$ regulatory T (Treg) cells is established during pregnancy. Paternal antigen-specific Treg cells accumulate during pregnancy, and seminal plasma priming plays an important role in expanding paternal antigen-specific Treg cells in mouse models. Although paternal-antigen specific Treg cells have not been identified in humans, recent studies suggest that antigen-specific Treg cells exist and expand at the feto-maternal interface in humans. Studies have also revealed that reduction of decidual functional Treg cells occurs during miscarriage with normal fetal chromosomal content, whereas insufficient clonal expansion of decidual Treg cells is observed in preeclampsia. In this review, we will discuss the recent advances in the investigation of mechanisms underlying Treg cell-dependent maintenance of feto-maternal tolerance.

Keywords: miscarriage, preeclampsia, pregnancy, regulatory T cells, seminal plasma

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INTRODUCTION

Feto-maternal tolerance protects the fetal tissues from rejection and leads to a successful pregnancy (1–7). After implantation of the blastocyst in the uterine endometrium, trophoblasts start to invade the endometrial tissue, and uterine spiral artery. Maternal lymphocytes such as $CD4^+$ T cells, $CD8^+$ T cells, and $CD16^-CD56^{bright}$ natural killer (NK) cells express activation markers on their surfaces, suggesting that maternal lymphocytes recognize trophoblasts or fetuses (8). Interaction with maternal immune regulation and trophoblast-derived tolerogenic molecules induces a tolerogenic environment at the feto-maternal interface. Considering the maternal immune system, regulatory T cells (Treg cells) play an essential role in the maintenance of allogenic pregnancy (9–12). $CD4^+CD25^+Foxp3^+$ regulatory T (Treg) cells regulate the T cell response. Treg cells are necessary to sustain tissue homeostasis and establish immune tolerance (13), and are also related to tumor growth and organ transplantation tolerance (14). Previous studies in mouse models have demonstrated that paternal antigen-specific Treg cells are expanded systemically and locally during pregnancy (15–17). Seminal plasma primes the induction of paternal antigen-specific Treg cells (17, 18). Treg cells also increase systemically and locally during human pregnancies (12, 19), whereas paternal antigen-specific Treg cells have not been identified in humans. Recent studies show that target-specific, clonally expanded Treg cells are expanded at the feto-maternal interface in human pregnancies (20). In the first part of this review, we discuss mechanisms by which Treg cells induce feto-maternal tolerance and highlight antigen-specific Treg cells by introducing recent important findings. Following that, we will attempt to analyze the relationship between maldistribution and dysfunction of Treg cells and implantation failure, recurrent pregnancy loss, and preeclampsia in humans.

Maternal Immune Cells at the Feto-Maternal Interface

Maternal immune cells in the reproductive tissues first come into contact with paternal antigens when seminal fluid is ejaculated into the vagina during intercourse. Seminal fluid is composed of seminal plasma and sperm. Maternal immune cells recognize paternal antigens which are contained in the seminal plasma. Sperm reach the fallopian tube and fertilize the oocyte present there. After fertilization, the blastocyst migrates to the uterus while undergoing cell cleavage and finally attaches to the decidua. During the implantation period, the blastocyst adheres to and starts invading the uterine endometrium. In human pregnancy, the cells of the trophoblast differentiate into villous and extravillous trophoblasts (EVTs), forming the placenta. EVTs invade the decidua and myometrium. Subsequent to implantation, EVTs further penetrate the maternal spiral artery and finally replace the vascular lumen (21, 22). The feto-maternal interface is thereby formed, and EVTs and maternal immune cells contact each other (23). EVTs escape from maternal immune cells by controlling the major histocompatibility complex (MHC) and expressing immune suppressive molecules. The maternal immune system also dynamically changes to induce tolerance against fetal tissues (**Figure 1**).

Villous trophoblasts lack the surface expression of MHC class I and class II. EVTs do not express polymorphic HLA-A, B, whereas they express HLA-C and non-polymorphic HLA-E, G, and F (24–29). Maternal $CD8^+$ T cells and NK cells can directly recognize paternal HLA-C, and $CD4^+$ T cells can indirectly recognize it. On the other hand, HLA- E and G protect EVTs from NK-cell mediated cytotoxicity (30, 31). HLA-G positive EVTs regulate T cell activation through the induction of tolerogenic dendritic cells (DCs) (32) and directly cause the expansion of Treg cells (33). Furthermore, trophoblasts suppress maternal immune cells via the expression of indoleamine 2,3-dioxygenase (IDO) (34, 35), the secretion of inhibitory cytokines, such as IL-10 and TGF-β (36), and the expression of programmed death ligand (PD-L I) (37).

Considering maternal immune cells in the decidua, Treg cells and $CD56^{bright}CD16^-$ uterine NK (uNK) cells play an important role in the maintenance of feto-maternal tolerance (3, 4, 38–41) (**Figure 1**). Treg cells, which are discussed in detail at a later part, can recognize fetal antigens via maternal antigen presenting cells (APCs) and induce tolerance in an antigen-specific manner. Compared with ordinary peripheral blood (pb) NK cells that have high cytotoxicity, uNK cells ($CD56^{bright}CD16^-$ NK cells) produce many cytokines and their cytotoxic activities are low (42). Rather, uNK cells play an important role in uterine angiogenesis and spiral artery remodeling (23, 38, 43–46). The expression patterns of cell surface NK receptors in uNK cells differ from those of pbNK cells. For example, killer immunoglobulin-like receptor (KIR) and natural killer group 2 (NKG2) receptors are expressed at high levels on uNK cells (47). When the NKG2A receptor recognizes HLA-E on EVTs, an inhibitory signal suppresses the cytotoxicity of uNK cells (26). When KIR2DL on uNK cells interacts with HLA-G, the uNK cell activity is suppressed (30, 33).

$CD8^+$ cytotoxic T cells can recognize the fetal antigen directly via HLA-C on EVTs and indirectly via maternal APCs (**Figure 1**). A previous report showed that fetal antigen-specific $CD8^+$ cytotoxic T cells (CTLs) are detected in maternal peripheral blood during human pregnancies (48, 49). Viral antigen-specific decidual CTLs that can cross-react against allo-antigens are also reported (50, 51). CTLs in the decidua have distinct phenotypes and functions compared with those in peripheral blood. T-cell immunoglobulin mucin-3 (Tim-3) and programmed cell death-1 (PD-1) are negative immune regulatory molecules. The expression of $Tim\text{-}3^+PD\text{-}1^+CD8^+$ T cells was higher in the human decidua than in peripheral blood. EVTs promote enrichment of $Tim\text{-}3^+PD\text{-}1^+CD8^+$ T cells in an HLA-C dependent manner, suggesting that decidual $CD8^+$ T cells would not attack trophoblasts. Furthermore, maternal $Tim\text{-}3^+PD\text{-}1^+CD8^+$ T cells recognize PD-L I expressed on EVTs, resulting in trophoblast antigen-specific tolerance (52). Highly differentiated resident memory $CD8^+$ T cells are observed in the decidua. This subset shows a lower expression of perforin and granzyme B (53). These reports suggest that antigen-specific CTLs exist at the feto-maternal interface, but their cytotoxic activity is controlled by the placental tissue (53).

How Do Paternal Antigen-Specific Treg Cells Function in Allogenic Pregnancy?

Previous studies suggest that Treg cells play an essential role in the induction of paternal antigen-specific tolerance in allogenic pregnancy in mice. Paternal MHC-specific tolerance during allogenic pregnancy was demonstrated by Tafuri et al. where a paternal MHC-bearing tumor graft was not rejected during pregnancy with the conceptus MHC being identical to the tumor graft, but was rejected in the postpartum period (54).

Aluvihare et al. (11) demonstrated that Treg cells are necessary for allogenic pregnancy in mice, but not necessary for syngeneic pregnancy. When T cell-depleted BALB/C nu/nu female mice were mated with C57BL/6 male mice (allogenic pregnancy) after transfer of total lymphocytes, pregnancies were normally maintained. On the other hand, mating after transfer of Treg cell-depleted lymphocytes resulted in fetal loss, suggesting that Treg cells are essential for the maintenance of allogenic pregnancies (11). Adoptive transfer of $CD4^+CD25^+$ Treg cells from mice with an allogenic pregnancy prevented fetal rejection in an abortion-prone mouse model during allogenic pregnancy, if the transfer was conducted before day 4.5 of gestation (9). Furthermore, depletion of Treg cells using anti-CD25 monoclonal antibodies induced implantation failure and abortion in allogenic pregnancies, but did not induce any pregnancy complications during the late stages of pregnancy (10). These findings suggest that Treg cells induce allo-antigen-specific tolerance and are necessary from implantation through early pregnancy periods in mice.

Where and how do fetal antigen-specific Treg cells expand during pregnancy? Previous studies have demonstrated the existence of fetal antigen-specific Treg cells and their distribution in mouse models (**Table 1**). Kahn and Baltimore showed that the H-Y-specific suppressive capability of Treg cells in splenocytes

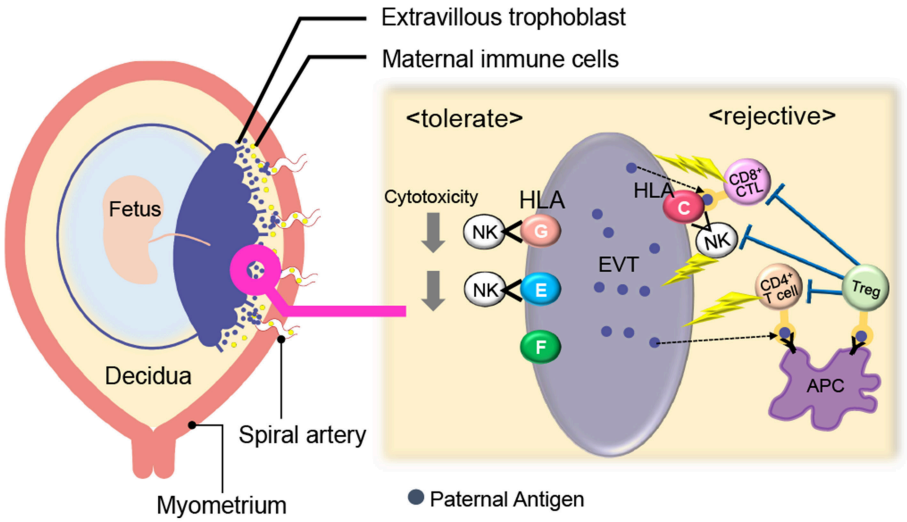


FIGURE 1 | Immunological balance at the feto-maternal interface during early pregnancy. EVTs did not express polymorphic HLA-A, B whereas HLA-C and non-polymorphic HLA-E, G, and F were expressed. Maternal $CD8^+$ T cells and NK cells can directly recognize paternal HLA-C and $CD4^+$ T cells can indirectly recognize it. HLA- E and G protect EVTs from NK-cell mediated cytotoxicity. Treg cells can recognize fetal antigens via maternal antigen presenting cells (APCs) and induce tolerance in an antigen-specific manner. EVT, Extravillous trophoblast; NK, natural killer cell; Treg; regulatory T cell; APC, antigen-presenting cell.

escalated more during pregnancy than before pregnancy (15). Rowe et al. demonstrated that Treg cells specific for the 2W1S antigen, which is derived from the mouse MHC-Eα chain, expanded in the systemic lymph nodes during the 1st pregnancy and rapidly re-accumulated during the 2nd pregnancy (16). Furthermore, Shima et al. reported the local distribution of paternal antigen-specific Treg cells during the implantation period and after pregnancy (**Figure 2**). DBA/2 mice have the Mls 1a super antigen, which is recognized by Vβ6 of the T cell receptor β chain. When BALB/C female mice were mated with DBA/2 male mice, CD4+CD25+Vβ6+ Treg cells, which can be regarded as Mls 1a-specific Treg cells, increased in the uterine-draining lymph nodes one day before implantation. This phenomenon was not observed when BALB/C female mice were mated with seminal vesicle-excised DBA/2 male mice. The local fetal antigen-specific Treg cells might be expanded at the draining lymph nodes by seminal plasma-priming and migrate to the uterus after pregnancy. After implantation, the Vβ6+ Treg cell population in the uterus increased day by day during pregnancy, but that in peripheral lymph nodes and spleen did not (17). Therefore, accumulation of paternal antigen-specific Treg cells is regulated in an organ-specific manner.

Paternal Antigen-Specific Treg Cells in Human Pregnancies

During pregnancy in humans, the systemic and local expansion of the Treg cell pool is observed from the 1st trimester and reaches a maximum in the 2nd trimester (12, 19). Although fetal antigen-specific regulatory T cells and their systemic and local expansion were observed in a mouse model, direct detection of fetal antigen-specific Treg cells is difficult in humans due to heterogenic MHC expression and limited knowledge concerning the physiological target peptide of Treg cells (55). However, the existence of fetal antigen-specific Treg cells in human pregnancies was indirectly suggested in some reports.

Decidual Treg cells, but not peripheral blood Treg cells, showed higher suppression toward self-fetal cord blood than 3rd party cord blood, suggesting that fetal antigen-specific Treg cells might exist at the feto-maternal interface during human pregnancy (56). Among human Treg cell subsets, $CD4^+CD45RA^-FoxP3^{high}$ comprises effector Treg cells which are memory type T cells with a high suppressive capability, and $CD4^+CD45RA^+FoxP3^{low}$ comprises naïve Treg cells with a relatively lower suppressive capability (57, 58). Effector Treg cells are the most dominant among Treg cells in both peripheral blood and decidua in the late gestation stage of human pregnancies (59). To demonstrate if expansion of the effector Treg cell pool is a reflection of clonal expansion of antigen-specific Treg cells, we conducted single-cell-based T cell receptor (TCR) repertoire analysis of $CD4^+CD25^+CD45RA^-CD127^{low}$ effector Treg cells in human pregnancies (**Figure 3**). Our study was the first to reveal that clonally expanded effector Treg cells were observed only in the decidua, but not in the peripheral blood (**Figure 4A**). Clonally expanded effector Treg cells were higher in the 3rd trimester than in the 1st trimester (**Figure 4A**). On the other hand, the common clonotypic effector Treg cells between the decidua and peripheral blood were rarely observed (20). Therefore, decidual effector Treg cells might recognize some antigens expressed at the feto-maternal interface and proliferate upon antigen stimulation. However, effector Treg cells in the peripheral blood expand nonspecifically. Interestingly, the same clonotypic decidual effector Treg cells repeatedly appeared in previous and subsequent pregnancies in three cases: two ended with paired normal term deliveries and the third ended with paired miscarriages (20). TCRβ varies, with over

TABLE 1 | Paternal antigen-specific Treg cells in mouse models.

Target Antigen	PA-specific Treg cells	Method	Existence of PA-antigen specific Treg cells	References
H-Y antigen derived peptide (Dby)	MHC classII I-A^b H-Y ($Dby_{608-622}$)restricted Treg cells	MLR	Systemic	(15)
I-A^b restricted $2W1S_{52-68}$ peptide	MHC classII I-Ab $2W1S_{52-68}$ restricted Treg cells	Direct detection by tetramer	Syetemic	(16)
Mls 1a superantigen	CD4+CD25+Vβ6+ Treg cells (Vβ6 of the T cell receptor β chain recognize Mls 1a superantigen	Direct detection by anti-Vβ6 antibody	Uterine-draining lymph nodes, Uterus	(17)

PA-specific Treg cells, Paternal antigen-specific Treg cells; MLR, Mixed lymphocyte reaction.

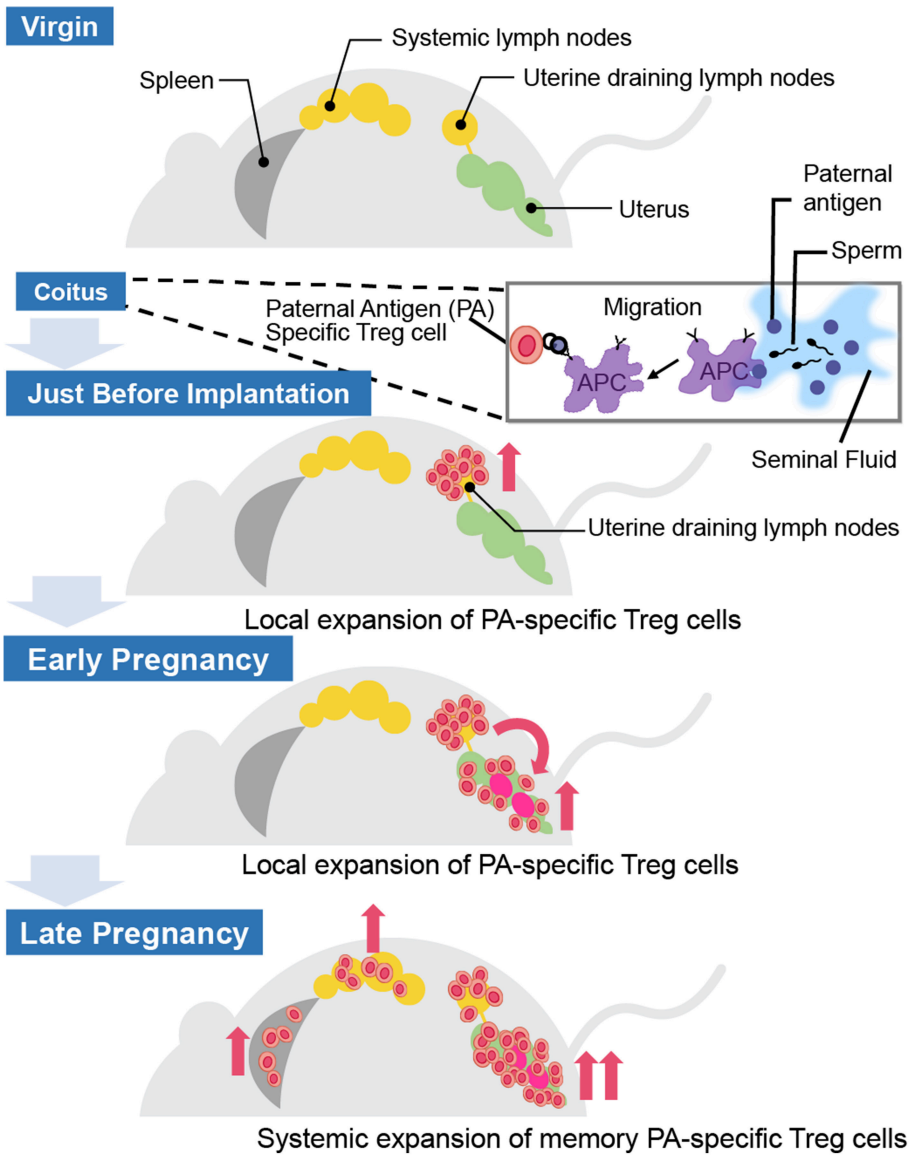


FIGURE 2 | Distribution of paternal antigen-specific Treg cells in mice. When a female mouse is mated with an allogenic male mouse, paternal antigen-specific Treg cells increase in the uterine-draining lymph nodes one day before implantation due to seminal plasma priming. After implantation, the paternal antigen-specific Treg cells population in the uterus increases day by day during the course of the pregnancy, but not in the peripheral lymph nodes and the spleen. APC, antigen-presenting cell.

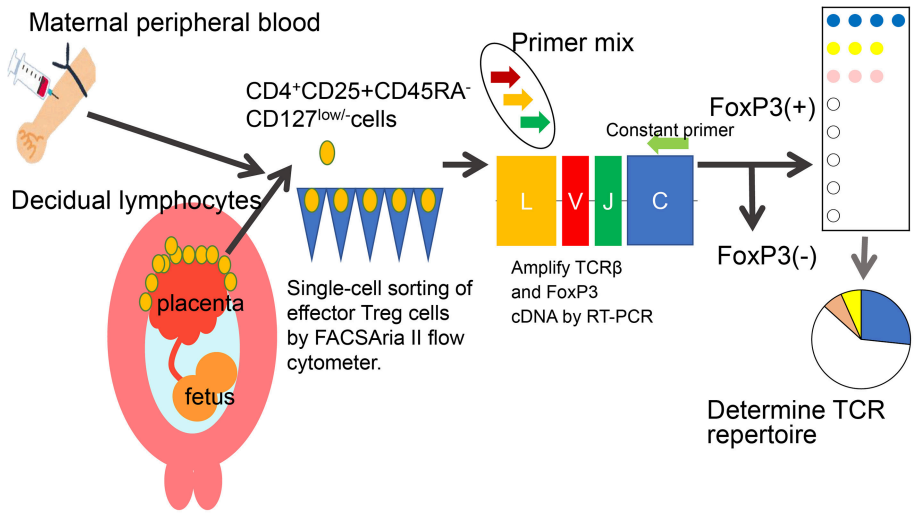


FIGURE 3 | Single-cell based TCR repertoire analysis method. To study the clonality of effector Treg cells, a single-cell based T cell receptor (TCR) repertoire analysis method was used. Paired samples of maternal peripheral blood mononuclear cells and decidual lymphocytes were obtained. CD4+CD25+CD45RA-CD127low/- effector Treg cells were single-cell sorted. The cDNAs of complementarity determining lesion 3 (CDR3) in TCRβ chain and FoxP3 were amplified by RT-PCR. The nucleotides and amino acid sequences of CDR3 were analyzed.

2×10^7 patterns estimated in young humans (60), thus these same clonotypic Treg cells might be repeatedly recruited by the same antigens at the feto-maternal interface rather than by accidental coincidence. Furthermore, clonal populations of decidual effector Treg cells were lower in the 3rd trimester in preeclampsia cases than in normal pregnancies (**Figure 4B**). However, while the effector Treg cell pool was reduced, clonal populations were not reduced in 1st trimester miscarriage cases (20) (**Figure 4B**). Taken together, these data indicate that fetal antigen-specific Treg cells might be recruited and expand in a fetal antigen-specific manner at the feto-maternal interface, and polyclonally expand in systemic circulation during human pregnancy. Clonally expanded decidual Treg cells might be important in the maintenance of feto-maternal tolerance, especially in the 3rd trimester.

Which Peptides Are Recognized by Treg Cells at the Feto-Maternal Interface?

Paternal MHC and minor antigen-derived peptide-specific Treg cells were identified in mouse models as previously described (15–17). A recent study showed that non-inherited maternal antigen (NIMA)-specific Treg cells enforce tolerance in a mouse model (61). NIMAs are peptides derived from polymorphic genes such as MHC and are expressed in the mother but not in offspring. Microchimerism, developed during pregnancy and breast feeding, enables transfer of maternal cells to the offspring. Microchimeric maternal cells persist for a long time and tolerance for NIMAs persists during this time. Kinder et al. demonstrated that the paternal MHC-derived antigen was identical to the NIMA, and the rapid expansion of NIMA-specific Treg cells contributed to successful pregnancy (61). NIMA-matched organ transplantation presents a lower risk of rejection than NIMA-mismatched transplantation in humans (62–65). Thus, theoretically, NIMA-specific tolerance might be induced during human pregnancy.

In humans, a polymorphic HLA-C mismatch pregnancy indicates T cell activation and Treg cell expansion (66). In an oocyte donation (OD) pregnancy, in which the fetus is a total allograft, the match level of HLA-A, B, C, DR, and DQ between the mother and offspring was higher, and fewer pregnancy complications were observed (67). Trophoblasts lack surface expression of HLA-class II molecules, but contain them intracellularly. Considering microchimerism between the mother and fetus, peptides derived from HLA-class II can act as epitopes presented by maternal APCs. Trophoblasts contain intracellular HLA-class II and release HLA-DR molecules upon stimulation by IFN-γ (68). Cell surface expression of HLA-DR in syncytiotrophoblasts and the presence of HLA-DR in syncytiotrophoblast-derived extracellular vesicles were observed in preeclampsia (69). Seminal plasma also contains soluble HLA molecules (70–72). Additionally, human EVTs express minor histocompatibility antigens, such as HY, HA, and ACC (73). HY antigen-specific $CD8^+$ T cells were observed during human pregnancy (48). Even these minor histocompatibility antigens mediate graft-vs.-host disease after organ transplantation (73); however, fetal tissues are not rejected. Taken together, allogenic-HLA-derived, and minor histocompatibility antigen-derived peptides presented by maternal APCs might be recognized by $CD4^+$ conventional T cells and Treg cells. The main target antigens of decidual $CD4^+$ conventional T cells, CTLs, and Treg cells are yet unclear. Further investigation is required to reveal the antigen-specificity of each T cell type and their regulation at the feto-maternal interface.

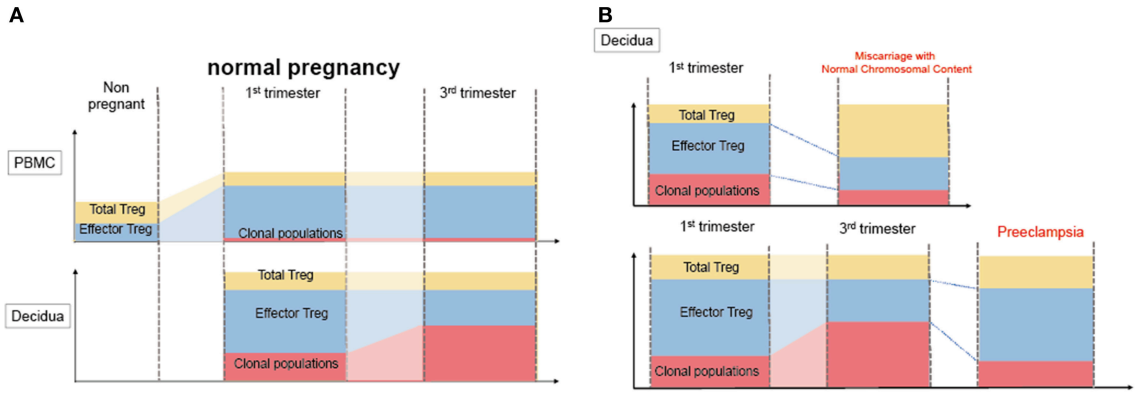


FIGURE 4 | Distribution of clonally expanded effector Treg cells in humans. **(A)** Frequencies of total Treg cells, effector Treg cells and clonal populations of effector Treg cells in normal pregnancy. The systemic and local expansion of the Treg cells and effector Treg cells pool is observed during pregnancies in humans. Clonally expanded effector Treg cells increase in decidua, but not in peripheral blood. Clonal populations of effector Treg cells more increase in 3rd trimester than 1st trimester. **(B)** Clonally expanded decidual effector Treg cells in miscarriage and preeclampsia. In decidua, effector Treg cells pool decreased in miscarriage with normal chromosomal content than 1st trimester normal pregnancy, whereas the frequency of clonal populations of effector Treg cells does not significantly decrease. On the other hand, clonal populations of decidual effector Treg cells decreased preeclampsia than 3rd trimester normal pregnancies.

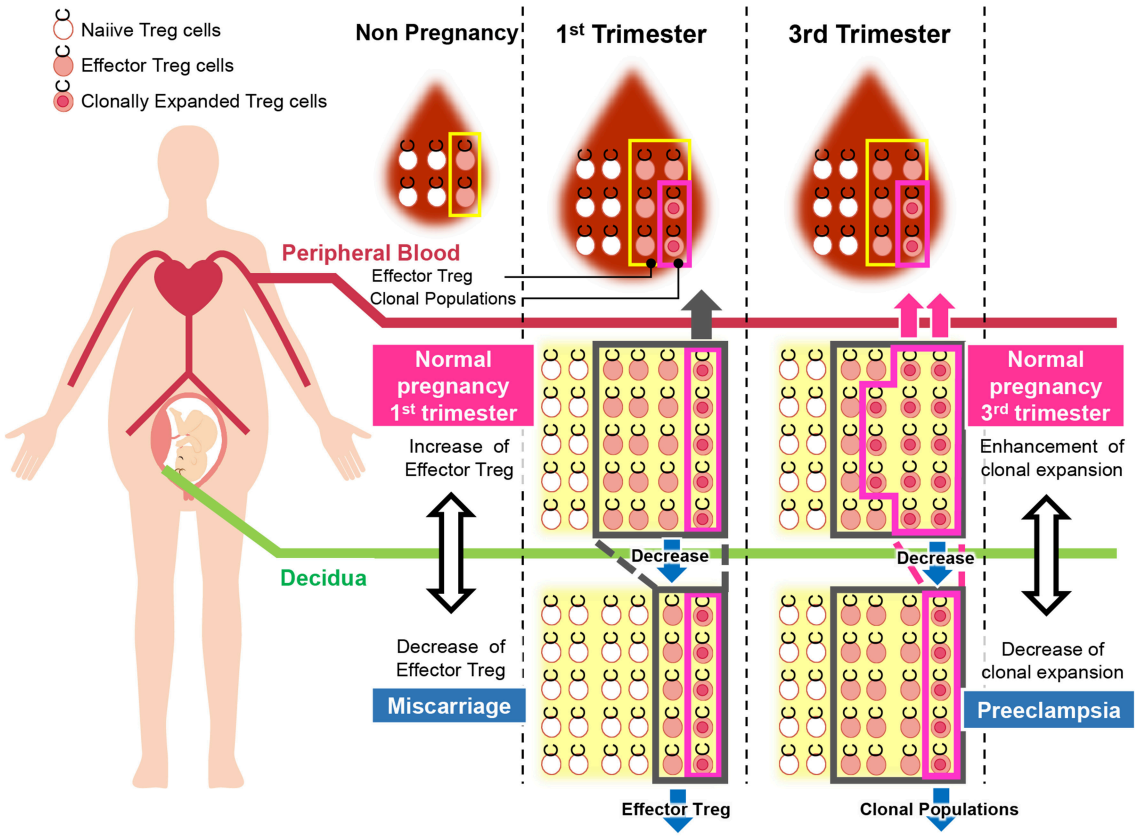


FIGURE 5 | Pathological change in Treg cells during pregnancy in humans. During pregnancy, Treg cell pools both in the peripheral blood and decidua expand. Clonally expanded effector Treg cells are observed only in the decidua, but not in the peripheral blood. Clonally expanded effector Treg cells are higher in the 3rd trimester than in the 1st trimester. In miscarriage cases with normal chromosomal fetal content, the number of decidual effector Treg cells decreases. On the other hand, clonal populations of decidual effector Treg cells decrease in cases of preeclampsia.

What Is the Origin of Treg Cells at the Feto-Maternal Interface?

There are two types of Treg cells in terms of origin: naturally occurring Treg (nTreg) cells, which originate in the thymus, and inducible Treg (iTreg) cells, which arise from conventional $CD4^+$ T cells in peripheral tissues (74). Conserved noncoding sequence 1 (CNS1) is a FoxP3 enhancer and is necessary for developing iTreg cells. Interestingly, only placental mammals have CNS-1, while marsupials and monotremes do not. CNS-1 knockout female mice with allogenic pregnancies showed increased fetal resorption (75). Thus, iTreg cells might be necessary to maintain allogenic pregnancy in placental mammals. On the other hand, nTreg cells are the dominant population (∼95%) among decidual Treg cells in the 1st trimester of human pregnancy, and the proportion of nTreg cells is similar between normal pregnancy and miscarriage (76). However, decidual iTreg cells significantly decreased in preeclampsia (77). To maintain human pregnancy, nTreg cells might be important in early stage pregnancy, and iTreg cells might also be important in late-stage gestation. Further study is necessary to confirm this possibility.

Pregnancy Complications and Treg Cells in Humans

Maldistribution and functional impairment of Treg cells were reported in implantation failure, miscarriage, and preeclampsia in humans. Contrarily, Treg cells are necessary in the implantation period and early gestation, but not in late gestation in mice (10).

Multiple factors, including Treg cell impairment, are thought to be related to implantation failure in humans. Treg cell transcription factor FoxP3 mRNA expression in the uterine endometrium is decreased in primary unexplained infertility (78, 79). A decrease in Treg cells in the peripheral blood in the late follicular phase predicts failure of artificial insemination by the donor (AID) sperm (80). These findings support the hypothesis that maldistribution of Treg cells impairs implantation in humans. Additionally, exposure to seminal plasma raises the success rate of IVF-ET pregnancy (81). Further investigation can validate the evidence that priming with the seminal plasma results in Treg cell-mediated tolerance and can rescue implantation failure.

Disturbance of Treg cell-mediated tolerance might be one of the etiologies of miscarriage. Previous studies reported that Treg cells were decreased in the peripheral blood and decidua in miscarriage cases (12, 82–84). Impaired suppressive capability of Treg cells in recurrent miscarriage cases has also been observed (85–88). Effector Treg cells and nTreg cells were decreased in the case of miscarriage with a normal karyotyped fetus compared to that in the 1st trimester of a normal pregnancy or in the case of miscarriage with an abnormal karyotyped fetus (76, 89). On the other hand, the clonally expanded population of Treg cells showed no significant difference between these groups (20) (**Figure 5**). These findings suggest that the number of nTreg cells and effector Treg cells is more important than antigen-specific Treg cell recruitment during the 1st trimester. The total Treg cell volume that regulates excessive inflammation might be important for the maintenance of the early gestation phase of pregnancy. A previous report demonstrated that high dose immunoglobulin therapy improved the live birth rate for refractory recurrent miscarriage cases with four or more consecutive miscarriages (90). Other studies also showed the effectiveness of anti-TNF-alfa inhibitor therapy (91). These medications have the potential to suppress immune activity in an antigen-nonspecific manner; therefore, these therapies might benefit patients with recurrent miscarriage with a reduced decidual effector Treg cell pool. In humans, peripheral blood Treg cells and decidual Treg cells form clonotypically different populations, and the migration of Treg cells from systemic circulation to the decidua has not yet been shown (20). Thus, these findings might explain why immunization therapy using white blood cells from the patient's partner is not effective for treating recurrent miscarriage (92).

Preeclampsia, which is defined as hypertension concomitant with proteinuria or placental dysfunction occurring in mid to late gestation, is a major cause of maternal and fetal morbidity and mortality. Chronic inflammation due to activation of neutrophils and NK cells, elevation of pro-inflammatory cytokines, and dysfunction of Treg cells is also thought to contribute to the pathogenesis of preeclampsia (93). Epidemiological findings provide the hypothesis that failure in maintaining paternal antigen-specific tolerance is related to the development of preeclampsia. First pregnancy, pregnancy following a partner change, and a pregnancy interval of more than ten years raise the risk of preeclampsia (94–96). Long-term condom usage and AID pregnancy also elevate the risk of preeclampsia, suggesting insufficient paternal antigen-specific tolerance mediated by seminal plasma priming (70, 97, 98). OD pregnancy, in which the fetus is completely allogenic and no priming effect has occurred, is associated with a high risk of preeclampsia (6, 98). Basic research on Treg cells supports this hypothesis.

Previous reports show that Treg cell pools decrease in the peripheral blood and decidua in preeclampsia (77, 99–108). Some reports demonstrate functionally impaired Treg cells in preeclampsia, where Treg cell apoptosis can be easily induced (109). Other reports showed that effector Treg cells decreased in the peripheral blood (108). Hsu et al. demonstrated that the function of decidual APCs was impaired in preeclampsia, resulting in fewer peripherally induced Treg cells (iTreg cells) than in normal pregnancies (77). Elevation of soluble endoglin (sEND), which is a co-receptor of TGFβ, results in the capture of circulating TGFβ, resulting in a systemic decrease of the Treg cell pool. It might also disturb the conversion of conventional Treg cells to iTreg cells (6).

So far, it has not been clarified whether a decreased total volume of Treg cells or decreased paternal antigen-specific Treg cells are related to the pathogenesis of preeclampsia. Our study reported for the first time that clonal expansion of decidual Treg cells was impaired in preeclampsia, suggesting that paternal antigen-specific tolerance might be insufficient. The frequencies of clonal populations of decidual effector Treg cells were 20.9% (15.4–28.1%) in the 3rd trimester during normal pregnancy and 9.3% (4.4–14.5%) in pregnancies with preeclampsia (**Figure 5**). Both early onset and late onset preeclampsia showed the same tendency (20). Our result is compatible with epidemiological

evidence that inadequate paternal antigen-specific tolerance raises the risk of preeclampsia. Paternal antigen-specific Treg cells are more important in the late gestation period of pregnancy than in early gestation (**Figure 3**). Decidual iTreg cells and clonal populations of decidual effector Treg cells decreased in preeclampsia (20, 77). The main population of decidual effector Treg cells during early pregnancy was that of nTreg (76), and the clonal population of decidual effector Treg cells did not decrease. These findings suggest that clonally expanded Treg cells might be iTreg cells. This point requires clarification in the future.

In terms of the clinical applications of these findings, oocyte donation after HLA matching with maternal or paternal HLA might reduce the risk of preeclampsia. Encouraging seminal plasma exposure might play a protective role for high risk patients.

CONCLUSION

Treg cell-mediated feto-maternal tolerance is important in the maintenance of allogenic pregnancy. Paternal antigen-specific Treg cells are expanded systemically and locally during mouse pregnancy. Seminal plasma priming induces paternal antigen-specific Treg cells. Although paternal antigen-specific Treg cells have not been identified in humans, clonal expansion of decidual effector Treg cells implies that antigen-specific tolerance by Treg cells might be induced during human pregnancy. A reduced amount of decidual Treg cells might be related to the pathogenesis of miscarriage, and the failure of decidual Treg cell clonal expansion might be related to the pathogenesis of preeclampsia in humans.

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Feto-Maternal Microchimerism: The Pre-eclampsia Conundrum

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Feto-maternal microchimerism (FMM) involves bidirectional cross-placental trafficking during pregnancy, leading to a micro-chimeric state that can persist for decades. In this manner a pregnant woman will harbor cells from her mother, as well as, cells from her child. Historically, eclampsia, a severe disorder of pregnancy provided the basis for FMM following the detection of trophoblast cells in the lungs of deceased women. Bi-directional cell trafficking between mother and fetus is also altered in pre-eclampsia and has been suggested to contribute to the underlying etiology. FMM has been implicated in tolerance promotion, remission of auto-inflammatory disorders during pregnancy, or the development of autoimmune conditions post-partum. The underlying mechanism whereby the host immune system is modulated is unclear but appears to involve HLA class II molecules, in that incompatibility between mother and fetus promotes remission of rheumatoid arthritis, whereas feto-maternal HLA compatibility may assist in the post-partum initiation of scleroderma. Couples having a high degree of HLA class II compatibility have an increased risk for pre-eclampsia, while the occurrence of scleroderma and rheumatoid arthritis is greater in pre-eclamptic cases than in women with normal pregnancies, suggesting a long term autoimmune predisposition. Since pregnant women with pre-eclampsia exhibit significantly lower levels of maternally-derived micro-chimerism, the question arises whether pre-eclampsia and post-partum development of autoimmune conditions occur due to the failure of the grandmothers cells to adequately regulate an inappropriate micro-chimeric constellation.

Keywords: feto-maternal microchimerism, pre-eclampsia, non-inherited-maternal-antigens, cell-free DNA, autoimmunity

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FMM AND PRE-ECLAMPSIA: HISTORICAL OBSERVATIONS

Granted that pre-eclampsia, a severe life-threatening disorder of pregnancy characterized by hypertension, proteinuria and organ failure is proposed to arise from dysfunctional placentation (1). It is hardly surprising that many key observations concerning feto-maternal cell trafficking and ensuing micro-chimerism were made in this context (2, 3).

In this regard, it is generally accepted that the first evidence of FMM was made at the turn of the nineteenth century by Georg Schmorl; a German pathologist in his examination of pregnant

women who had succumbed to eclampsia (2, 4). Eclampsia is the fulminant form of pre-eclampsia where very severe symptoms are accompanied by seizures (1). In his report, Schmorl documented the occurrence of multi-nucleate trophoblast cells in the lungs of 14 out 17 cases with eclampsia. It is noteworthy that this feature was absent in 4 pregnant women who had died from other causes, suggestive that it may be linked to the underlying pathology of eclampsia (2, 4). Due to the potential role of the placenta as a key aetiological trigger in pre-eclampsia, the pivotal findings of Schmorl relating FMM to altered placentation proved to be worthy of more than passing interest and formed the basis of numerous other studies examining trophoblast deportation, the release of trophoblast micro-particles or even cell-free DNA from the placenta in cases with eclampsia or pre-eclampsia (5–7). Consequently, it was worthwhile reappraising Schmorl's widely-cited seminal report in translated form (4).

These were, however, not the only early reports suggesting that pre-eclampsia may be associated with altered feto-maternal cell trafficking. In 1905 Diehl postulated that pre-eclampsia may result from increased transfusion of incompatible fetal blood cells into the maternal compartment (2). This hypothesis fitted with the then current concept of pre-eclampsia being a form of *pregnancy toxemia*, triggered by toxins, possibly of placental origin during pregnancy (8). Further evidence supporting enhanced feto-maternal bleeding in pre-eclampsia was provided by epidemiological observations suggesting that the frequency of immunization due to Rhesus D incompatibility was greater in pregnancies affected by pre-eclampsia than in those with healthy deliveries (9). Since the Rhesus D antigen is expressed exclusively on cells of the erythrocyte lineage (10), such maternal immunization could not be due to the increased presence of placental trophoblast but would of necessity involve the increased presence of fetal red blood cells in the maternal circulation. This vital aspect was subsequently confirmed by the use of the then novel Kleihauer-Betke stain, which permitted the detection of fetal erythrocytes in maternal blood samples (11). This indicated that their proportion was indeed greater in pregnancies affected by pre-eclampsia (12). These data thereby provided further insight into the placental lesion in pre-eclampsia, indicating that it involved both increased trophoblast shedding, as well as leakage of fetal blood cells across the villous barrier (2, 3).

FMM: THE QUESTION OF FETAL CELLS PERSISTENCE

Possibly one of the most exciting subsequent developments was the detection of fetal leucocytes in mitogen stimulated maternal blood samples, wherein male fetal cells could be detected by the presence of a Y chromosome (13). While this finding was confirmed in a number of ensuing studies, these did highlight the possible longevity of such fetal leucocytes as they could be detected for a considerable period post-partum (14–16). Indeed, the question arose whether such persisting fetal cells could contribute to the presence of male leucocytes in maternal blood samples from pregnancies with a female fetus (14–16).

In a landmark finding the research group of Diana Bianchi reported on the detection of circulatory fetal cells with stem cell-like characteristics for a period of almost three decades post-delivery (17). Subsequent studies have revealed that fetal cell persistence is a frequent event occurring both in mouse and man, and that this affects a variety of tissues including the brain (18–20). As we shall observe in the continuation of this discourse, the longevity of trafficking fetal cells provided the impetus for a number of other investigative routes, particularly with regard to the development of autoimmune conditions since women are more prone to develop them post-partum (21).

FMM: THE QUEST FOR NON-INVASIVE PRENATAL DIAGNOSIS LEADS TO NEW DEVELOPMENTS

The advent of amniocentesis and karyotyping revolutionized obstetrical practice by facilitating the prenatal assessment of fetal chromosomal anomalies, such as trisomy 21 occurring in Down syndrome (22). A caveat of such invasive prenatal diagnostic procedures, especially that of chorionic villous sampling performed earlier in gestation, was the risk of injury to mother and potential loss of the unborn fetus (23–25). Consequently, the need was voiced for safe efficacious alternatives, thereby fuelling the quest for methods permitting non-invasive prenatal diagnosis. It was therefore a foregone conclusion that the prior reports of FMM would provide the basis for the development of such novel prenatal tests (26). Thus, most of these early studies focussed either on the detection of fetal cells in maternal blood while a few examined for the presence of trophoblast cells in the cervix of pregnant women (27). Due to their scarcity, fetal cells in maternal blood had to be enriched with most centers using either flow cytometric or magnetic cell sorting approaches (28).

The fetal erythroblast, also termed nucleated red blood cell, emerged as the target cell of choice for fetal cells in maternal blood-based strategies. This was largely due to its abundance in the fetal circulation, its short half-life that precluded any issue pertaining to longevity, as well as the availability of suitable markers for enrichment (CD71) and identification (fetal and embryonic hemoglobin) (26, 28). By the use of multi-color FISH (fluorescent *in-situ* hybridization) early studies suggested that it may be possible to detect the most common fetal aneuploidies by this approach (28, 29), while the use of single cell PCR permitted the detection of Mendelian disorders i.e., hemoglobinopathies (30, 31). These encouraging results promoted the assessment of this challenging route in two independent NIH and EU funded studies (32, 33). Unfortunately, both of these multicenter studies revealed that the paucity of fetal erythroblasts in maternal circulation rendered their use impractical in daily clinical routine since the required level of specificity and sensitivity could not be attained (32, 33).

During these explorations our group did make two pertinent observations with regard to feto-maternal cell trafficking in preeclampsia. In the first study, by the use of male singleton pregnancies and multi-color FISH for the X and Y chromosomes we could irrevocably demonstrate a significantly increased presence of male fetal erythroblasts in the maternal circulation in cases that manifest pre-eclampsia when compared to matching healthy control pregnancies (34). Furthermore, in an examination of maternal blood samples collected from an at-risk cohort we observed an increase in male fetal erythroblasts in samples from cases that subsequently developed pre-eclampsia (35). Therefore, these results confirmed that the underlying placental lesion in pre-eclampsia facilitated leakage across the usually tight feto-maternal barrier and that this defect was an early event in the course of this disorder occurring prior to clinical manifestation of symptoms (2, 3).

FMM: NOT RESTRICTED TO CELLS BUT ALSO INCLUDES CELL-FREE DNA

During the "NIH NIFTY" study that explored the use of fetal cells for non-invasive prenatal diagnosis the discovery of fetal cell-free DNA (cfDNA) in maternal plasma or serum was reported by Dennis Lo and colleagues in Oxford using Y chromosome specific PCR (36). The basis for the Oxford study were reports on the presence of tumor-derived cfDNA in cancer patient sera (37, 38). In an extension of their original findings they observed that maternally-derived cfDNA fragments could be detected in cord blood samples indicating that FMM was not restricted to the cross placental traffic of cells but could also involve cellular cfDNA fragments (39).

The use of real-time PCR permitted a rapid and precise assessment of the concentration of cfDNA in maternal blood samples. This indicated a progressive increase during pregnancy, peaking at term and ending with rapid clearance post-partum (40). Due to our interest in pre-eclampsia we performed several detailed investigations using qRT-PCR assays. These indicated that manifestation of pre-eclampsia was associated with a significant increase in the concentration of both fetal and maternally-derived cfDNA, which correlated with disease severity and being greatest in cases complicated with HELLP (hemolysis, elevated liver, low platelet) syndrome or eclampsia (41). Furthermore, we observed that when the blood samples were drawn early during pregnancy prior to the onset of clinical symptoms only the level of fetal cfDNA but not maternal cfDNA was elevated (42, 43). Since fetal cfDNA was determined to arise from the placenta this provided further evidence that the underlying etiology of pre-eclampsia involved placental lesions that occurred early in the cascade of events leading to clinical manifestation (3). It is still unclear whether or not elevations in the amount of fetal cfDNA contribute to the development of pre-eclampsia or other pregnancy-related disorders, such as preterm labor (44). The reason for this was that by its hyper-methylated status, fetal cfDNA could trigger an inflammatory response via TLR (toll like receptor) activation, thereby playing a crucial initiatory role (44). Unfortunately no clear data exists to support this enticing hypothesis (45). The question as to the source of elevated maternal cfDNA molecules was more complex to answer and took a rather unexpected turn when it was finally revealed to be derived from excessive generation of neutrophil extracellular traps (NETS) in pre-eclampsia (46, 47).

The advantage of cfDNA over fetal cells in maternal blood for prenatal diagnostic approaches quickly became apparent, particularly when examining for fetal genetic loci absent from the maternal genome, such as the Y chromosome for male fetuses, or the Rhesus D gene in Rhesus D pregnant women (48). Furthermore, the exploitation of the post-genomic advent of massive parallel sequencing permitted the reliable detection of fetal chromosomal abnormalities, such as trisomies, thereby ushering this long sought goal into clinical practice (48, 49).

FMM: RHEUMATOID ARTHRITIS—AMELIORATION, PREVENTION OR PROMOTION?

Amongst the autoimmune rheumatic disorders, rheumatoid arthritis probably responds most favorably to pregnancy in that many cases exhibit amelioration or remission whilst women affected by systemic lupus erythematosus frequently have poor pregnancy outcome characterized by high rates of pre-eclampsia or even fetal loss (50, 51).

Since the beneficial effect of pregnancy on rheumatoid arthritis disease status was determined not to be due to the action of immune-suppressive hormones, Lee Nelson and colleagues examined whether an HLA-based interaction between mother and fetus could contribute to disease improvement (52, 53). For their study they focussed on HLA Class II antigens due to the predisposing effect of certain alleles on rheumatoid arthritis incidence. In their analysis of 57 pregnancies from 41 women with rheumatoid arthritis remission was noted in 22 and amelioration in 12 instances. In 12 pregnancies no improvement of rheumatoid arthritis symptoms were noted (52). Their data indicated that a disparity in MHC class II alleles existed between mother and fetus in those pregnancies exhibiting a remission or improvement of rheumatoid arthritis symptoms. These were most pronounced for HLA-DRB1, DQA and DQB. Limited allelic differences were noted in the 12 rheumatoid arthritis cases where no improvement of symptoms was offered by pregnancy. This association between MHC class II disparity and disease activity was also evident in instances in women having successive pregnancies where rheumatoid arthritis symptoms were reduced in one instance but not the other. At the time that these studies were conducted, no conclusions could be drawn as to the operative mechanism although the question of trafficking fetal cells and FMM were raised (52).

Pregnancy may, however, not only afford some temporary respite from rheumatoid arthritis but may prevent or reduce the onset of this debilitating disorder post-partum (54). In

order to examine whether pregnancy provided a protective effect against the development of rheumatoid arthritis, Guthrie and colleagues examined 310 women with newly diagnosed rheumatoid arthritis in comparison to 1,418 control women (54). This study indicated that the occurrence of rheumatoid arthritis was indeed lower in women who had been pregnant and delivered healthy babies by a factor of almost 40% when compared to non-parous women. It was furthermore observed that no reduction in the risk of developing rheumatoid arthritis was evident in women who had been pregnant but had not continued with the pregnancy until delivery (54). There was also no evidence for a cumulative protective effect offered by multiple deliveries. In contrast it appeared that the greatest protective effect offered by pregnancy was in a period of 5 years or less after delivery, and that this effect diminished significantly by 15 years post-partum. The authors proposed that the protective effect offered by a completed pregnancy may be attributable to persisting fetal cells. This could account for the diminished influence observed in pregnancies not completed to term, as cross-placental cell trafficking would be limited in these instances. The mechanism whereby micro-chimeric fetal cells would favorably modulate the maternal immune system to prevent rheumatoid arthritis onset may be by the provision of disparate MHC class I alleles as described above. Furthermore, in parous women deemed to be at high risk for rheumatoid arthritis due to the presence of two copies of HLA alleles, such as certain HLA-DRB1 loci, micro-chimeric fetal cells may be able to offset disease development by the provisional protective HLA molecules, such as the "DERAA" HLA-DRB1 locus. Evidence that such a mechanism may be operative is provided by the report of Pietsma and colleagues who observed that the protective "DERAA" locus in the form of a non-maternally inherited allele (NIMA) lead to a diminished risk for rheumatoid arthritis (55). The NIMA mechanism is discussed in more detail below.

On the other hand, FMM could assist with the initiation of an auto-inflammatory condition by provision of the necessary genetic background (56). An example of this is the "shared epitope" of certain HLA-DRB1 alleles associated with an increased incidence of rheumatoid arthritis (57). This "shared epitope" is a highly similar five amino acid peptide sequence contained in the HLA-DRB1*04 allele. In an elegant study Yan and colleagues set out to address whether FMM could contribute to the development of rheumatoid arthritis in women who were genetically "shared epitope" negative (58). In this study they examined 52 cases with rheumatoid arthritis and 34 healthy controls; 84% of cases and 92% of controls had at least one live birth. The mean age of cases was 51 and that of controls 42 years. By the use of "shared epitope" specific PCR assays they assessed the degree of microchimerism for such alleles in their study and control groups. Although some degree of "shared epitope" specific signals could be detected in both cohorts, the extent was significantly greater in cases with rheumatoid arthritis (58). In addition, the level of FMM for these "shared epitopes" was higher in rheumatoid arthritis cases than controls. Therefore, FMM, can contribute to the subsequent development of an autoimmune condition i.e., rheumatoid arthritis by the provision of necessary HLA haplotypes it via the inheritance of fetal cells or maternal cells during pregnancy (58).

FMM: CELLS FROM GRANDMA ARE MISSING IN PRE-ECLAMPSIA

During pregnancy, the pregnant woman exhibits a complex micro-chimeric phenotype, hosting cells from her current fetus, cells from previous pregnancies as well as cells from her mother (56, 59). Following the discovery of persisting fetal cells and their possible role in autoimmune conditions like systemic sclerosis, Maloney and colleagues investigated the behavior of trafficking maternal cells. Their examination, which employed HLA specific PCR assays as well as the use of XY FISH to detect female cells in male offspring revealed that cells of maternal origin can persist for numerous decades well into adulthood (59). In most instances maternal cells were HLA class I and II disparate from those of the host offspring (59). To study whether trafficking maternal cells play a role during pregnancy, Gammil et al. examined maternal blood samples collected in each trimester of pregnancy and post-partum (60). In some instances, they also had access to samples drawn prior to conception. By the use of quantitative PCR for specific HLA loci they were able to assess the extent of microchimerism attributable to trafficking maternal cells. In their examination of 86 maternal blood samples obtained from 27 healthy pregnant women with normal deliveries, no trafficking maternal cells were detected pre-conception or in samples obtained in the first trimester of pregnancy. On the other hand, such cells could be detected in 16% of second trimester, 29% of third trimester and 14% of post-partum samples. The degree of trafficking maternal cells microchimerism was greatest in samples collected close to term (60) (**Figure 1**). A startling finding made during this study was that no evidence of microchimerism due to trafficking maternal cells could be detected in any of the samples obtained from 20 pregnant women with manifest pre-eclampsia, in contrast to matching healthy control pregnant women, where such micro-chimeric cells could be detected in 30% of cases (60). This result could have profound implications since trafficking maternal cells are potentially important immune modulators due to their expression of NIMA (see below).

FMM: THE ROLE OF NIMA

A number of early reports indicated that cross-placental cell traffic was not restricted to the fetus but could also be derived from the mother. Pioneering works performed by Owen and colleagues in 1954 observed that Rh-negative girls born from Rh-positive mothers develop very low amount of antibodies to Rh suggesting the existence of a tolerance mechanism to NIMA (61). Later and in the setting of solid organ transplantation tolerance, Claas et al. observed that 50% of the patients receiving several blood transfusions showed selective anergy to non-inherited HLA haplotypes (62). Similarly, Burlingham and colleagues describe that graft survival between

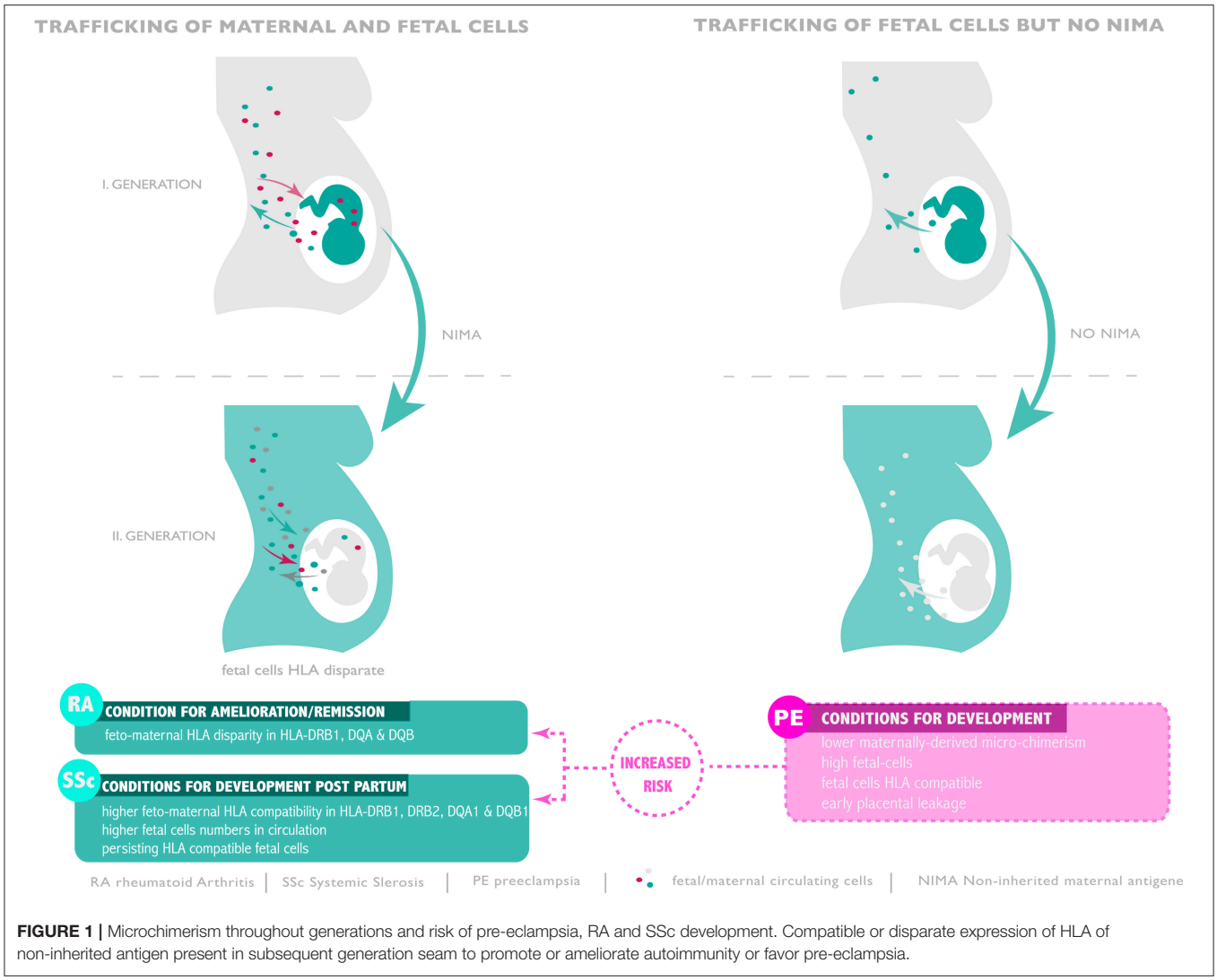


FIGURE 1 | Microchimerism throughout generations and risk of pre-eclampsia, RA and SSc development. Compatible or disparate expression of HLA of non-inherited antigen present in subsequent generation seam to promote or ameliorate autoimmunity or favor pre-eclampsia.

siblings who are mismatched with the recipient for one HLA haplotype results in higher graft survival when the donor has maternal HLA antigens not inherited by the recipient than when the donor has paternal antigens not inherited by the recipient suggesting and highlighting an important role for NIMA (63). More works performed in this direction conclude that the risk of graft vs. host disease among NIMA-matched stem cell transplants is reduced, suggesting possible clinical benefits of NIMA-specific tolerance that persists in individuals through to adulthood (64, 65). Post-natal persistence of genetically foreign chimeric maternal cells in offspring was originally described in infants with severe combined immune deficiency. In a study of 121 infants with defective T and B lymphocyte development, 40% had engrafted maternal T cells and a similar proportion developed clinically apparent graft-vs.-host disease caused by anti-fetal-allo-immunity (66, 67). Mother to offspring transfer and persistence of maternal cells is likely an unavoidable by-product of a porous placental interface. In this scenario, post-natal persistence of NIMA-specific tolerance represents an expendable developmental remnant of immune suppressive mechanisms essential for *in utero* survival (68). Very interesting is the role of NIMA during fetal life: Kinder and colleagues showed that the developmental exposure to foreign maternal cells primes the expansion and accumulation of NIMA-specific immune suppressive regulatory T cells that help establishing better tolerance (68, 69). The persistence of those regulatory T cells could then reinforce fetal tolerance during the next generation pregnancies sired by males with overlapping MHC haplotype specificity, conferring a reproductive advantage.

Taken together, genetic fitness is not restricted to chromosome transmission but is expanded through vertical transfer and survival of tolerogenic cells that establish microchimerism in offspring favoring in return the preservation of NIMA. On the other hand, cross-generational reproductive advantages that preserve post-natal retention of microchimeric maternal cells

may also perpetuate auto-inflammatory or autoimmune diseases in offspring (56).

FMM: SYSTEMIC SCLEROSIS—TRIGGERED BY PERSISTING FETAL CELLS?

Unlike rheumatoid arthritis where the risk is diminished post-partum, systemic sclerosis, an autoimmune disorder characterized by graft-vs.-host disease like symptoms has a strong predilection in women post-partum (21). It was therefore hypothesized that persisting fetal cells may play a role in initiation of systemic sclerosis (21). In order to address this possibility, a group of 40 women who had previously given birth to a son were recruited. Of these, 17 were affected by systemic sclerosis, 7 were sisters of affected cases and 16 were healthy matching controls. The degree of fetal cell persistence in the circulation was assessed by a quantitative PCR assay specific for the Y chromosome. This analysis indicated that the median level of male positive cells was significantly higher in the circulation of systemic sclerosis cases than in matching controls, with intermediate levels being recorded in the 7 siblings (21). Since rheumatoid arthritis remission or amelioration during pregnancy involves a disparity in HLA class II, the role of such an interaction between mother and fetus was examined also in the systemic sclerosis cases. This indicated that a high degree of compatibility between mother and child existed for HLA $DRB1_1$, $DRB1_2$, DQA1, and DQB1 in systemic sclerosis cases (21). Since this feature was not evident in the healthy controls group, it is possible that microchimerism due to persisting HLA compatible fetal cells may contribute to the development of systemic sclerosis post-partum (**Figure 1**).

FMM: WHAT IS THE INFLUENCE OF PRE-ECLAMPSIA ON INCIDENCE OF SYSTEMIC SCLEROSIS OR RHEUMATOID ARTHRITIS?

As our report on elevated trafficking of fetal cells in pre-eclampsia (34) was published at around the same time as that of persisting fetal cells in systemic sclerosis (21), we queried whether the incidence of systemic sclerosis was higher in women who had pre-eclamptic pregnancies (26). Unfortunately, we did not have the correct epidemiological data set at our disposal at the time and so this hypothesis lay resting for almost two decades to finally be addressed in two recent reports (70, 71). In the first van Wyk and colleagues examined whether a relationship existed between the incidence of pre-eclampsia and subsequent development of systemic sclerosis (70). In their study cohort ($n = 103$), the incidence of systemic sclerosis post-first delivery was in mean after 27 years. The authors also determined that systemic sclerosis occurred with greater frequency in women who had pregnancies affected by pre-eclampsia or fetal growth restriction. A limitation of this study was that no information was available concerning potential HLA compatibility, a facet which would have been most interesting to investigate; on one hand in view of previous findings reporting on FMM (21), on the other hand due to reports indicating that pre-eclampsia may occur with greater frequency in instances where a high degree of MHC compatibility for the HLA-DR, -DP, and -DQ alleles occurs between spouses (72). In the second study, which made use of the Danish national register from which the pregnancy outcome and co-incidence of systemic sclerosis for 778,758 women was obtained (71). This indicated that the occurrence of pre-eclampsia is associated with a 69% risk of developing systemic sclerosis. Once again, unfortunately no details concerning HLA types could be obtained.

In the instance of post-partum development of rheumatoid arthritis, the issue is somewhat more complex with a major difference existing with regard as whether the pregnancy was healthy or disturbed by pre-eclampsia. As discussed above, it is widely accepted that prior pregnancy has a beneficial effect on reducing the subsequent development of rheumatoid arthritis. Indeed, it has even been suggested that parity may serve as a vaccine to prevent post-partum rheumatoid arthritis, especially in the first 5 years after the last delivery (54). This is in stark contrast to what is observed if the pregnancy was affected by pre-eclampsia, where an alternate scenario emerges in that such a constellation may favor post-partum rheumatoid arthritis. Evidence of such a feature was initially obtained by epidemiological data mining of the Danish National Patient Register where a retrospective examination suggested that pre-eclampsia was associated with an increased risk for post-partum development of rheumatoid arthritis (73). This feature was confirmed in a subsequent more detailed examination of 55,752 pregnant women of which 169 developed rheumatoid arthritis during the follow up period (74). The incidence of pre-eclampsia was greater in the rheumatoid arthritis study group (6.5%) than in the control cohort (3.6%) suggesting that pre-eclampsia lead to an increased risk for rheumatoid athritis (R.H = 1.96). On the basis of these findings the authors argued that rheumatoid arthritis may be associated with a long pre-clinical phase prior to symptom manifestation, a feature which may affect pregnancy outcome and thereby promote pre-eclampsia, or that a shared predisposition for pre-eclampsia and rheumatoid arthritis may exist (74) (**Figure 1**). In an independent study Ma and colleagues examined whether adverse pregnancy outcome as measured by extreme ($\leq$1,000 g) or very low birth weight ($\leq$1,500), a common feature of severe pre-eclampsia, was associated with subsequent clinical manifestation of rheumatoid arthritis (75). In their study they examined 202 cases with rheumatoid arthritis and 1,102 controls. This analysis indicated that both extreme and very low birth weight had a 3- to 5-fold greater risk of rheumatoid arthritis, particularly for the rheumatoid factor positive form (75).

FMM: THE PRE-ECLAMPSIA CONUNDRUM AND THE ROLE OF NIMA

The incidence of pre-eclampsia, particularly the severe form involving a defect in placenta development and spiral artery

modification is associated with a high degree of HLA class II compatibility between spouses specifically for the HLA-DR, -DP, and -DQ alleles (72). Since trans-placental fetal cell traffic is enhanced in cases with pre-eclampsia (34), this would lead to a high level of microchimerism involving HLA class II compatible fetal cells of the type previously observed in cases with systemic sclerosis, autoimmune condition characterized by graft-vs.-host disease-like lesions (21). In this context it is worth noting that previous studies have suggested that pre-eclampsia exhibits traits of an autoimmune condition with placental features akin to graft-vs.-host disease (76). The question, hence, arises if a histo-compatible FMM state contributes to the etiology of pre-eclampsia. It is also plausible that the presence of this high grade pro-autoimmune microchimeric setting in pre-eclampsia, involving a greater than normal dosage of fetal cells, contributes to the subsequent increased risk for systemic sclerosis and rheumatoid arthritis occurring years later in an extended post-partum period (71, 74).

As pointed out above, microchimerism in a pregnant woman not only involves cells from the current fetus, but also that of any previous pregnancies as well as trafficking maternal cells the proband inherited from her mother (60). Due to their expression of NIMA, these trafficking maternal cells are important in inducing tolerance. A striking feature of pre-eclampsia is the apparent lack of trafficking maternal cells (60). It is therefore possible that the immunological effect of the microchimeric imbalance occurring in pre-eclampsia due to an influx of fetal cells may be further skewed by the apparent lack of trafficking maternal cells in the proband. Consequently, there is no mechanism in place to check or dampen the deleterious action of FMM derived haplo-identical or similar HLA alleles. Since pregnancy is an inflammatory process characterized by basal activation of circulatory neutrophils (77), this could in combination with a micro-chimeric aberrancy provide the necessary stimulus for the dysregulation observed in pre-eclampsia. In a similar manner, the combination of a lack or reduced level of trafficking maternal cells and high grade fetal microchimerism of disease associated HLA alleles could contribute to post-partum onset of rheumatoid arthritis or systemic sclerosis.

SUMMARY AND CONCLUSIONS

While feto-maternal exchange in pregnancy was previously viewed as rare and innocuous event, a vast body of work in the interim has shown that it is a common feature of mammalian pregnancy being readily discernible from mouse to man. The effects of this exchange are far reaching and can persist into adulthood and possibly even old age. In this review we have highlighted the potential anomaly regarding FMM occurring in pre-eclampsia and how this could contribute to the etiology of this enigmatic disorder, but also to the development of autoimmune diseases years post-partum. Should an imbalance between trafficking maternal cells and newly acquired fetal microchimerism prove to be a pivotal trigger driving these developments, then it is highly likely that an altering of the microchimeric milieu could be used to modulate or steer the maternal immune system away from this destructive direction. Evidence that such a mechanism may be viable is provided by reports indicating that the incidence of pre-eclampsia is reduced in women who have received blood transfusions, an event known to lead to a microchimeric state (78). It could therefore transpire that we are on the cusp of new era in the field of microchimerism, whereby this phenomenon provides the basis for new cell-based therapies geared at immune modulation and if so, what better target than pre-eclampsia.

AUTHOR CONTRIBUTIONS

SH and SR wrote the manuscript. LV prepared the figure. SH, PH, SvB, LV, NT, IH, OL, and SR discussed and revised the manuscript.

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

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REVIEW
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Memory T Cells in Pregnancy

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Adaptations of the maternal immune response are necessary for pregnancy success. Insufficient immune adaption is associated with pregnancy pathologies such as infertility, recurrent miscarriage, fetal growth restriction, spontaneous preterm birth, and preeclampsia. The maternal immune system is continuously exposed to paternal-fetal antigens; through semen exposure from before pregnancy, through fetal cell exposure in pregnancy, and through microchimerism after pregnancy. This results in the generation of paternal-fetal antigen specific memory T cells. Memory T cells have the ability to remember previously encountered antigens to elicit a quicker, more substantial and focused immune response upon antigen reencounter. Such fetal antigen specific memory T cells could be unfavorable in pregnancy as they could potentially drive fetal rejection. However, knowledge on memory T cells in pregnancy has shown that these cells might play a favorable role in fetal-maternal tolerance rather than rejection of the fetus. In recent years, various aspects of immunologic memory in pregnancy have been elucidated and the relevance and working mechanisms of paternal-fetal antigen specific memory T cells in pregnancy have been evaluated. The data indicate that a delicate balance of memory T cells seems necessary for reproductive success and that immunologic memory in reproduction might not be harmful for pregnancy. This review provides an overview of the different memory T cell subtypes and their function in the physiology and in complications of pregnancy. Current findings in the field and possible therapeutic targets are discussed. The findings of our review raise new research questions for further studies regarding the role of memory T cells in immune-associated pregnancy complications. These studies are needed for the identification of possible targets related to memory mechanisms for studies on preventive therapies.

Keywords: pregnancy, reproduction, memory T cell, immunologic memory, literature review

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INTRODUCTION

Immune tolerance toward paternal-fetal antigen is crucial for reproductive success since dysfunctional tolerance is implicated in the pathophysiology of pregnancy complications as infertility, recurrent miscarriage, fetal growth restriction, spontaneous preterm birth, and preeclampsia (1–4). In reproduction, the maternal immune system is exposed to paternal-fetal antigens (**Figure 1**). Firstly, the male antigen is introduced to the maternal immune system through semen exposure even before pregnancy (5). Secondly, paternal-fetal antigens are exposed at the fetal-maternal interface in pregnancy since the maternal immune cells in blood are in direct contact with fetal trophoblast cells in the placenta (6, 7). Additionally, in pregnancy, there is trafficking of

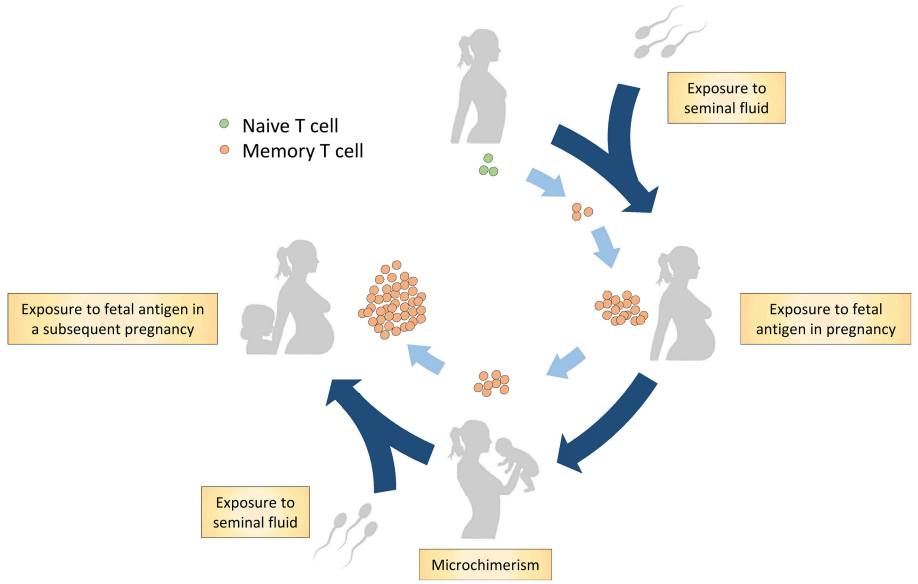


FIGURE 1 | Hypothesis on generation of the memory T cell population in reproduction through paternal-fetal antigen exposure. Firstly, naive T cells are exposed to the male antigen through antigens in seminal fluid. A subsequent encounter with the antigens occurs during pregnancy through exposure to fetal antigens on trophoblast cells and through microchimerism. Postpartum, the maternal immune system remains exposed to fetal antigens through microchimerism. In addition, postpartum, memory T cells are possibly exposed to paternal antigens through exposure to seminal fluid. In a subsequent pregnancy, the maternal memory T cells likely reaccumulate and respond to the cognate paternal-fetal antigens.

fetal cells expressing paternal-fetal antigens to maternal tissues at low levels which can recirculate in the maternal blood for years after pregnancy (8, 9). This phenomenon is called microchimerism (8, 9). It has been shown that the exposure of the maternal immune system to paternal-fetal antigens induces a memory T cell population with paternal-fetal antigen specificity (10–12).

The memory lymphocyte population is comprised of memory T lymphocytes (T cells) and memory B lymphocytes (B cells) (13, 14). Memory T cells are the most studied and appear to be the most important memory cell population in reproduction. Memory cells enable the immune system to protect the body from pathogens efficiently by generating a more adequate immune response to a known antigen, making it unnecessary to elicit a new response to an antigen that was encountered before (15). This process forms the basis for vaccination which is widely used to prevent infectious diseases and more recently to fight cancer and auto-immune diseases (16–18). In general, a more aggressive immune response toward pathogens is protective for health since the pathogen is cleared faster, however, the same aggressive response toward paternal- or fetal antigens would be disastrous for fetal and maternal health. Indeed, most studies of memory T cell populations in reproduction indicated that memory T cell subsets may exhibit a different function, proliferation pattern and migratory abilities toward paternal antigens in healthy pregnancies as compared with their function, proliferation and migratory abilities toward other antigens (12, 19, 20). In fact, specific memory cell populations have been shown to be involved in generating immune tolerance, rather than immune rejection, toward paternal-fetal antigens (12, 21–23).

In recent years, the implication and relevance of memory T cells in pregnancy and complications of pregnancy have been revealed. Major conceptual breakthroughs were seen in the T cell field, showing the role of memory T cells in reproductive fitness in mouse studies (11, 12, 21). Since increasing numbers of human studies on memory T cells have been published, this review gives an overview of the current literature on the different memory T cell subtypes and their adaptation in pregnancy and the implication of memory T cells in different complications of pregnancy. We will mainly focus on human studies and refer to mouse studies if needed. Current research gaps, controversies, and possible therapeutic targets will also be discussed.

MEMORY T CELLS

The memory T cell population is formed during a primary antigen response (24). In the primary response, antigens are presented to T cells through major histocompatibility complex (MHC) molecules (25). Depending on the type of MHC molecule, either type I or type II, CD8 positive or CD4 positive T cells respectively are activated through the T cell receptor (TCR) on the cell membrane (25). Additional co-stimulatory molecules can connect to co-stimulatory receptors on the T cell such as CD28 and CD70, for extra induction of the T cell response (25, 26). Depending on the cytokine environment, $CD4^+$ cells differentiate into either different T helper (Th) subsets (Th1, Th2, and Th17) which help in inducing/activating immune responses through secretion of cytokines, or into T regulatory (Treg) cells which exert regulatory effects on other immune cells after activation (27). After the primary response, most $CD4^+$ cells die,

but some $CD4^+$ cells differentiate into $CD4^+$ memory T cells (24, 28). $CD8^+$ cells also differentiate into different subpopulations; i.e., effector $CD8^+$ cells which are ready to release cytotoxic cytokines or induce apoptosis via cell surface interaction, and a small population of regulatory $CD8^+$ cells which exhibit an immune regulatory function (29). Once the pathogen is cleared, most $CD8^+$ cells die, however some proliferate into memory $CD8^+$ cells (29).

Several memory T cell subsets are known, and can be distinguished by various markers (**Tables 1**, **2**). The main markers are CD45RO expression, and lack of CD45RA expression (52, 53). The $CD45RO^+CD45RA^-$ phenotype has been linked to long living memory T cells (52, 53). It should be noted that CD45RO expression and lack of CD45RA expression are not conclusive markers for memory T cells, since their expression does not predict long time survival and rapid effector function upon secondary exposure *per se* (54). In addition, it has been shown that $CD45RO^+$ T cells can be reprogrammed and go back to a $CD45RO^-$ naive phenotype (55, 56). So far there are no other reliable markers of phenotype memory T cells in clinical experiments, therefore, phenotypic characterization of the memory cell population by CD45RO expression is widely used. Memory $CD4^+$ and $CD8^+$ cells can be divided into subsets based on their migration pattern, cytokine secretion abilities, and protein expression profile. The main memory cell subsets are the central memory (CM) cells and the effector memory (EM) cells, although the number of subsets is expanding rapidly (**Tables 1**, **2**). The CM cell subset differentiates into effector cells upon secondary antigen exposure and is characterized by CCR7 expression which makes them home to secondary lymphoid organs (53, 57). The EM cell subset is characterized by their presence in peripheral tissue and direct pro-inflammatory effector function upon secondary antigen encounter with the cognate antigen (53). Below, an overview of the current knowledge of the various memory T cell subsets in pregnancy is reviewed (**Supplementary Material**).

$CD4^+$ MEMORY CELLS IN PREGNANCY

Within the $CD4^+$ memory cell population, a subdivision has been made based on migration pattern and effector function; i.e., $CD4^+$ effector memory ($CD4^+$ EM) cells, $CD4^+$ central memory ($CD4^+$ CM) cells, $CD4^+$ tissue resident memory ($CD4^+$ TRM) cells, $CD4^+$ T follicular helper memory ($CD4^+$ FHM) cells, $CD4^+$ regulatory memory cells, and $CD4^+$ memory stem cells (58–65).

It has been known for many years that pregnancy and some pregnancy complications affect the general $CD4^+$ memory T cell population. In 1996, it was shown that general $CD4^+$ memory cell ($CD4^+CD45RO^+$) proportions in peripheral blood were lower from the second trimester onwards until 2–7 days postpartum compared to proportions in non-pregnant controls (66). These findings have been followed up by studies in preeclampsia (67–69), gestational diabetes (70), and preterm labor (71) in which higher proportions of total memory T cells in peripheral blood have been found compared to healthy pregnant controls. Early studies also showed $CD4^+CD45RO^+$ memory cells in the decidua and showed that CD45RO expression on $CD4^+$ cells is upregulated in the decidua compared to $CD4^+$ cells in peripheral blood (72, 73). Later, Gomez-Lopez et al. suggested a role for $CD4^+$ memory cells in human term parturition by showing an increase of $CD4^+$ memory cells ($CD4^+CD45RO^+$) using immunohistochemistry on choriodecidual tissue from women in spontaneous labor at term compared to women with term scheduled cesarean sections (74). The early data already indicated that memory T cells are affected by pregnancy and its complications. In more recent years, studies have focused on specific subsets of $CD4^+$ memory cells. These data are reviewed per memory T cell subset below.

$CD4^+$ Effector Memory Cells in Pregnancy

Th1, Th2, and possibly Th17 effector cells can differentiate into $CD4^+$ EM cells (75–77). $CD4^+$ EM cell characterization is based on the lack of expression of lymph node homing receptors CC-chemokine receptor-7 (CCR7) and CD62L (L-selectin), which enables them to migrate to peripheral tissue (59). EM cells are the memory cells with the fastest immune response on a secondary encounter. Within several hours after re-stimulation with a memorized antigen, $CD4^+$ EM cells produce a variety of cytokines as interferon-gamma (IFN-gamma), tumor necrosis factor (TNF), interleukin-4 (IL4), and IL5 (53, 77, 78). A specific subtype of $CD4^+$ EM cell can re-express CD45RA after antigen stimulation (TEMRA) (79). These cells are poorly studied and there are no published investigations on $CD4^+$ TEMRA cells in reproduction to our knowledge.

In the second and third trimesters of pregnancy, two studies showed higher $CD4^+$ EM cell ($CD45RA^-CCR7^-$ and $CD45RO^+CCR7^-$) proportions in peripheral blood, compared to proportions of these cells in non-pregnant women (23, 30), while another study found decreased numbers of $CD4^+$ EM cells in peripheral blood during pregnancy (34). Differences between the studies could be due to the fact that that hormonal fluctuations during the menstrual cycle were not taken into account in the latter study. Not only is the proportion of $CD4^+$ EM cells increased during pregnancy, these cells also showed increased expression of CD69 (30), as well as decreased expression of programmed death-1 (PD-1) (23). This suggests that there is increased activation of $CD4^+$ EM cells, and that these cells are less susceptible to apoptosis. The increase of $CD4^+$ EM cells is not only seen during pregnancy, but also years after when $CD4^+$ EM cell proportions remained increased, i.e., at gestation levels as compared with women that have never been pregnant (30). These cells also showed increased CD69 expression after pregnancy, which could suggest persistent activation through exposure to antigen. This could be related to microchimerism, although it remains to be investigated whether the increased EM cell proportion is due to an increase in cells specific for paternal-fetal antigens.

Whereas, in blood the proportion of $CD4^+$ EM cells of the total $CD4^+$ cell population was about 20–30% (19, 30, 31), locally, in the decidua, the proportion of $CD4^+$ EM cells ($CD45RA^-CCR7^-$) was higher with 50–60% of the total $CD4^+$ cell population being EM cells (19, 31). This may indicate

TABLE 1 | $CD4^+$ memory T cells in pregnancy.

Memory T cell		Markers (human)	Cytokines	Findings in pregnancy	Findings in complications of pregnancy
$CD4^+$	EM	$CD45RO^+$, $CD45RA^-$, $CD44^+$, $CCR7^-$, $CD62L^-$, $CD28^+$	IFN-gamma$^+$, TNF$^+$, IL4$^+$, IL5$^+$	- Higher proportion in peripheral blood in pregnancy (23, 30) - Higher proportions in decidua compared to peripheral blood (19, 31) - Increased IFN-gamma, IL4, PD-1, Tim-3, CTLA-4, and LAG-3 expression in decidua compared to peripheral blood (19) - Lower PD-1 expression in peripheral blood in pregnancy (23) - Higher proportion and higher activated proportion in peripheral blood postpartum compared to nulliparous women (30)	- Comparable proportions in peripheral blood in preeclampsia and healthy controls (32) - Higher proportions in peripheral blood in women with recurrent miscarriages compared to healthy controls (not specified CD4/CD8) (33)
	TEMRA	$CD45RO^-$, $CD45RA^+$, $CCR7^-$, $CD62L^-$, $CD28^-$	Perforin$^+$, granzyme B$^+$	Not studied in pregnancy	Not studied in complications of pregnancy
	CM	$CD45RO^+$, $CD45RA^-$, $CD44^+$, $CCR7^+$, $CD62L^+$, $CD28^+$	IL2$^+$, IFN-gamma$^-$, and TNF$^-$	- Higher proportion in peripheral blood compared to menstrual blood (31) - Comparable proportions and HLA-DR and CD38 expression in peripheral blood in non-pregnant and pregnant women (23, 30, 34) - Higher proportions in decidua compared to peripheral blood (31) - Higher proportion and higher activated proportion in peripheral blood postpartum compared to nulliparous women (30)	- Higher proportion in peripheral blood in preeclampsia compared to healthy controls (32) - Comparable CD27, CD28, and CD127 expression in peripheral blood in preeclampsia and healthy controls (32) - Higher proportions in peripheral blood in women with recurrent miscarriages compared to healthy controls (not specified CD4/CD8) (33, 35)
	TRM	$CD45RO^+$, $CD45RA^-$, $CCR7^-$, $CD62L^-$, $CD69^{+/-}$, $CD103^{+/-}$	IFN-gamma$^+$, IL17$^+$	Not studied in pregnancy	Not studied in complications of pregnancy
	Treg memory	$CD45RO^+$, $CD45RA^-$, $CD44^+$, $CD25^+$, $CD127^-$, Foxp3$^+$, CTLA4$^+$	IL10$^+$, TGFB$^+$	- Higher proportions in the decidua compared to peripheral blood (36) - Strong increase in peripheral blood in the first trimester (37) - Comparable proportions in peripheral blood in 3rd trimester and non-pregnant women (34) - Higher immune regulating capabilities upon stimulation with umbilical cord blood (36) - HLA-DR$^+$ proportion in pregnancy showed decreased suppressive activity compared to HLA-DR$^+$ proportion in non-pregnant women (37) - Differentiated from RTE Treg cells in first trimester peripheral blood (38) - Remain high during pregnancy, decreased with onset of labor, and at preconception levels postpartum (38) - In mice, paternal-specific Treg memory is generated in gestation, remaining at lower levels postpartum, and lowering resorption rates in a subsequent pregnancy (12, 39, 40)	- Higher proportion in peripheral blood in preeclampsia compared to healthy controls (32) - Higher proportion of RTE Treg cells in peripheral blood in preeclampsia differentiate into CD31$^+$ Treg memory cells (38). - CD31$^+$ Treg memory cells in peripheral blood in preeclampsia have decreased immune suppressive capacity compared to CD31$^+$ memory Treg cells in healthy women (38). - HLA-DR$^-$ memory Treg cells were increased in gestational diabetes with dietary adjustment (41) - HLA-DR$^-$ memory Treg cells were strongly increased in gestational diabetes with insulin therapy (41)
	FHM	CXCR5$^+$, $CD45RA^-$, $CD45RO^+$, $CD62L^+$, $CCR7^+$, FR4$^+$	IL21$^+$, IL10$^+$	- Increased in the uterus and placenta toward late gestation in mice (42)	- Higher PD-1$^+$CCR7$^+$ and PD-1$^+$ICOS$^+$ proportions in decidual tissue but not in peripheral blood from spontaneous miscarriages compared to elective terminations in healthy controls (43)
	Memory stem cell	$CD45RO^-$, $CD45RA^+$, $CCR7^+$, $CD62L^+$, $CD28^+$, $CD27^+$, $CD95^+$, IL2RB$^+$	IFN-gamma$^-$, IL2$^{+/-}$	Not studied in pregnancy	Not studied in complications of pregnancy

EM, effector memory; TEMRA, effector memory CD45RA revertant; CM, central memory; TRM, tissue resident memory; Treg, T regulatory; FHM, follicular helper memory; CCR7, CC-chemokine receptor 7; CD62-L, L-selectin; Foxp3, forkhead box P3; CXCR5, chemokine receptor type 5; FR4, folate receptor-4; IFN-gamma, interferon-gamma; TNF, tumor necrosis factor; IL, interleukin; TGFB, transforming growth factor B; PD-1, programmed death-1; Tim-3, T cell immunoglobulin and mucin domain 3; CTLA-4, cytotoxic T lymphocyte antigen; LAG-3, lymphocyte activation gene 3; HLA-DR, Human Leukocyte Antigen-DR; ICOS, inducible T cell co-stimulator; RTE, recent thymic emigrant.

TABLE 2 | $CD8^+$ memory T cells in pregnancy.

Memory T cell		Markers (human)	Cytokines	Findings in pregnancy	Findings in complications of pregnancy
$CD8^+$ EM	General	$CD45RO^+$, $CD45RA^-$, $CD44^+$, CCR7-, CD62L-	Combination of cytokines produced by EM1, EM2, EM3, and EM4	- Higher proportions in peripheral blood postpartum compared to nulliparous women (30) - Higher proportion in decidua compared to peripheral blood (19, 22, 31) - Comparable proportions and PD-1^+ and PDL-1^+ proportions in peripheral blood in pregnant and non-pregnant women (30, 34) - Higher CD38 and HLA-DR expression in peripheral blood in 3rd trimester compared to non-pregnant women (34, 44) - Higher proportions of IFN-gamma$^+$ and $IL4^+$ in the decidua compared to peripheral blood (19, 22) - Higher expression of PD-1, Tim-3, CTLA-4, and LAG-3 in the decidua compared to peripheral blood (19, 45, 46) - Elevated gene expression in genes involved in chemotaxis, co-inhibitory receptors, T cell activation, galectin 1, and the IFN-gamma pathway, in decidua compared to peripheral blood (19, 46) - Comparable gene expression in decidua in 1st and 3rd trimester (46)	- Comparable proportions in peripheral blood in preeclampsia and healthy controls (32) - Higher proportions in peripheral blood in women with recurrent miscarriages compared to healthy controls (not specified CD4/CD8) (33)
	EM1	$CD45RO^+$, $CD45RA^-$, $CD44^+$, $CCR7^-$, $CD62L^-$, $CD27^+$, $CD28^+$, $CD127^+$	Granzyme K^+, Granzyme B^-, Perforin $^{+/-}$, IFN-gamma$^+$, $IL4^+$, $IL5^+$	- Higher proportions in the decidua compared to peripheral blood (19, 22)	- Comparable proportions in peripheral blood in preeclampsia and healthy controls (32) - Lower proportions in pregnant CMV+ women compared to pregnant CMV- women (44)
	EM2	$CD45RO^+$, $CD45RA^-$, $CD44^+$, $CCR7^-$, CD62L-, $CD27^+$, $CD28^-$, $CD94^+$	Perforin$^{+/-}$, Granzyme $B^{+/-}$, IFN-gamma$^+$	- Higher proportions in the decidua compared to peripheral blood (19, 22) Lower proportion of perforin$^+$ and granzyme B^+ in the decidua compared to peripheral blood (22, 47)	- Comparable proportions in peripheral blood in preeclampsia and healthy controls (32)
	EM3	$CD45RO^+$, $CD45RA^-$, $CD44^+$, $CCR7^-$, $CD62L^-$, $CD27^-$, $CD28^-$, $CD94^+$	Perforin$^+$, Granzyme B^+, IFN-gamma$^+$	- Higher proportions in the decidua compared to peripheral blood (19, 22) Lower proportion perforin$^+$ and granzyme B^+ in the decidua compared to peripheral blood (22, 47)	- Comparable proportions in peripheral blood in preeclampsia and healthy controls (32) - Higher proportions in pregnant CMV+ women compared to pregnant CMV- women (44)
	EM4	$CD45RO^+$, $CD45RA^-$,$CD44^+$, $CCR7^-$, $CD62L^-$, $CD27^-$, $CD28^+$, $CD127^+$	Granzyme K^+, Granzyme B^-, Perforin $^{+/-}$, IFN-gamma$^+$	- Higher proportions in the decidua compared to peripheral blood (19, 22)	- Comparable proportions in peripheral blood in preeclampsia and healthy controls (32) - Higher proportions in pregnant CMV+ women compared to pregnant CMV- women (44)
	TEMRA	$CD45RO^-$, $CD45RA^+$, $CCR7^-$, $CD62L^-$, $CD28^-$		- Higher proportions in the decidua compared to peripheral blood (22) Higher CD38 expression in peripheral blood in pregnancy compared to non-pregnant women (44)	- Higher proportions in pregnant CMV+ women compared to pregnant CMV- women (44)

(Continued)

TABLE 2 | Continued

Memory T cell	Markers (human)	Cytokines	Findings in pregnancy	Findings in complications of pregnancy
CM	$CD45RO^+$, $CD45RA^-$, $CD44^+$, $CCR7^+$, $CD62L^+$, $CD28^+$, $CD27^+$, $CD127^+$	$Perforin^-$, $Granzyme\ B^-$, $IFN\text{-}gamma^-$, $IL2^+$	- Low proportions in decidua, peripheral blood and menstrual blood (22, 31) - Higher proportions in peripheral blood compared to menstrual blood (31) - Comparable proportions and $CD38^+$, $CD28^+$, and $CD27^+$ proportions in peripheral blood in pregnant and non-pregnant women (23, 30, 34) - Higher $HLA\text{-}DR^+$ expression in peripheral blood in 3^{rd} trimester compared to non-pregnant women (34)	- Comparable $CD28^+$ proportions in peripheral blood in preeclampsia and healthy controls (32) - Higher proportions in peripheral blood in women with recurrent miscarriages compared to healthy controls (not specified CD4/CD8) (33)
TRM	$CD45RO^+$, $CD45RA^-$, $CD103^{+/-}$, $CD69^+$, $CCR7^-$, $CD62L^-$, $CD49A^+$	$Granzyme\ B^+$, $IFN\text{-}gamma^+$, $perforins^+$	- Present in the reproductive tract (48, 49) - In the reproductive tract do not require IL15 for maintenance and work independently from $CD4^+$ cells (48–50)	- Comparable proportions in endometrial tissue from women with recurrent miscarriages compared to healthy controls (51)
Treg memory	Unknown	Unknown	- Not studied in pregnancy	- Not studied in pregnancy
FHM	$CD45RO^+$, $CXCR5^+$	$IL21^+$, $IL4^+$, $IFN\text{-}gamma^+$	- Not studied in pregnancy	- Not studied in pregnancy
Memory stem cell	$CD45RO^-$, $CD45RA^+$, $CCR7^+$, $CD62L^+$, $CD28^+$, $CD27^+$, $CD95^+$,	$IFN\text{-}gamma^+$, $IL2^+$	- Not studied in pregnancy	- Not studied in pregnancy

EM, effector memory; TEMRA, effector memory CD45RA revertant; CM, central memory; TRM, tissue resident memory; Treg, T regulatory; FHM, Follicular Helper Memory; CCR7, CC-chemokine receptor 7; CD62-L, L-selectin; CXCR5, chemokine receptor type 5; IFN-gamma, interferon-gamma; IL, interleukin; PD-1, programmed death-1; PDL-1, programmed death ligand-1; Tim-3, T cell immunoglobulin and mucin domain 3; CTLA-4, cytotoxic T lymphocyte antigen; LAG-3, lymphocyte activation gene 3; CMV, cytomegalovirus; HLA-DR, human leukocyte antigen-DR.

accumulation of $CD4^+$ EM cells in the decidua, although it can also be simply due to the fact that naive T cells do not accumulate in peripheral tissue (80). Important for the function of memory T cells is the expression of co-stimulatory molecules like CD28 (81). Such molecules are important for the recall response of memory T cells (82). Interestingly, within the $CD4^+$ EM cell population in the decidua, the proportion of the EM subset not expressing co-stimulatory molecules is highly increased compared to peripheral blood (19), suggesting that the $CD4^+$ EM cells in the decidua may not be able to mount a secondary response comparable to $CD4^+$ EM cells in peripheral blood. Despite this, increased IFN-gamma and IL4 expressions were found in decidual $CD4^+$ EM cells compared to $CD4^+$ EM cells in peripheral blood *in vitro* following mitogen stimulation (19). This may be related to the high local progesterone concentrations at the fetal maternal interface (19). The decidual EM cells were not only able to respond to mitogen stimulation, they were also able to respond to fetal antigens (19). The fact that the decidual EM cells are able to respond to fetal antigens and other stimuli suggests that there are extrinsic or intrinsic mechanisms at the fetal-maternal interface to suppress these cells. One of these mechanisms could be the presence of Treg cells (83, 84). Another mechanism may be the expression of immune inhibitory checkpoint receptors on decidual $CD4^+$ EM cells (19). Activation of these receptors inhibit immune responses to avoid autoimmunity and chronic inflammation (85). Increased expression of the immune inhibitory checkpoint receptors PD-1, T cell immunoglobulin and mucin domain 3 (Tim-3), cytotoxic T lymphocyte antigen 4 (CTLA-4), and lymphocyte activation gene 3 (LAG-3), on $CD4^+$ EM cells in the decidua was found as compared to peripheral blood (19). These findings are in line with Wang et al. who showed that the majority of $CD4^+$ EM cells ($CD44^+CD62L^-$) in first trimester decidual tissue from healthy terminated human pregnancies, expressed Tim-3 and PD-1 (86). A role for such immune inhibitory check point receptors in pregnancy has been shown in mouse studies (86). Blocking the Tim-3 and PD-1 pathway (not on $CD4^+$ EM cells specifically) in healthy pregnant mice showed that lower expression of Tim-3 and PD-1 increased fetal resorption rates (86). These studies propose a regulatory function for $CD4^+$ EM cells locally that could be favorable for fetal-maternal immune tolerance and prevent pregnancy loss.

The current data on $CD4^+$ EM cells in women with uncomplicated pregnancy outcomes show that during pregnancy $CD4^+$ EM cells may accumulate in the decidua and remain present at higher levels and higher activated proportions in peripheral blood postpartum (30). In addition, the $CD4^+$ EM cell population in the decidua has a different phenotype with increased IFN-gamma expression, however the $CD4^+$ EM cell population also has increased expression of immune inhibitory proteins compared to peripheral blood (19). To understand the relevance and function of $CD4^+$ EM cells in fetal-maternal tolerance and their role in the postpartum period, further research should focus on their general and more specifically on their antigen specific function, since none of the studies has shown antigen specific tolerance induction by $CD4^+$ EM cells yet.

Unfortunately, until now, $CD4^+$ EM cells have been hardly studied in complications of pregnancy. $CD4^+$ EM cells were studied in preeclampsia by Loewendorf et al. who performed flow cytometric analyses on peripheral blood and a swab from the intrauterine cavity during cesarean sections (32). They did not find differences in levels of $CD4^+$ EM cells between healthy and preeclamptic women in peripheral blood or in lymphocytes isolated from the intra uterine swab (32). However, since the specific tissue of origin of the cells from the swab cannot be defined, caution should be taken when interpreting these results. In non-pregnant women suffering recurrent spontaneous miscarriages, higher proportions of EM cells were observed in peripheral blood compared to non-pregnant fertile controls (33). This study did not further specify the $CD4^+$ or $CD8^+$ status or phenotype. With the proposed relevance of $CD4^+$ EM cells in fetal-maternal tolerance it would be of great value to gain knowledge on $CD4^+$ EM cells in complications of pregnancy.

$CD4^+$ Central Memory Cells in Pregnancy

$CD4^+$ CM cells circulate in the blood and are home to lymph nodes through expression of lymph node homing receptors CCR7 and CD62L (57–59). $CD4^+$ CM cells secrete IL2 and only very low levels of effector cell cytokines (28, 53). Upon secondary antigen exposure, or spontaneously, in the presence or absence of polarizing cytokines, $CD4^+$ CM cells differentiate into Th1, Th2, and $CD4^+$ EM cells, and produce effector cytokines as IFN-gamma and IL4 (53, 87–89). Furthermore, CM cells can quickly cause expansion of the antigen specific T cell population (89).

During pregnancy, as for $CD4^+$ EM cells, $CD4^+$ CM cells are studied mainly in the circulating blood and less at the fetal-maternal interface. One study looked at $CD4^+$ CM cells ($CD45RA^-CCR7^+$) in decidual tissue at the end of pregnancy and showed that proportions of $CD4^+$ CM cells were higher compared to peripheral blood from non-pregnant women (31). Another study evaluated first trimester decidual tissue from terminated healthy pregnancies, and showed that about 40% of $CD4^+$ CM cells ($CD44^+CD62L^+$) were $Tim\text{-}3^+$ and $PD\text{-}1^+$ (86). This appears to be a subset of $CD4^+$ EM cells that have a strong suppressive capacity on proliferation and preferentially produce Th2 type cytokines (86). Since blocking of PD-1 and Tim-3 in pregnancies in mice induced fetal loss (86), the $Tim\text{-}3^+PD\text{-}1^+$ $CD4^+$ EM cells may be important for maintaining normal pregnancy.

A number of studies in pregnancy observed that the proportions of $CD4^+$ CM cells ($CD45RA^-CCR7^+$) in peripheral blood are comparable between women in the second or third trimester of pregnancy and in healthy non-pregnant women (23, 30, 34). However, it seems that after pregnancy the $CD4^+$ CM cell proportions in peripheral blood are increased, since $CD4^+$ CM cells were higher in women after pregnancy compared to pregnant women and compared to women that have never been pregnant (30). Whether the $CD4^+$ CM cells are activated in the circulation of pregnant women remains to be established, since expression of the activation marker CD69 was higher during pregnancy as compared with non-pregnant women (30), whereas expression of the activation markers HLA-DR and CD38 was not affected in the $CD4^+$ CM cell population ($CCR7^+CD45RO^+$) in

peripheral blood from 3rd trimester pregnant women compared to non-pregnant women (34). This higher $CD69^+$ proportion of $CD4^+$ CM cells in pregnancy remained high in women after pregnancy compared to women who have never been pregnant (30).

To date, $CD4^+$ CM cells are investigated in two complications of pregnancy, i.e., preeclampsia and miscarriages. In preeclampsia, slightly, but significantly higher proportions of $CD4^+$ CM cells ($CD45RO^+CCR7^+$) were found in peripheral blood from preeclamptic women compared to healthy pregnant women (32). Proportions of $CD4^+$ CM cells isolated from a swab from the intrauterine cavity during a cesarean section did not show differences between preeclamptic and healthy pregnant women (32). This study also analyzed expression of co-stimulatory molecules, CD28, CD27, and the survival receptor CD127 (IL7 receptor alpha chain), on $CD4^+$ CM cells (32). Only a difference in CD28 expression was found: in an intrauterine swab from preeclamptic women, $CD4^+$ CM cells expressed lower levels of CD28 compared to healthy pregnant women (32). In peripheral blood this difference was not observed (32). In women suffering from recurrent spontaneous miscarriages, higher levels of CM cells ($CD45RO^+CD62L^+$) have been found in peripheral blood compared to fertile women (33). It was not specified whether these CM cells were from the $CD4^+$ or the $CD8^+$ lineage. Part of this increase is likely due to $CD4^+$ CM cells, since another study reported higher levels of $CD4^+$ CM cells ($CD4^+CD45RA^-CCR7^+$) in peripheral blood from women suffering from recurrent miscarriages compared to women with proven fertility and women with no previous pregnancies (35).

As indicated above, Tim-3 and PD-1 expression on memory cells may be important for a healthy pregnancy. This suggestion is in line with the finding of decreased proportions of $Tim\text{-}3^+PD\text{-}1^+$ $CD4^+$ cells in decidua from patients who had undergone miscarriage (86). Unfortunately, these $CD4^+$ cells were not stained for memory cell markers. Further studies on $CD4^+$ CM cells in pregnancy complications in blood and at the fetal-maternal interface are needed in order to be able to show that these cells may play a role in the physiology of pregnancy and the pathophysiology of complications.

$CD4^+$ Regulatory Memory Cells in Pregnancy

Treg cells have potent immunosuppressive properties. They produce IL10 and transforming growth factor beta (TGFB), and have the capability of suppressing $CD4^+$, $CD8^+$, and B cell proliferation and cytokine secretion as well as inhibiting effects on dendritic cells and macrophages (90–93). It was long assumed that Treg cells did not survive the contraction phase of the immune response and undergo apoptotic cell death (64). Nevertheless, a long time surviving memory Treg cell subset has now been shown to persist after antigen exposure (12, 64, 94, 95). There is increasing evidence that memory Treg cells regulate the EM immune response on a secondary encounter with a memorized antigen (64). Treg memory function is implicated in many different pathological and physiological contexts such as auto-immune diseases (96), respiratory disorders (97), hepatitis (98), and pregnancy (12). Treg memory cells are complex to study, since no conclusive markers for a long-living Treg cell population are known (64). Identification of the Treg memory cell pool is therefore performed by combining Treg cell markers as [forkhead box p3 ($Foxp3^+$), $CD25^+$, and $CD127^-$ (99)] with memory cell markers [as $CD45RO^+$ and $CD45RA^-$ (52, 53)] (64).

In rodent models, Treg cells with fetal antigen specificity and a memory phenotype have been shown to accumulate in gestation and impact reproductive success in subsequent pregnancies (12, 39, 40). Rowe et al. developed a mouse model that demonstrated an increase of fetal antigen specific Treg memory cells at mid-gestation in first pregnancies that remained present at lower levels postpartum (12). The Treg memory cell population expanded substantially with accelerated kinetics in a following pregnancy as compared with the first pregnancy (12). This expansion resulted in decreased resorption rates compared to Treg memory cell ablated mice (12). The fetal antigen specific memory Treg cells, as shown by Rowe et al. seem important at mid gestation and it is hypothesized that they might be especially valuable in subsequent pregnancies to set boundaries for a secondary EM cell response toward paternal-fetal antigens (12). Chen et al. showed that in early gestation in mice (during implantation) self-antigen specific memory Treg cells and not fetal antigen specific memory Treg cells are recruited to the reproductive tract and create the tolerant environment for the implantation of the blastocyst (39).

Similar to mouse studies, in early pregnancy fetal maternal immune tolerance is probably not exclusively managed by memory Treg cells, since higher naive Treg cell subsets were associated with successful *in vitro* fertilization (IVF)/intracytoplasmic sperm injection (ICSI) treatment (37). Schlossberger et al. distinguished naive Treg ($CD45RA^+CD25^+Foxp3^+$) and memory Treg ($CD45RA^-CD25^+Foxp3^+$) subsets in blood samples from women undergoing IVF/ICSI treatment and observed higher proportions of naive Treg cells in women who became pregnant compared to the women who did not (37). This finding could indicate that in (preparation for) early pregnancy, higher levels of naive Treg cells are important for successful pregnancy. It could be speculated that these higher levels of naive Treg cells might be able to proliferate into antigen experienced memory Treg cells which are possibly beneficial in late pregnancy. This hypothesis needs to be tested in further studies, but would be in line with findings in mouse studies, in which the paternal antigen specific Treg memory cells were important at mid gestation (12).

The same group followed up on this study and showed that in healthy pregnant women, in early pregnancy (1st trimester) the decrease in naive Treg cells is most likely due to a decreased output of thymic Treg cells, since a decrease in recent thymic emigrant Treg cells was found in early pregnancy (38). They also showed that the increase in memory Treg cells in early pregnancy seems to be due to a differentiation of the recent thymic emigrant Treg cells, since an increased proportion of $CD45RA^-CD31^-$ memory Treg cells was found (38), which returned to normal non-pregnancy levels over the course of pregnancy (38). In line with their previous publication (37), this group showed again

that the suppressive capacity of the naive Treg cells is increased during pregnancy and the suppressive capacity of the memory Treg cell population is decreased during pregnancy (38). At the end of pregnancy, the proportion of $CD4^+$ Treg memory cells ($CD45RA^-Foxp3^+$) in peripheral blood were present at comparable levels as in non-pregnant women (34, 38), which may suggest that memory Treg cells either undergo apoptotic cell death or reside in tissues toward the end of pregnancy. Thus, $CD4^+$ memory Treg cells are found to be favorable for pregnancy in mice, differentiate from recent thymic emigrant Treg cells in early human pregnancy, and circulate in peripheral blood. Studies on their presence and function at the fetal-maternal interface during pregnancy and studies postpartum and during a second pregnancy are lacking.

$Foxp3^+$ Treg cells are implicated in the pathophysiology of many complications of pregnancy as, preeclampsia (4, 100), recurrent miscarriage (101), and infertility (4, 102), however the potential role of the memory cell subset of the Treg cell population in different complications is not well studied. In preeclampsia, there was a decrease of naive Treg cells and an increase in memory Treg cells as compared with healthy pregnancy (32, 103). Although the naive Treg population in preeclampsia showed decreased suppressive activity compared with healthy pregnancy, this was not the case for the memory cell population (103). Further studies are needed to evaluate the role of the memory Treg population in preeclampsia.

In women with gestational diabetes, phenotypic characterization of memory Treg cell subsets showed that the proportion of naive Treg cells ($CD45RA^+HLA\text{-}DR^-CD127^+Foxp3^+$) was lower in women with gestational diabetes compared to healthy pregnant women, independently of whether diabetes was treated with a diet or insulin (41). The proportion of memory Treg cells, on the other hand, increased in gestational diabetes (41). Within the memory Treg cell population $HLA\text{-}DR^+$ and $HLA\text{-}DR^-$ memory Treg cells are distinguished (104), in which $HLA\text{-}DR^+$ memory Treg cells have a more differentiated phenotype, are more suppressive and secrete lower amounts of pro-inflammatory cytokines as compared with $HLA\text{-}DR^-$ memory Treg cells (104). Whereas, $HLA\text{-}DR^-$ memory Treg cells were increased in gestational diabetes with dietary adjustment, $HLA\text{-}DR^+$ memory Treg cells were strongly increased in gestational diabetes treated with insulin therapy compared to healthy pregnant women (41). Whether this is an effect of the insulin treatment or a reflection of the pathophysiology of the disease is not known.

In summary, studies on memory Treg cells in complications of pregnancy show that memory Treg cells might be beneficial for reproductive success in subsequent pregnancies in mice (12), however human studies are inconclusive so far. The fact that some studies find higher memory Treg cell levels in pregnancy complications such as preeclampsia and gestational diabetes (32, 41, 103), whereas others find that lower levels prior to embryo transfer in IVF/ICSI treatment are associated with pregnancy success (37), could indicate specific roles depending on the phase of pregnancy. Identification of more conclusive markers for memory Treg cell function and longevity is a priority to fully elucidate the role of memory Treg cells in reproduction. In addition, since previous studies were mostly performed in peripheral blood, studies on memory Treg cells should also focus on the fetal-maternal interface, as it is known that memory T cells not only reside in peripheral tissues but also in the decidua (48, 74).

$CD4^+$ Follicular Helper Memory Cells in Pregnancy

$CD4^+$ follicular helper cells, which are located mainly in lymphoid organs and in particular in the germinal centers of lymphoid organs, also have a memory cell subset, called $CD4^+$ FHM cells (63, 105). They are known to assist B cells in their differentiation process and produce IL10 and IL21 (106). $CD4^+$ FHM cells are recognized by CXCR5, CD62L, CCR7, and Folate receptor 4 (FR4) (106). Contrary to the effector T follicular helper subset, $CD4^+$ FHM cells exhibit low B-cell lymphoma 6 (Bcl-6) expression (63, 106, 107). Bcl-6 is a transcriptional suppressor of GATA3, TBET, and RORGT, and is of major importance for T follicular helper functioning and maintenance (63). Within the $CD4^+$ FHM cell population, different $CD4^+$ FHM cell subsets can be distinguished based on PD-1, CCR7, and inducible T cell co-stimulator (ICOS) expression (63, 106, 107).

One mouse and one human study reported on $CD4^+$ FHM cells in pregnancy (42, 43). In mid gestation, in mice after allogeneic mating, T follicular helper cells ($CD4^+CXCR5^+PD\text{-}1^+/ICOS^+$) were shown to accumulate in the uterus and placenta (42). These $CD4^+$ T follicular helper cells could be $CD4^+$ FHM cells, since they showed an activated memory ($CD44^+$) phenotype. This putative $CD4^+$ FHM population increased abundantly toward late gestation, but this study also showed that programmed death ligand-1 (PDL-1) blockage induced abortion and increased the putative $CD4^+$ FHM cell accumulation even further (42). The study does suggest that $CD4^+$ FHM cells may be implicated in fetal-maternal tolerance and that excessive abundance might be associated with pregnancy loss (42).

In accordance with the suggestion that increased numbers of $CD4^+$ FHM cells may be implicated in pregnancy loss, a human study in recurrent miscarriage found higher decidual $CD4^+$ FHM cells ($CXCR5^+PD\text{-}1^+CCR7^-$ and $CXCR5^+PD\text{-}1^+ICOS^+$) in spontaneous miscarriage decidual tissue compared to tissue from elective terminations in healthy women (43). In peripheral blood, the proportions of $CD4^+$ FHM cells ($CXCR5^+PD\text{-}1^+CCR7^-$ and $CXCR5^+PD\text{-}1^+ICOS^+$) did not differ between the groups, implying a local response (43). In summary, the current data that exist on $CD4^+$ FHM cells in complications of pregnancy may suggest that pregnancy loss is associated with abundance of $CD4^+$ FHM cells. Thorough research is necessary to increase fundamental knowledge on the function of TFH memory cells in normal and complicated pregnancies.

$CD4^+$ Tissue Resident Memory Cells in Pregnancy

In the classification of memory T cells, $CD4^+$ TRM cells are distinguished from circulating cells (108, 109). Since no conclusive defining markers for the TRM cells from the $CD4^+$ compartment are known, they are difficult to investigate (62, 108,

109). Occasionally, the markers for $CD8^+$ TRM cells are used to study TRM cells in the $CD4^+$ compartment, although it is unclear whether this is correct (**Table 2**) (108, 109). To the best of our knowledge, no literature on $CD4^+$ TRM cells in reproduction is published yet.

$CD4^+$ Memory Stem Cells in Pregnancy

$CD4^+$ memory stem cells are a rare kind of memory T cell that cannot be classified according to the general differentiation of naive and memory cells using the CD45 isoforms (65). Long living cells with a naive phenotype ($CD45RA^+CCR7^+CD27^+$), but with antigen specificity and effector function, were shown in human blood years after Epstein Barr Virus infection (110). The so-called T memory stem cells exhibit almost all conventional memory cell like properties as high CXCR3, CD95, and IL2 receptor beta expression (65, 111), however they lack CD45RO expression and show similar recirculation patterns as naive T cells (65). Studies have shown that $CD4^+$ memory stem cells play a role in auto-immune diseases, Human Immunodeficiency Virus (HIV) and immune protection from a range of infections (65). To our knowledge, $CD4^+$ memory stem cells have not been studied in reproduction yet.

$CD8^+$ MEMORY CELLS IN PREGNANCY

Similar to $CD4^+$ memory cells, $CD8^+$ memory cell subsets are distinguished according to their migration pattern, cytokine secretion abilities, and protein expression (**Table 2**) (112–114). $CD8^+$ memory cells are divided in several subpopulations: $CD8^+$ effector memory cells ($CD8^+$ EM), $CD8^+$ central memory cells ($CD8^+$ CM), $CD8^+$ tissue resident memory cells ($CD8^+$ TRM), $CD8^+$ follicular helper memory cells ($CD8^+$ FHM), and $CD8^+$ memory stem cells (58–65, 115, 116). $CD8^+$ memory cells with regulatory properties are described, however there is no consensus on existence of a $CD8^+$ Treg memory subset (45). Most $CD8^+$ memory cells are generated from antigen experienced effector cells over the course of an immune response (113, 117, 118), however some $CD8^+$ memory cells may arise directly from naive T cells (119, 120). $CD8^+$ memory cells form the first line of defense in mucosal tissues and are able to produce effector cytokines and granzymes, IFN-gamma and perforin upon stimulation without the need for co-stimulatory signals (53, 121).

It has been known for many years that $CD8^+$ memory cells are present in the decidua during pregnancy (73). Higher $CD45RO^+$ proportions of $CD8^+$ cells were found in first trimester decidua compared to peripheral blood at the same time of pregnancy (72, 73). Furthermore, the proportion of $CD8^+$ memory ($CD8^+CD45RO^+$) cells in peripheral blood did not differ between pregnant and non-pregnant women (30, 73). In a further study, the $CD8^+$ memory T cell population was found to be influenced by seminal fluid (122). Using immunohistochemistry, $CD8^+$ memory cells ($CD3^+CD8^+CD45RO^+$) were shown to be increased in the stroma and epithelium of human cervix biopsies taken 12 h after unprotected coitus compared to biopsies after a period of abstinence and biopsies after coitus with condom use (122). Although this shows that memory $CD8^+$ cells are generated as a response toward seminal fluid, it is unknown whether these cells are specific to the paternal antigen. Furthermore, their role in preparation for pregnancy and fetal-maternal tolerance is not known. More recent studies have focused on evaluating the different $CD8^+$ memory cell subsets in reproduction. This is reviewed per subset below.

$CD8^+$ Effector Memory Cells in Pregnancy

$CD8^+$ EM cells express CD45RO, but lack CCR7 expression and are therefore bound to circulate in peripheral blood and non-lymphoid tissue (28, 112). $CD8^+$ EM cells rapidly produce effector cytokines as IL4, IL5, and IFN-gamma upon secondary encounter with the cognate antigen and therewith generate immediate protection (24). The $CD8^+$ EM cells express co-stimulatory molecules CD27 and CD28, which are gradually lost with differentiation of $CD8^+$ EM cells (47). Using these molecules, the $CD8^+$ EM cell population can be subdivided in 4 EM cell subtypes; i.e., EM1 ($CD27^+CD28^+$), EM2 ($CD27^+CD28^-$), EM3 ($CD27^-CD28^-$), and EM4 ($CD27^-CD28^+$) (**Table 2**) (47), with EM-1 being the most prominent in peripheral blood (about 70%) (47, 123). Next to a different immune phenotype, these subsets may exert different functions (47).

CD45RA expression on $CD8^+$ T cells is widely known to be lost on antigen exposure, however on one highly differentiated subpopulation of $CD8^+$ EM cells, CD45RA is again expressed despite previous antigen exposure (47, 121). These $CD8^+$ memory cells are terminally differentiated and called CD45RA revertant effector memory cells ($CD8^+$ TEMRA or sometimes abbreviated EMRA) (47, 121). $CD8^+$ TEMRA cells exhibit great cytolytic activity, but lack expansion abilities and CCR7 expression, disabling them to migrate to secondary lymphoid tissue (47, 121).

Increasing evidence shows that $CD8^+$ EM cells are involved in the establishment of functional immune tolerance toward the fetus (20, 46, 124). In peripheral blood, the total $CD8^+$ EM cell population was similar in healthy non-pregnant women compared to healthy pregnant women in the 2nd and 3rd trimesters (30, 34). Several studies showed an altered activation marker profile on $CD8^+$ EM cells in pregnancy. Higher expression of $CD38^+$ on $CD8^+$ EM ($CD45RO^+CCR7^-$) and $CD8^+$ TEMRA ($CCR7^-CD45RA^+$) cells was found in peripheral blood from pregnant women in the 3rd trimester compared to non-pregnant women (34, 44). Moreover, higher HLA-DR expression, but comparable CD69 expression, were found on $CD8^+$ EM cells ($CD45RO^+CCR7^-$) in peripheral blood in pregnant women compared to non-pregnant women (30, 34). Interestingly, although during pregnancy the proportions of $CD8^+$ EM cells in blood were not different from the proportion in non-pregnant women, higher proportions of $CD8^+$ EM cells ($CD45RO^+CCR7^-$) were found in peripheral blood from women postpartum compared to women who have never been pregnant (30). The higher expression of some of the activation markers on $CD8^+$ EM cells in pregnancy suggest that $CD8^+$ EM cells are activated in peripheral blood in pregnancy. A similar expression of inhibitory molecules PD-1 and PDL-1 on $CD8^+$ EM cells

was found, suggesting that their effector function remains the same (23).

Approximately half of the $CD8^+$ cells in the decidua were found to be $CD8^+$ EM cells ($CD45RA^-CCR7^-$), which is about two-fold higher than the proportion of these cells in peripheral blood (19, 22, 31). This may be due to preferential accumulation of these cells in the decidua, but as for naive $CD4^+$ cells, it may also be due to the fact that naive $CD8^+$ cells do not accumulate in peripheral tissues (80). Not only does the proportion of $CD8^+$ EM cells differ between peripheral blood and the decidua, also substantial differences in phenotype, gene expression and function between these cells in peripheral blood and decidua have been observed (19, 22, 31, 46). $CD8^+$ EM cells ($CD45RA^-CCR7^-$) in the decidua have shown increased IFN-gamma and IL4 secretion abilities and reduced perforin and granzyme B expression compared to $CD8^+$ EM cells in peripheral blood (19, 22). Whether these specific functionalities of the decidual $CD8^+$ EM cells contribute to fetal-maternal immune tolerance remains to be established.

More evidence for altered functionality of $CD8^+$ EM cells in the decidua compared to peripheral blood was found by a study showing elevated expression of inhibitory check point receptors PD-1, Tim-3, CTLA-4, and LAG-3 on decidual $CD8^+$ EM cells compared to $CD8^+$ EM cells in peripheral blood (19, 45, 46). The higher Tim-3 and PD-1 expression on decidual $CD8^+$ T cells might be the result of interaction with trophoblasts, since co-culturing $CD8^+$ T cells with trophoblasts induced upregulation of Tim-3 and PD-1 (45), suggesting that trophoblasts may induce a function change, i.e., tolerance in $CD8^+$ EM cells in the decidua. In accordance with the increased expression of activation markers, inhibitory check point receptors, and cytokine production in decidual $CD8^+$ EM cells is the elevated gene-expression of several genes that was found in decidual $CD8^+$ EM cells compared to $CD8^+$ EM cells in peripheral blood (19, 46). Genes involved in chemotaxis, inhibitory receptors, T cell activation, Treg cell differentiation and genes associated with the IFN-gamma pathway were found higher in decidual $CD8^+$ EM cells compared to peripheral blood $CD8^+$ EM cells (19, 46). The different characteristics of decidual $CD8^+$ EM cells vs. peripheral blood $CD8^+$ EM cells might be beneficial for immune tolerance at the fetal maternal interface.

The question arises whether the changes in $CD8^+$ EM cells are due to the appearance of fetal specific $CD8^+$ EM cells. H HY tetramers are used to detect maternal T cells with specificity for Y-chromosome encoded HY-protein expressed by a male fetus (125). The proportion of HY-specific $CD8^+$ cells (not further specified which memory subtype) in peripheral blood in early pregnancy was 0.035% of the $CD8^+$ population, which almost tripled toward the end of pregnancy (10). The majority of the HY-specific $CD8^+$ memory cell population in peripheral blood and decidua showed an effector memory phenotype, being either $CD8^+$ EM ($CCR7^-CD45RA^-$) or $CD8^+$ TEMRA ($CD45RA^+CCR7^-$) (10, 125). Upon stimulation with male cells, the HY-specific T cells were cytotoxic and secreted IFN-gamma (10). The HY specific $CD8^+$ cells in the decidua expressed higher PD-1 and CD69 as compared with peripheral blood (19).

In preeclampsia, $CD8^+$ EM cell proportions and their CD27 and CD28 expression were comparable to $CD8^+$ EM cell proportions in healthy women in peripheral blood and in a swab from the intrauterine cavity (32). Contrary to preeclampsia, in non-pregnant women following recurrent spontaneous miscarriages, higher proportions of EM cells (not specified whether from the $CD4^+$ or $CD8^+$ cell compartment) were observed in peripheral blood compared to fertile non-pregnant controls (33). Lissauer et al. found that $CD8^+$ EM cell subsets are present at different proportions in pregnancy in women with latent CMV infection (44). They found that in CMV seropositive women the proportion of $CD8^+$ TEMRA cells ($CD45RA^+CCR7^-$) was higher and that the $CD8^+$ EM cell population was more differentiated with higher EM3 ($CD28^-CD27^-$) and EM4 ($CD28^+CD27^-$) phenotypes and lower EM1 ($CD28^+CD27^+$) compared to CMV seronegative pregnant women (44). With the proposed important role for $CD8^+$ EM cells in successful pregnancies, it is worthwhile to investigate $CD8^+$ EM cells and their function in peripheral blood and in the decidua in complications of pregnancy to further evaluate their role in reproduction.

$CD8^+$ Central Memory Cells in Pregnancy

$CD8^+$ CM cells have little effector function and need to be converted to other cell types before effector functions can be induced (53, 112). In contrast to $CD8^+$ EM cells, they are highly proliferative upon stimulation and express the lymph node homing receptor CCR7, which allows these cells to migrate to secondary lymphoid tissue (57). $CD8^+$ CM cells have the ability to generate a diverse progeny, with different types of daughter cells like $CD8^+$ EM cells and effector cells (126). The main cytokine produced by $CD8^+$ CM cells is IL2, but they also produce low levels of IFN-gamma and TNF (112).

In reproduction, $CD8^+$ CM cells are less well studied than $CD8^+$ EM cells, this could be explained by their low prevalence, as the proportions of $CD8^+$ cells with a CM phenotype in the decidua and peripheral blood are low (about 5% of $CD8^+$ cells) (22, 30, 31). Three studies showed that $CD8^+$ CM cell proportions in peripheral blood are not altered by pregnancy (23, 30, 34). CD38, CD28, and CD27 expression on the $CD8^+$ CM cell population was also found to be similar in peripheral blood in pregnant and non-pregnant women (23), although HLA-DR expression on $CD8^+$ CM cells was found higher in peripheral blood from women in the third trimester compared to non-pregnant women (34). Investigation of male-fetus specific $CD8^+$ CM cells in peripheral blood using HY-dextramer staining, revealed that very low proportions of HY specific $CD8^+$ cells have a $CD8^+$ CM phenotype (10, 19). This could suggest that fetal antigens do not reach the secondary lymphoid tissue, less HY-specific $CD8^+$ CM cells develop, and less HY-specific $CD8^+$ CM cells recirculate into peripheral blood (10). Whether less HY-specific $CD8^+$ cells develop is not known.

Whether $CD8^+$ CM cells are present at different proportions in decidual tissue compared to peripheral blood remains

to be established, since one study did not find differences, while another study found significantly lower $CD8^+$ CM cell ($CD45RA^-CCR7^+$) proportions in decidual tissue compared to peripheral blood (22, 31). A possible explanation for the discrepancy could be methodological, as only one of the studies used a viability stain. Granzyme B and perforin are very low expressed by $CD8^+$ CM cells and no differences have been found for granzyme B and perforin expression when comparing decidual and peripheral blood $CD8^+$ CM cells (22).

In preeclampsia, $CD8^+$ CM cell proportions and their CD28 and CD27 expression were comparable to the proportions in healthy women, both in peripheral blood and in a swab from the intrauterine cavity (32). In peripheral blood from non-pregnant women suffering from recurrent spontaneous miscarriage, higher proportions of $CD8^+$ CM cells ($CD45RO^+CD62L^+$) were found compared to non-pregnant fertile women (33). However, as $CD4^+$ or $CD8^+$ cell phenotype was not identified, it is not sure if this finding reflects a difference in $CD8^+$ CM cells.

$CD8^+$ Tissue Resident Memory T Cells in Pregnancy

Tissue resident memory (TRM) cells are a distinct subpopulation of $CD8^+$ memory cells which reside in peripheral tissues, including endometrium and decidua (49, 51). After the primary immune response, $CD8^+$ TRM cells reside in peripheral tissues awaiting a secondary encounter without recirculating in peripheral blood or lymph nodes (127–129). Upon reactivation, $CD8^+$ TRM cells produce IFN-gamma, granzyme B, and perforins (127). TRM cells are typically identified by the expression of different surface markers as CD103, CD69, and CD49A (127, 130–133). $CD8^+$ TRM cells are found in the entire reproductive tract and in contrast to $CD8^+$ TRM cells in the kidney, skin and salivary gland, do not require IL15 for maintenance of the cell population (48, 49, 134).

Next to this, $CD8^+$ TRM cells in the reproductive tract seem to be able to recruit circulating memory T cells, independently from their cognate antigen, into mucosal tissue of the reproductive tract and convert them to TRM cells (50). These data are suggestive of a well-functioning first line of defense of memory T cells in the reproductive tract. Presumably, TRM cells, $CD4^+$ or $CD8^+$, are the first memory T cells the male antigens on spermatozoa will encounter. Despite their presence in the reproductive tract, little information is available on their function and presence during pregnancy. One study looked at $CD8^+$ TRM in endometrial tissue and showed the presence of high proportions of memory $CD8^+$ cells in endometrial tissue, which was similar in women with recurrent miscarriages and control women (51). Part of these $CD8^+$ memory T cells expressed CD103, indicating that the cells may be $CD8^+$ TRM cells (51). The proportion of $CD8^+$ memory cells expressing CD103 was similar in women with recurrent miscarriages and control women (51). However, the percentage of $CD8^+$ memory cells expressing CD69, a TRM marker, was decreased in women with recurrent miscarriages as compared with control women (51). This might suggest a decrease in $CD8^+$ TRM cells in women with recurrent miscarriage.

$CD8^+$ Regulatory Memory, $CD8^+$ Follicular Helper Memory, and $CD8^+$ Memory Stem Cells in Pregnancy

The regulatory memory, follicular helper memory, and the memory stem cell subsets are relatively well studied in the $CD4^+$ cell compartment but only to a limited extent in the $CD8^+$ cell compartment. $CD8^+$ cells with immune regulatory abilities are described in literature (135–137), however, the existence of a memory cell subset within the $CD8^+$ Treg cell population is still uncertain (45). $CD8^+$ cells with expression of follicular helper cell marker CXCR5, and memory cell marker CD45RO, are identified in germinal centers of human tonsils, and were found to support B cells (116, 138). The presence of such $CD8^+$ follicular helper memory cells are only very recently confirmed and are not studied in pregnancy yet (116). $CD8^+$ memory stem cells, as for their $CD4^+$ counterpart, are antigen specific memory cells with a naive phenotype and are mostly studied in oncology settings (115, 139–141). Research on $CD8^+$ regulatory memory, $CD8^+$ FHM, and $CD8^+$ memory stem cells in pregnancy will be of interest, but more knowledge on their functioning in general is needed before studying their role in reproduction.

MEMORY T CELLS IN PREGNANCY AS POSSIBLE THERAPEUTIC TARGETS

Literature shows that memory T cells are likely implicated in fetal-maternal tolerance before, during and after pregnancy. Firstly, it has been shown that exposure to seminal fluid before pregnancy induces a memory T cell population in the ectocervix (122). Even though there is no evidence yet that these memory cells are paternal-antigen specific, this could be a mechanism that contributes to tolerance toward paternal-fetal antigens. This mechanism is supported by existing epidemiologic data showing an association between a longer period of exposure to seminal fluid of the future father and a lower risk of preeclampsia (142–144). Generating paternal specific memory T cells as a therapeutic target, through paternal cell immunization before conception seems obvious and has indeed been carried out by several studies (145, 146). Studies are however small, but a meta-analysis of 7 small studies showed an improvement in clinical pregnancy rate following IVF treatment when seminal plasma is used as an adjunct treatment (average pregnancy rate increased from 25% in the control group to 29% in the seminal plasma treated group), with no significant increases in live birth or ongoing pregnancy rate (146). Since in these studies, timed intercourse or deposition of untreated semen in the vagina before IVF was used, it is not known whether the positive effect of semen is due to the seminal plasma itself or to paternal-fetal antigen exposure. This should be subject of future research. In order to potentially achieve better results, additional options for priming may be tested. An additional option could be a prime and pull method, by first eliciting an immune response to recruit T cells into the reproductive tract, followed by topical vaccination, a method that has been shown to be effective in genital herpes prevention (147).

Secondly, this review indicates that tolerance mechanisms involving memory T cells are in place during pregnancy. Various

alterations in memory T cell function and levels have been shown, which together likely ensure tolerance; for instance, $CD4^+$ Treg memory cells may play an important role, while also low responsive PD-1^+Tim-3^+ $CD8^+$ memory cells are present at the fetal-maternal interface, which may also be important. These different tolerating mechanisms and their interactions should be further investigated, while it is also important to focus on their alterations in complications of pregnancy. The lack of knowledge on these mechanisms in healthy pregnancy and how they are affected in complications of pregnancy, makes therapeutic options using immune modulation of memory T cells to treat pregnancy complications not feasible yet.

Thirdly, after pregnancy, maternal immune cells are exposed to fetal-paternal antigens through microchimerism and possibly through semen exposure (8, 9). Since $CD4^+$ memory T cells are known to require low-levels of antigen exposure for long term maintenance (60, 148, 149), it is proposed that microchimerism and semen exposure are ways to ensure persistence of the fetal-paternal specific $CD4^+$ memory cell population (150–152). Thorough investigations on possible beneficial effects of memory T cells on reproductive success and of microchimerism on memory T cell populations should point out whether this could bring forward another possible therapeutic target. Lowering pregnancy complication rates through priming and enhancing the maternal memory T cell repertoire in parous women could be considered for future therapies. These could involve similar approaches as therapeutic options before pregnancy.

CONCLUSIONS

To conclude, a delicate balance of memory T cells seems necessary for successful pregnancy and memory T cells might not be harmful for pregnancy, but in fact, they may induce tolerance. Memory T cells show different phenotypes, dynamics, and functioning in uncomplicated pregnancies compared to memory T cells outside the reproductive context. Together, these mechanisms may induce tolerance toward fetal antigens during pregnancy. More research on memory T cells in pregnancy is needed to better understand the function of these cells in pregnancy and to develop therapeutic strategies for pregnancy complications based on memory T cells.

AUTHOR CONTRIBUTIONS

TK organized financial support, built the search strategy, performed the literature study, and wrote the first draft of the manuscript. AL organized financial support, built the search strategy, performed the literature study, and reviewed and edited the manuscript. SS, MF, and JP reviewed and edited the manuscript, organized financial support, and supervised the project.

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SUPPLEMENTARY MATERIAL

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MicroRNA-Mediated Control of Inflammation and Tolerance in Pregnancy

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Gestational age-dependent immune intolerance at the maternal-fetal interface might be a contributing factor to placental pathology and adverse pregnancy outcomes. Although the intrauterine setting is highly choreographed and considered to be a protective environment for the fetus, unscheduled inflammation might overwhelm the intrauterine milieu to cause a cascade of events leading to adverse pregnancy outcomes. The old paradigm of a sterile intrauterine microenvironment has been challenged, and altered microflora has been detected in gestational tissues and amniotic fluid in the absence of induction of significant inflammation. Is there a role for endotoxin tolerance at the maternal-fetal interface? Endotoxin tolerance is a phenomenon in which tissues or cells exposed to the bacterial product, particularly lipopolysaccharide, become less responsive to subsequent exposures accompanied by decreased expression of pro-inflammatory mediators. This could also be related to trained or experienced immunity that leads to the successful outcome of subsequent pregnancies. Adaptation to endotoxin tolerance or trained immunity might be critical in preventing rejection of the fetus by the maternal immune system and protecting the fetus from excessive maternal inflammatory responses to infectious agents; however, to date, the exact mechanisms contributing to the establishment and maintenance of tolerance at the maternal-fetal interface remain incompletely understood. There is now extensive evidence suggesting that microRNAs (miRNAs) play important roles in the maintenance of a healthy pregnancy. miRNAs not only circulate freely in extracellular fluids but are also packaged within extracellular vesicles (EVs) produced by various cells and tissues. The placenta is a known, abundant, and transient source of EVs; therefore, our proposed model suggests that repeated exposure to infectious agents induces a tolerant phenotype at the maternal-fetal interface mediated by specific miRNAs mostly contained within placental EVs. We hypothesize that impaired endotoxin tolerance or failed trained immunity at the maternal-fetal interface will result in a pathological inflammatory response contributing to early or late pregnancy maladies.

Keywords: microRNA, immune tolerance, endotoxin tolerance, extracellular vesicles, pregnancy, maternal-fetal interface, innate immunity

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INTRODUCTION

Our current understanding of immunity at the maternal-fetal interface has been shaped by acceptance of periodic concepts and approaches formed based on tissue-allograft studies in non-pregnant individuals, male or female, and by the paradigm suggesting the uterus as a sterile organ (1, 2). However, our contemporary understanding of the role of the placenta, hormones, and novel cellular controls warrants re-evaluation of old paradigms associated with fetal immune-protection in both hostile and normal intrauterine microenvironments (3, 4). The decidua, the maternal tissue in pregnancy, is replete with immune cells that should intrinsically harm the fetus; however, normal pregnancy ensues, suggesting that the placenta transforms these cells into a specialized, pregnancy compatible milieu (5). What remains incompletely understood is why the placenta fails to counter challenges from intrauterine infections or sterile inflammation. This is further complicated by the observations that some women can experience a successful pregnancy despite repeated exposure to infectious agents, particularly endotoxins, and other environmental factors. This review discusses recent observations that might explain successful pregnancy outcomes despite repeated intrauterine infections and potentially provide important insights into the role of the placenta via secretion of extracellular vesicles (EVs).

Despite constant exposure to hygienic challenges and infection during pregnancy, most of the 4 million annual deliveries in the United States have a successful outcome without presenting any clinical evidence of intrauterine infection (6). In this regard, the onus can be placed on the placenta to counter inflammation and ensure trained immunity for a successful pregnancy (7–10); however, it remains unclear how the placenta programs these pregnancy compatible events. The placenta is a transient organ that supports fetal growth and development by ensuring respiratory gas exchange, regulates maternal-fetal nutrient transport, provides protection for the fetus against maternal immunity, and acts as a transient endocrine organ by producing hormones, such as estrogen, progesterone, human chorionic gonadotropin, and human placental lactogen, while protecting the fetus from external infectious and immune threats (11, 12). Increasing evidence suggests that molecular exchanges occur between maternal and fetal systems to enable adaptation of maternal physiology for growing fetal requirements during gestation, with the focus placed on placenta-derived EVs as a medium of placental communication with maternal physiology (13–15). An important question arises from these observations and concerns whether placental EVs play a role in protecting the fetus from infectious agents.

Abbreviations: CD, cluster of differentiation; DAMP, damage associated molecular patterns; EV, extracellular vesicle; IL, interleukin; IRAK, interleukin-1 receptor associated kinase; miRNA, microRNA; MV, microvesicles; MVB, multivesicular body/late endosome; MyD88, myeloid differentiation primary response 88 protein; NF-κB, transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells; PAMP, pathogen associated molecular patterns; PLAP, placental alkaline phosphatase; PRR, pattern recognition receptors; TLR, toll-like receptor; TNF, tumor necrosis factor; TRAF6, TNF-receptor associated factor 6.

Among various models of immune tolerance, microRNAs (miRNAs) are implicated as mediators, as well as markers of immune tolerance in various tissues; however, the mechanisms associated with miRNA-mediated immune tolerance in gestational tissues are not well-understood. Because miRNAs are also included as cargo in EVs, do placental EVs and their embedded miRNA cargo play a role in immune tolerance at the maternal-fetal interface? This review focuses on immune tolerance at the maternal-fetal interface and reviews the role of miRNA in mediating immune tolerance via EVs in gestational tissues.

MATERNAL-FETAL INTERFACE IMMUNITY AND ITS ROLE IN ADVERSE PREGNANCY OUTCOMES

Pregnancy is a period of physiological stress accompanied by a vital balance between proinflammatory and anti-inflammatory stimuli. Disruption of this delicate balance at the maternal-fetal interface has been linked to various adverse pregnancy outcomes. Starting from exposure to seminal antigens at coitus, to implantation, an active inflammatory period facilitates implantation and initial pregnancy (16). The cytokines and chemokines in seminal fluid play a role in attracting regulatory T cells (Tregs) to the microenvironment (17). Female dendritic cells recognize fetal antigens and cross-present seminal-fluid antigens that transform effector $CD4^+$ T cells into Tregs, which are then recruited to the endometrium. Tregs and decidual natural killer (dNK) cells increase during early pregnancy and play a vital role in implantation and maintenance of pregnancy (17–19). Although *in vitro* fertilization (IVF) pregnancies involving fertilized eggs with washed semen do occur, Treg recruitment to the implantation site appears to be a physiologically helpful and critical event for implantation and pregnancy maintenance (20, 21). dNK cells and Tregs are integral cellular components that contribute to normal placentation and vascular support at the maternal-fetal interface, while playing an important role in preventing fetal rejection (22).

This period of inflammatory activity during implantation is subsequently followed by a period of relative inflammatory quiescence during mid-pregnancy to enable fetal growth and development (17, 23). Dysregulated immunity with excessive maternal inflammation further into pregnancy contributes to impaired angiogenesis, especially of the spiral arteries, resulting in gestational vascular disorders, such as preeclampsia (24–27). The concept of trained immunity and inflammation tolerance might be applicable in the case of preeclampsia, as subsequent pregnancy with the same partner within a few years of first pregnancy is not always associated with recurrence of the pregnancy complication (28, 29).

Pregnancy can be divided into three phases of inflammatory milieu: implantation (inflammatory), active gestation (anti-inflammatory), and parturition (inflammatory) (17, 23). At parturition, labor is induced by an inflammation-associated cascade of events that result in delivery. Premature activation of this process results in preterm labor and delivery. Why

are the placenta or its EVs and miRNAs unable to blunt this premature activation of inflammation? It is possible that inflammation tolerance (not necessarily immune tolerance) escapes the placenta due to detrimental activation of dNK cells and Tregs, or that EVs carry an inflammatory cargo that does not counter inflammation.

TYPES OF INFLAMMATION AT THE MATERNAL-FETAL INTERFACE: INFECTION-MEDIATED OR STERILE?

A fetus in the intrauterine environment is believed to be in a relatively protected state. Occasionally, microbes gain passage into the intrauterine milieu by ascending infection or transplacental routes, with the transplacental transmission of infection from mother to fetus capable of occurring antenatally or perinatally (12). Pregnant women with altered immunity secondary to various stressors, including infection, can potentially experience preterm delivery. Romero et al. showed that despite evidence of preterm birth being linked to infection, preterm birth without infection is more common (30). Furthermore, antibiotics alone are ineffective at preventing preterm birth related to infection, suggesting that preterm birth induced by infection is mediated by inflammation rather than the organism *per se* (31). One possible explanation for antibiotic failure is that inflammatory damage has already been established, and detrimental pathways have been initiated and cannot be controlled by poorly timed treatment with antibiotics (32). Our understanding of inflammatory signals also suffers from a poor definition of sterile inflammation and its role in programming preterm birth. Therefore, it is important to understand the mechanistic differences between sterile inflammation and infection-mediated inflammation.

Pro-inflammatory stimuli can arise from both host (self) and alien (non-self) sources, also described as "danger" and "stranger" stimuli (23), that include damage-associated molecular patterns (DAMPs; also known as alarmins) and pathogen-associated molecular patterns (PAMPs), which interact with a group of pattern-recognition receptors (PRRs) expressed on the cell surface. Sterile pathways that trigger inflammation include host factors (e.g., tissue injury, cell death, and environmental factors, such as low oxygen tension and elevated uric acid) that act via DAMPs (25). These differ from infectious triggers (e.g., bacteria or viruses) that act via PAMPs (23). However, pathological inflammatory processes can be triggered by either sterile or infection-mediated pathways.

DAMPs are a group of endogenous intracellular molecules released in the early stages of unplanned cell death to signal cell injury (33). The most common DAMPs include uric acid, high-mobility group box 1, interleukin (IL)-1α, and cell-free DNA. High-mobility group box 1 levels increase in multiple animal models along with sterile inflammation, as well as in human models of acute organ injury, autoimmune diseases, and cancer (34–37). High concentrations of uric acid, a byproduct of cell death, can form monosodium urate crystals in the presence of extracellular sodium and induce acute inflammation. Upon exposure to foreign antigens and as the non-specific first line of defense against foreign microorganisms, monocytes differentiate into macrophages capable of phagocytosing the offending agents. Monosodium urate crystals, upon phagocytosis by antigen-presenting cells, can interact with the NALP3 inflammasome to convert pro-IL-1β to IL-1β, thereby resulting in an inflammatory response (23) recognized as pyroptosis. Inflammasome activation has been shown in both intra-amniotic infections as well as sterile intra-amniotic inflammation with preterm labor (38). Furthermore, treating sterile inflammation by inhibiting inflammasome activation has also been shown to reduce preterm delivery in a mice model (39).

PRRs include Toll-like receptors (TLRs) 1 through TLR13, C-type lectins, scavenger receptors, and nucleotide-binding oligomerization-domain-like receptors, all of which operate transduction pathways resulting in cytokine-mediated inflammatory responses. PRRs are expressed in multiple human cells, including decidua, placenta, membranes, and myometrium, throughout pregnancy and in immune and non-immune cells (23, 40–42). The release of DAMPs secondary to tissue injury from hypoxia-ischemia, oxidative stress, vascular dysfunction, or other stressors is implicated in sterile inflammation that acts not only on the placenta but also on the uterus, cervix, fetal membranes, and the fetus. This inflammatory process needs to be tightly regulated since such inflammation left unchecked can cause extensive tissue injury, septic shock, and death. In the placenta, this resultant maternal-fetal inflammation is suggested to contribute to various adverse pregnancy outcomes, including placental dysfunction, preeclampsia, intrauterine growth restriction, and preterm labor (23). Therefore, attenuation of this inflammation at the maternal-fetal interface can play a key role in fetal health and survival.

INDUCTION OF INNATE IMMUNITY VIA microRNAs AND SHAPING OF IMMUNE TOLERANCE: FROM MICE TO HUMANS

The systemic immune system is regulated and dominated by T lymphocytes and adaptive immunity. On the other hand, the decidual leukocyte population in the pregnant uterus is replete with NK cells (65–70%) and antigen presenting cells (macrophages and dendritic cells 10–20%), both contributors to innate immunity (4, 5). Other cell types have also been described to a lesser extent at the maternal-fetal interface, in varying numbers at different stages of pregnancy, and in certain pathological conditions (43, 44), including innate lymphoid cells, other T cell subsets and B cells. Innate immunity at the maternal-fetal interface is of a specialized variety, wherein NK cells and macrophages are pregnancy compatible and support local vascular activity. Prolonged exposure to microbial products, such as lipopolysaccharide (LPS) induces a form of innate immunity that resembles trained immunity (memory) and blunts subsequent responses to unrelated pathogens [referred to as endotoxin tolerance] (45). What is the molecular basis of endotoxin tolerance, and does this occur in the female reproductive tract? Recent pioneering work by Seeley et al.

(45) suggests that repeated exposure to LPS induces tolerance-associated miRNAs (miR-221 and miR-222) in macrophages, with this tolerance phenotype achieved through silencing of inflammatory genes and chromatin remodeling. Most studies of endotoxin tolerance have been undertaken in mouse models, as well as human cell and tissue models, resulting in links between endotoxin tolerance and protection against tissue injury and death (46, 47). Seeley et al. (45) suggest that in humans with sepsis, increased expression of these miRNAs is associated with immunoparalysis and organ damage; therefore, it is possible that a threshold level of tolerance-associated miRNAs needs to be maintained for a longer period of time in order to influence a better outcome in humans.

The molecular mechanisms associated with endotoxin tolerance are believed to be multifactorial, with multiple levels of negative feedback to blunt the inflammatory response. Mice deficient in IL-10 are more susceptible to endotoxic shock following repeated exposure to LPS, suggesting a role for IL-10 in endotoxin tolerance (48). In gestational tissues, endotoxin tolerance has been identified in mouse models, whereas human studies are lacking. During pregnancy, this adaptation due to tolerance might be critical to preventing fetal rejection by the maternal immune system, as well as protecting the fetus from excessive maternal inflammatory responses to various infectious and inflammatory agents. An increase in proinflammatory cytokine responses to bacterial insult can trigger undesirable consequences, including preterm labor and delivery, as well as fetal mal-development and inflammatory injury. Although bacterial exposure during pregnancy is associated with preterm delivery and intra-amniotic infections (49, 50), a wide array of organisms have been described in the maternal-fetal compartments in healthy pregnancies. Interestingly, the presence of an organism in the uterine environment is not always pathological, as previously reported in gene-amplification studies using amniotic fluid from women who delivered healthy newborns following an uncomplicated pregnancy (32, 51, 52).

IMMUNE TOLERANCE MEDIATED BY microRNA

miRNAs are small (18–22 nucleotides), non-coding RNA sequences that play an important role in regulating multiple cellular processes critical for development, differentiation, and organ function and are associated with marked biological consequences in health and disease (53). miRNAs act as negative regulators at the post-transcriptional level by binding to the 3′ untranslated region on target mRNA to inhibit the translation of respective proteins. Thousands of genes are regulated by miRNAs, with the list continuing to grow (53, 54). It is estimated that ∼60% of all protein-coding genes can be regulated by miRNA (54); therefore, it is now accepted that mutations that cause dysfunctional miRNA can potentially affect multiple disease conditions.

Multiple organs harbor specific miRNAs that play vital roles. miR-122 is associated with cholesterol and lipid metabolism in the liver (55), whereas the miR-1 and mir-133 families regulate heart development and play roles in cardiovascular disorders (56, 57). Additionally, numerous recently identified miRNAs have been implicated in a wide array of human diseases, including cardiovascular diseases, neoplasms, and liver and kidney disorders (58–60). Moreover, early gestational tissues, such as human blastocysts, express miRNA, which might be essential for successful implantation and subsequent survival by guiding processes that navigate the intrauterine environment. Euploid and aneuploid embryos exhibit differential expression of miRNAs, ultimately resulting in different eventual outcomes (61). Furthermore, the presence of miRNAs in breast milk and serum could represent markers or mediators of cell signaling. Maternal serum miR-191 is currently being investigated as a potential non-invasive candidate for aneuploidy, whereas other miRNAs, including miR-191, miR-372, and miR-645, have been implicated in IVF failure, and elevated levels of miR-25, miR-302c, miR-196a2, and miR-181a have been identified in degenerate embryos as compared with their levels in blastocyst embryos (62).

In addition to regulating multiple cellular processes, various miRNAs are also implicated in regulating the immune system, including the differentiation and function of innate immune cells (63, 64). miRNAs have also been shown to regulate immune responses to bacterial, viral and parasitic infections (65–67). For example, miR146a polymorphism has been linked to increased risk of malaria in pregnant women. Also, miR 221 negatively regulated innate antiviral response, while miR 34/449 family has been implicated in various viral infections [(66), review]. The role of miRNA in bacterial infections is being studied in various scenarios- from a suggested role in Helicobacter pylori and Epstein-Barr virus-induced cancers [(68), review], to pathogen triggered TLR pathway stimulation.

Multiple miRNAs are up-regulated via TLR-ligand stimulation in monocytes (69) and suggested to play a major role in mediating immune tolerance by regulating the TLR pathway through TLR-receptor transcription and/or creating feedback loops by suppressing key downstream molecules that in turn down-regulate TLR activation (70). Moreover, the pattern of miRNA expression is related to the type of TLR ligand involved in its stimulation, overall ligand concentration, and the type of cells being stimulated.

Increases in miR-146a levels in response to LPS stimulation was observed in THP1 cells (63, 69, 71, 72). Additionally, Nahid et al. (71) showed that exposure to high-dose LPS (1,000 ng/mL) increases levels of the inflammatory cytokines tumor necrosis factor (TNF)α, IL-1-receptor-associated kinase (IRAK)1, and TNF-receptor-associated factor (TRAF)6 along with a simultaneous increase in miR146a levels. On the other hand, when the same cells were primed with low-dose LPS (10 ng/mL), transient elevations in TNFα, IRAK1, and TRAF6 were observed along with upregulated miR146a levels, with subsequent exposure to high-dose LPS (1,000 ng/mL) resulting in a blunted TNFα response in the presence of upregulated miR146. Other studies suggested that elevated miR-146a levels are involved in regulating TLR signaling and cytokine production by downregulating the inflammatory response (73, 74). Furthermore, miR-146a induction is mediated by NF-κB via TLR-ligand stimulation (69). Conversely, miRNAs can directly

target TLRs in order to create a feedback loop that regulates the inflammatory response, with previous studies reporting that miR-105 targets the mRNA encoding the TLR2 receptor in order to attenuate its translation (75, 76). Additionally, miR-146a targets TRAF6 and IRAK activities to inhibit TLR4 signaling and downregulate NF-κB activation (69). By contrast, miRNAs, such as miR-155, can be significantly downregulated after the induction of endotoxin tolerance, suggesting cross-talk between miRNAs in order to maintain immune homeostasis and suppress proinflammatory responses. In addition to miRNAs, long non-coding RNAs are also altered upon endotoxin challenge and negatively regulate TLR signaling, thereby contributing to immune tolerance.

miRNAs are also being studied as diagnostic and therapeutic tools (77). A recent study suggests that decidual tissue from patients with recurrent spontaneous abortions shows decreased expression of miR-146a-5p (78). Similarly, quantification of 30 miRNAs from peripheral blood taken during the first trimester was used to predict preeclampsia, a late pregnancy complication (79). These observations support further study of miRNAs from gestational tissues and peripheral blood to investigate their role(s) in both endotoxin tolerance and pregnancy complications.

PLACENTA AND microRNA

The human placenta produces numerous miRNAs. miRNAs play roles in various key steps in pregnancy, including implantation, maintenance, and labor (80–84). miRNAs have been identified in trophoblasts and mostly originated from the two largest clusters on chromosomes 14 and 19 (C14MC and C19MC, respectively). Most miRNAs identified in primary trophoblasts originate from C19MC, which gives rise to 46 intronic miRNAs that are converted to 54 mature miRNAs (85). miRNAs from C19MC are found in human embryonic stem cells and play an important role in cell proliferation, invasion, and differentiation. C19MC expression is reduced in extravillous trophoblasts and several malignancies while the increase in C19MC expression confers resistance to viral infections (85, 86).

Interestingly, abundant expression of miRNA does not always translate to functional significance. A previous study reported the deletion of the miR379/410 cluster (from C14MC) without consequence (84). Additionally, miR-675 exhibits anti-proliferative effects by silencing *insulin-like growth factor receptor-1*, whereas miR-675 deletion is associated with placental overgrowth (87). Moreover, miR-378a-5p and miR-376c are involved in trophoblast proliferation and invasion regulated by the nodal signaling pathway (88, 89), and the miR17-92 cluster regulates primary human trophoblast differentiation (90). Furthermore, recently identified miR-155 inhibits trophoblasts invasion and is implicated in the pathogenesis of preeclampsia, and increased levels of plasma and placental miR-210 have been reported in association with preeclampsia (91). Although additional miRNAs continue to be discovered their precise clinical implications and roles remain elusive; however, a non-invasive sampling of such placenta-derived miRNAs is potentially useful for diagnosing placental dysfunction.

The majority of miRNAs function in the cell of origin by regulating mRNA levels and translation; however, some miRNAs are selectively secreted by cells into the extracellular space (mainly packaged within EVs) to regulate intercellular signaling of distant "target" cells. Analyses of miRNA from EVs show that EVs' miRNAs content is distinct from that found in the cytoplasm of donor cells from which they were derived. This suggests active miRNA sorting into these vesicles (92–95), although the exact mechanism remains unclear. Our unpublished data confirm that placental miRNAs play an important role in placental endotoxin tolerance resulting in blunted immune response to repeated exposure to LPS. Specifically, placenta-derived miR519c (derived from C19MC) was shown to inhibit TNFα gene expression in our placental explant model. miR519c is placenta-specific and produced by trophoblasts and can be released as free miRNA or packaged into EVs.

EXTRACELLULAR VESICLES IN GESTATIONAL TISSUES

EVs are membrane vesicles of various sizes that are secreted by almost every cell type and multiple organisms ranging from bacteria to humans. EVs are broadly classified according to size and origin into two different categories: exosomes and microvesicles (MVs; including microparticles and apoptotic bodies) (14, 96). MVs are larger membrane-derived vesicles >150 nm in size and secreted directly from the cell membrane. Exosomes are cell-secreted, membrane-derived nanovesicles, which represent a subpopulation of EVs measuring from 40 to 120 nm and with a density of between 1.13 and 1.19 g/mL (14). Exosomes arise from the endosomal compartment as intraluminal vesicles and are secreted by the fusion of endosomes or multivesicular bodies (MVBs) with the cell membrane. Additionally, exosomes can be described according to morphological characteristics (spherical or cup-shaped) and surface markers (CD63, CD9, CD81, and Tsg101) (14, 97). Various gestational tissues, including pre-implantation embryos, oviduct epithelium, placental trophoblasts, and endometrium, secrete EVs, with their source determined according to cell-specific markers. Previous studies suggest that miRNAs are protected from RNase degradation in serum by encapsulation within EVs, which act as carriers of regulatory RNA (53, 98).

THE ROLE OF EXTRACELLULAR VESICLES IN NORMAL PREGNANCY AND GESTATIONAL VASCULAR DISORDERS

The placenta releases EVs as early as the sixth gestational week, with this activity implicated in regulating maternal pregnancy physiology and fetal development, including pregnancy-induced hypertension, gestational diabetes, preterm labor, and delivery (14, 15, 97, 99). EVs (especially exosomes) play a vital role in the preparatory cross-talk between endometrium and embryo at the onset of pregnancy (83, 100–102). Trophoblast-derived EVs harbor molecules specific to placental physiology and cell-cell communication and that exert diverse effects on

maternal and embryonic compartments. These molecules include fibronectin, syncytin, galectin-3, human leukocyte antigen-G, and cytokines as well as bioactive lipids and/or miRNAs, capable of immunomodulation (103). Placental alkaline phosphatase (PLAP), a membrane protein of the placenta, is primarily produced by syncytiotrophoblasts and used as a marker to identify placenta-derived exosomes in maternal circulation (104). $PLAP^+$ exosomes have only been described in the peripheral circulation of pregnant women (13, 14), with the number of placental exosomes positively correlated with total exosomes concentrations during the first trimester in normal pregnancy (105). The total number of exosomes ($CD63^+$) and placental exosomes ($PLAP^+$) present in maternal plasma increases exponentially in the second and third trimesters (14). Although PLAP shows potential as a useful marker for measuring exosomes in normal pregnancy, its levels have not yet been quantified in pathological pregnancies. Furthermore, syncytiotrophoblast EVs are secreted into maternal circulation as early as the tenth gestational week, their numbers increasing by the third trimester; however, their excessive secretion has also been reported in association with preeclampsia (15).

EVs in pregnancy play a pro-coagulant role to a greater degree than that observed in non-pregnant women (103). Levels of EVs harboring the tissue factor antigen, which is involved in the first step of the coagulation cascade, increase during pregnancy, and a significant increase in EVs harboring tissue factor is related to gestational vascular disorders, suggesting a pro-coagulant immune profile in such situations. Moreover, EVs play a significant role in regulating the inflammatory milieu during pregnancy, as EVs from pregnant women show higher levels of inflammatory proteins. Furthermore, EVs from hypoxic trophoblasts exhibit a more intense inflammatory response to peripheral blood mononuclear cells than do EVs from normal trophoblasts. Additionally, placenta-derived EVs carry a functional Fas ligand and TNF-related apoptosis-inducing ligand molecules that convey signals for apoptosis, suggesting a role in establishing EV-mediated immune privilege on behalf of the fetus (106). It is also possible that placental EVs modulate the response to LPS in pregnant women. Unpublished data from our lab showed that exosomal miRNAs mediate endotoxin tolerance in the placenta after repeated LPS exposure. Cytochalasin-D, an inhibitor of exosomes release and uptake, blocked endotoxin tolerance and restored the proinflammatory response in placental explants treated with repeated doses of LPS, thereby suggesting that exosomes mediate endotoxin tolerance in the placenta.

EXTRACELLULAR VESICLE TARGETING OF SPECIFIC TISSUE AND CELLS

The process of EV formation is complex and aimed at secreting selectively prepared vesicles with their content and presenting surface markers geared toward specific target cells. Although the mechanisms of exosomes biogenesis and release continue to be investigated, little is known regarding how exosomes' content is

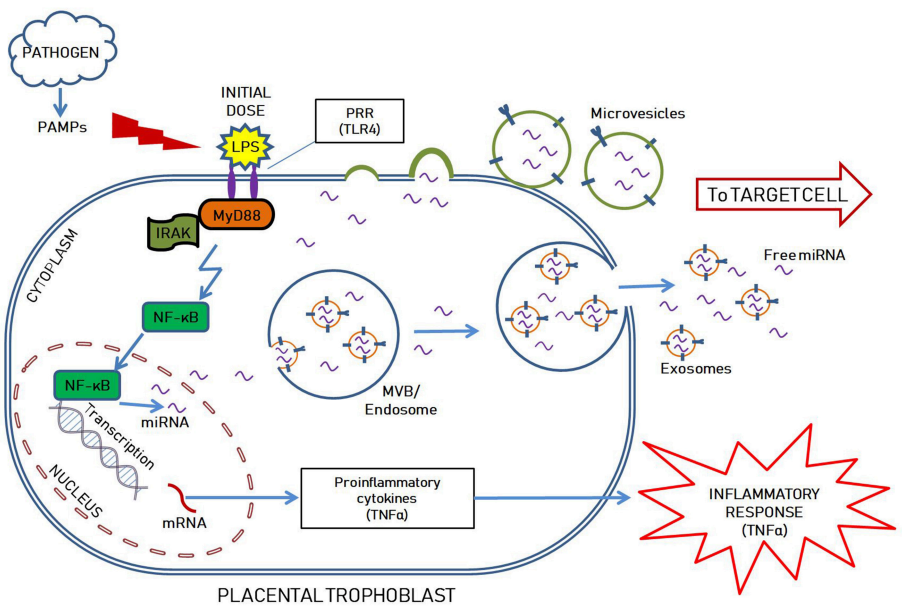


FIGURE 1 | Hypothetical illustration showing LPS mediated inflammatory response, possible mechanism of anti-inflammatory miRNAs mediated endotoxin tolerance as well as packaging within extracellular vesicles (EVs; including exosomes and microvesicles) in a placental trophoblast. The initial LPS dose stimulates TLR4 pathway in the placental trophoblast to prompt the initial inflammatory response, while also upregulating miRNA transcription. The miRNAs are either found freely in the cytoplasm and released outside the cells, or selectively packaged into extracellular vesicles by various mechanisms. Exosomes are formed intraluminally in the endosomal system within multivesicular bodies (MVB) while microvesicles are secreted by membrane derived vesicle formation. They are selectively packaged with nucleic acids (DNAs, mRNAs, miRNAs), proteins, lipids and carbohydrates, specific to the cell of origin and the intended target. The EVs and free miRNAs are transported to the target cell via the extracellular space.

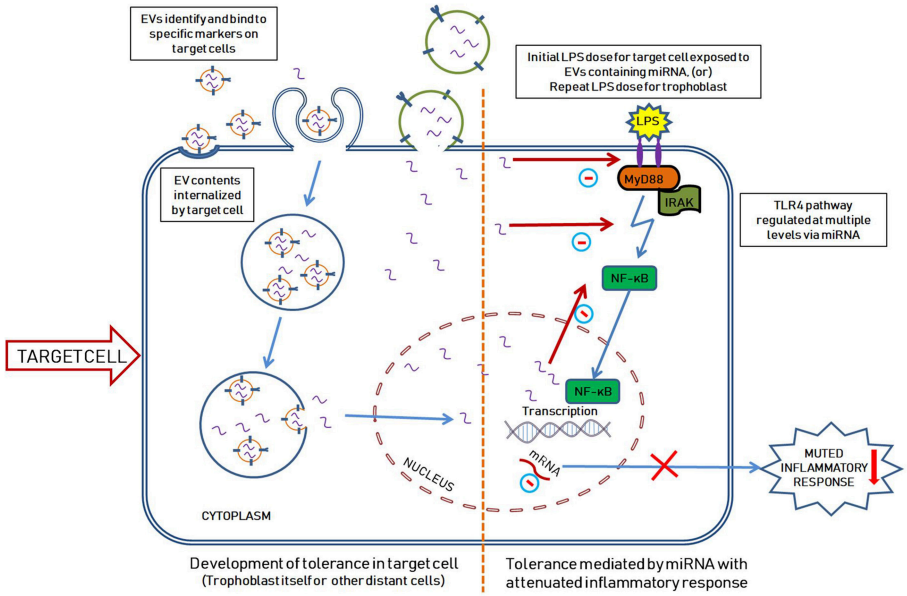


FIGURE 2 | Hypothetical illustration showing the development of endotoxin tolerance in a target cell mediated by miRNA packaged within the EVs. EVs carrying miRNAs (and other contents) are released from the original placental trophoblasts upon exposure to initial LPS dose as seen in **Figure 1**. The released EVs travel to the target cells where they are selectively internalized by different mechanisms as shown. Microvesicles are internalized into the cytoplasm by fusing with the cell membrane while exosomes are internalized into vesicular bodies, lipid rafts on cell surface or by directly binding with specific cell surface receptors (96). The target cell in this scenario can represent a distant cell (exposed to first dose of LPS) or the original trophoblast cell (exposed to a second dose of LPS). We hypothesize that the miRNAs within the EVs are released into the cytoplasm and enter the nucleus thereby negatively regulating the inflammatory pathway at multiple levels (including direct inhibition of TLR4, downregulating mRNA translation of TLR and negative feedback for NF-κB mediated TLR4 pathway). Upon exposure to a second dose of LPS, the original tolerized cell generates an attenuated inflammatory response (since it has high levels of anti-inflammatory miRNAs as a result form the first LPS exposure).

regulated as well as which cells they target. Cell-specific markers presented by exosomes are proposed to play a role in target-cell identification and interaction. Adaptor proteins reportedly recruit an exosome-associated helicase (MTR4) to unique RNA substrates, and exosome cofactors, such as the TRAMP-like protein complex, localize to the cytoplasm and recruit exosomes to specific viral RNA for degradation (107). Other examples of EV targets include cancer cells, which subsequently use EVs to target other organs, such as the lung and liver, to identify pre-metastatic niches based on specific integrin composition (108).

A PLACENTAL MODEL FOR microRNA-MEDIATED ENDOTOXIN TOLERANCE via EVs

Our lab demonstrated that endotoxin tolerance exists in placental tissues (109) and we proposed a model of miRNA-mediated placental immune tolerance packaged within EVs. An LPS stimulus primes placental trophoblasts to exhibit a proinflammatory response through the TLR4 pathway, which activates NF-κB to increase pro-inflammatory cytokine expression and release (such as TNFα) (**Figure 1**). Additionally, TLR4 activation increases the production of placenta-specific miRNAs (either free or packaged in EVs; **Figure 1**) likely also mediated by NF-κB (110, 111). Free miRNAs induce a feedback loop to down-regulate TLR4 signaling and alter related downstream processes, including the inflammatory response, as well as inhibiting mRNA translation of TLR receptors and directly attenuate TLR-receptor levels (**Figure 2**). The secreted EVs translocate to specific target cells/sites, such as placental trophoblasts (autocrine mechanisms), local gestational tissues (paracrine mechanisms), or other maternal and fetal compartments, via circulating peripheral blood or other extracellular fluid, according to specific chemo-attractants or cell-surface markers. EVs then interact with target tissue using cognate surface markers and release their contents, thereby potentially affecting several biological mechanisms, including protein biosynthesis and/or post-transcriptional regulation. The miRNAs within the EVs will reduce the ability of the target cells to produce TNFα in response to LPS exposure. The anti-inflammatory miRNAs implicated in endotoxin tolerance likely act using similar mechanisms, including negative feedback loop at the TLR receptor, to inhibit downstream regulators of the TLR pathway as well as decrease the transcription of pro-inflammatory molecules, such as TNFα (**Figure 2**).

Therefore, LPS insult of target cells/tissue harboring these anti-inflammatory miRNAs increases the readiness for subsequent LPS doses by attenuating the TLR response, thereby tipping the balance against a proinflammatory environment at the maternal-fetal interface (immune tolerance to repeated LPS dose). **Figure 3A**, shows a hypothetical response involving suppressed TNFα levels in the presence of miRNA-induced endotoxin tolerance. Unprimed cells challenged with LPS

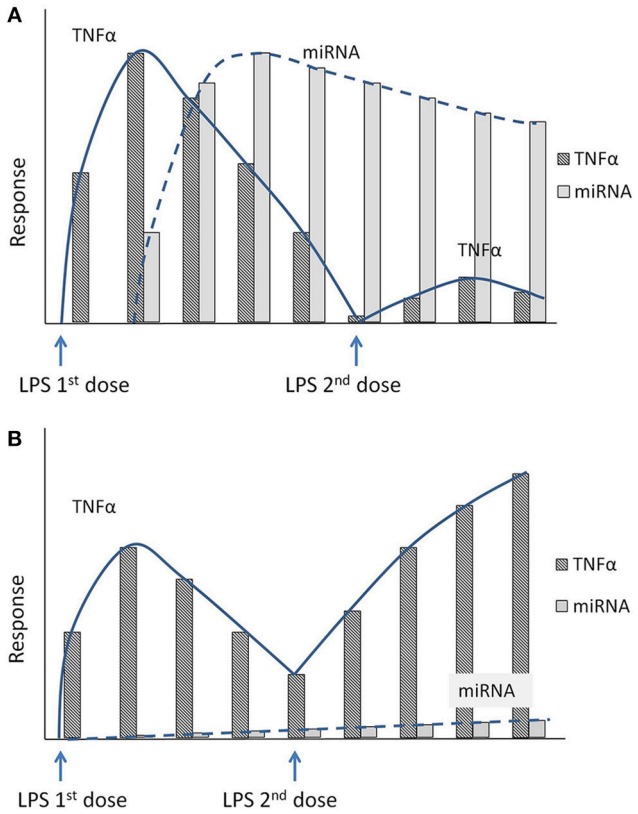


FIGURE 3 | (A) Hypothetical figure illustrates the inflammatory response to repeated LPS exposure. Exposure to the first dose of LPS increases the proinflammatory marker TNFα. Shortly following the initial dose of LPS, the cells also secrete specific anti-inflammatory miRNAs that accumulate in the cytoplasm. Upon exposure to second dose of LPS, the sustained high levels of the anti-inflammatory miRNAs will blunt the inflammatory response with decreased TNFα levels (endotoxin tolerance). **(B)** Hypothetical figure illustrates the inflammatory response in the absence of the anti-inflammatory miRNAs mediated endotoxin tolerance. In a knockout model of specific miRNA, the miRNA levels do not increase after initial exposure to LPS. A subsequent exposure to a second dose of LPS thus produces an unchecked inflammatory response with an exaggerated increase in proinflammatory cytokines (TNFα).

produced a prominent TNFα response followed by an increase in anti-inflammatory miRNA production, which corresponds to later down-regulation of TNFα (**Figure 3A**). As shown in **Figure 3A**, in the presence of sustained high anti-inflammatory miRNA expression, a second dose of LPS will lead to a muted TNFα production by the cells (*tolerized cells*). However, in the absence of these specific miRNAs, subsequent exposure to LPS would result in failure of immune tolerance to repeated infection resulting in exaggerated inflammation at the maternal-fetal interface (**Figure 3B**). We speculate that placentas from women with infection-induced preterm births will have reduced expression of specific placental anti-inflammatory miRNAs. This will lead to endotoxin tolerance failure and exaggerated inflammatory response to repeated infections that result in preterm births or other inflammatory diseases of pregnancy.

CONCLUDING REMARKS

In summary, we propose that miRNAs play a vital role in mediating immune tolerance at the maternal-fetal interface by attenuating immune responses following repeated exposure to inflammatory insult. Packaging and transport of miRNAs by EVs is suggested as a "smart" process specifically intended to address the potential requirements of the target cell/tissue. The success of this activity is dependent upon the homeostasis of the origin cell (placenta, in this case). Therefore, normal or pathologic conditions in the placenta might affect EVs composition and number, resulting in an altered response to endotoxins or other inflammatory stimuli. Additionally, we suggest that the target cells are not randomly selected and are, in fact, pre-identified according to specific markers presented on the EV surface to allow specific identification of target cell/tissue. Perturbations in this process can result in the failure of miRNA-mediated endotoxin tolerance and an imbalanced proinflammatory state leading to adverse pregnancy outcomes, including preterm labor or preeclampsia. The identification of placenta-specific miRNAs and EV markers will promote identification of novel molecules as potential biomarkers for further study of endotoxin tolerance, as well as possible molecular targets for controlling injury from failed immune tolerance.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Immune Checkpoint Molecules in Reproductive Immunology

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Immune checkpoint molecules, like CTLA-4, TIM-3, PD-1, are negative regulators of immune responses to avoid immune injury. Checkpoint regulators are thought to actively participate in the immune defense of infections, prevention of autoimmunity, transplantation, and tumor immune evasion. Maternal-fetal immunotolerance represents a real immunological challenge for the immune system of the mother: beside acceptance of the semiallogeneic fetus, the maternal immune system has to be prepared for immune defense mostly against infections. In this particular situation, the role of immune checkpoint molecules could be of special interest. In this review, we describe current knowledge on the role of immune checkpoint molecules in reproductive immunology.

Keywords: immune checkpoint molecule, reproductive immunology, pregnancy, immunotolerance, CTLA-4, TIM-3, PD-1

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INTRODUCTION

The activation of the immune system to eliminate harmful agents is usually followed by tissue damage at the site of the exposure. In order to keep this side effect of the immune response limited and localized, efficient immunoactivation of immune cells requires multiple incoming signals. Beside antigen recognition, co-stimulatory, survival, and proliferative signals, even environmental factors can determine the outcome of the immune response (1–4).

Immune checkpoint molecules are co-stimulatory receptors, occurring on the surface of several immune cells. After ligand binding, these regulators are capable of transducing inhibitory signals (5). CTLA-4, TIM-3, PD-1 are the most studied members from this group of cell surface receptors (5). The physiological role of immune checkpoints is to prevent a harmful immune attack against self-antigens during an immune response by negatively regulating the effector immune cells, e.g., by inducing T cell exhaustion (5, 6). Recent studies suggest that each checkpoint decreases immunoactivation through different intracellular signaling mechanisms (5, 7). Immune checkpoint regulators are thought to actively participate in the immune defense of infections, prevention of autoimmunity, transplantation, and tumor immune evasion (5, 7).

Pregnancy is a natural model of active immunotolerance, where maternal immune system simultaneously faces two challenges: beside acceptance of the semiallogeneic fetus, the maternal immune system has to be prepared for immune defense mostly against infections. In this particular situation, the role of immune checkpoint molecules could be of special interest. Therefore, this paper aims to review the literature presenting current knowledge about the role of immune checkpoint molecules in reproductive immunology.

CTLA-4

The first described inhibitory receptor CTLA-4 (Cytotoxic T-lymphocyte-associated protein 4) is predominantly and constitutively expressed intracellularly in regulatory T cells, and it is missing in naive conventional T cells (8, 9). Following activation, CTLA-4 is expressed on the cell surface of Tregs, but it can also be found on the cell surface of activated CD8+ or CD4+ T cells (10). The inhibitory effect of CTLA-4 results from the competition with the T cell activatory CD28 receptor to bind the B7 ligands CD80/CD86 present on the cell surface of antigen presenting cells (11). The ability of Treg cells to induce IDO expression in APCs through the CTLA-4-B7 binding was thought to be one of the major mechanism of immune suppression by these cells (12, 13). Interestingly, current thinking suggests, that the main function of CTLA-4 is not delivering negative signals through ligand binding but the removal of its ligands CD80/CD86 from the cell surface of APCs preventing thereby their binding to the costimulatory CD28 present on T cells (8, 14).

CTLA-4 in Murine Pregnancy

The significance of the CD80/86-CD28 activation pathway in T cells during fetal rejection was shown by blocking both ligands with mAb during the pregnancy of the abortion-prone murine model. The blockade resulted in the improvement of fetal survival with an increase of Th2 type cytokines at the maternal-fetal interface (MFI) and in the peripheral expansion of the CD4+ C25+ T cell population. Furthermore, CTLA-4 expression by T cells increased as well which was found to be significantly reduced at the MFI in abortion-prone matings (15, 16). Preventing binding of CD80/CD86 to CD28 is thought to be the way of action of CTLA-4 with similar beneficial effects in maternal-fetal tolerance. Blocking only CD86 using the same experimental setting resulted in the same observations (17). These findings support previous theories about CD80 might be the most functional ligand for CTLA-4. Blockade of the CD86 could turn off the co-stimulatory CD86/CD28 pathway while allowing a prolonged CD80/CTLA-4 interaction with all of the benefits (17–19).

Further evidence for the immunosuppressive capacity of CTLA-4 was delivered from experiments with the CTLA4Ig fusion protein. Using an adenoviral vector, CTLA4Ig was shown to be heavily expressed at the MFI. CTLA4Ig therapy of abortion-prone CBA/DBA matings could effectively improve pregnancy outcome by shifting serum cytokine levels toward Th2 bias and expanding regulatory T cell population at the periphery (20). Furthermore, the CTLA4Ig fusion protein significantly inhibited splenic lymphocyte proliferation and apoptosis of the fetoplacental unit (21). Interestingly, adoptive transfer of Treg with CTLA-4 blockade from normal pregnant mouse to CBA/DBA pregnancy didn't abolish the protective effect of Treg treatment without a blockade resulting in decreased abortion rates (22).

In another abortion-prone setting, in sonic stressed pregnant mice, decidual lymphocytes expressed decreased levels of CTLA-4, without any changes in CD28 expression suggesting the failure of the control of local immunoactivation. CTLA-4 expression by decidual lymphocytes of stressed animals could be enhanced by injections of the dipeptidyl peptidase IV inhibitor, a well-known terminator of T-cell activation (23, 24).

CTLA-4 in Human Pregnancy (Figure 1)

CTLA-4 at the Periphery

Although regulatory T cells increase in number in the periphery during early pregnancy, the enhanced CTLA-4 expression on the cell surface was not observed (10, 25). In contrast to these findings, the expression of one of the ligands of CTLA-4, namely CD86 showed an increased expression by peripheral DCs and monocytes in healthy pregnancy while CD80 expression patterns did not change (26).

CTLA-4Ig treatment of peripheral blood mononuclear cells resulted in a significantly higher IFN-γ secretion in normal

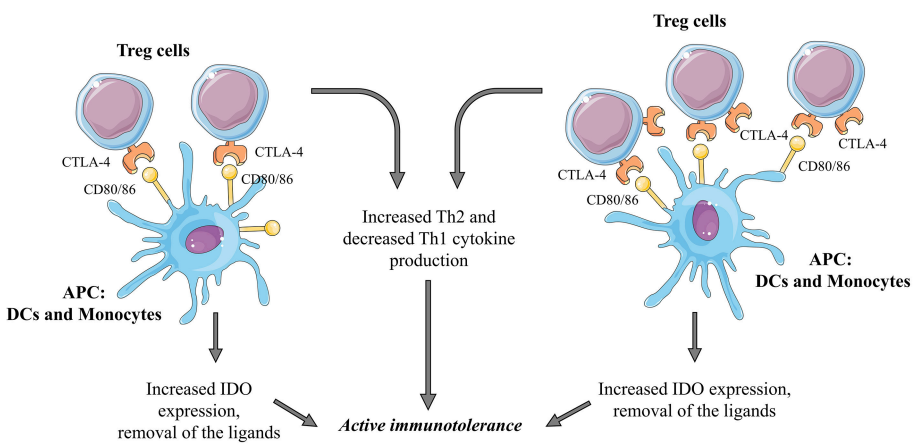


FIGURE 1 | Summarizing the possible role of the CTLA-4/CD80, CD86 pathway during human pregnancy.

pregnancy compared to non-pregnant condition (26). Despite the fact, that CTLA-4 is capable of inducing indoleamine 2,3 dioxygenase (IDO) expression in dendritic cells and monocytes through the induction of IFN-γ, there are conflicting data about whether CTLA-4Ig treatment could enhance IDO expression in DCs and monocytes in normal pregnancy (13, 26, 27).

CTLA-4 at the Maternal-Fetal Interface

Compared to the periphery, decidual Treg cells further increase in number, and the frequency of Treg expressing intracellular or surface CTLA-4 was also found to be elevated in the decidua (10, 27, 28). Interestingly, placental fibroblasts also express CTLA-4, but it is supposed to have non-immunological functions since fibroblasts are not directly in contact with maternal tissues (29). The CTLA-4 ligands, CD80, and CD86, are also present on decidual DCs and monocytes, and they show the same expression profile as in the periphery in normal pregnancy (26, 30). The decidual CTLA-4 expression is in a significantly positive correlation with decidual Th2 cytokine production and a negative correlation with decidual Th1 cytokine production suggesting remarkable immunosuppressive effects locally (30).

CTLA-4Ig treatment of decidual lymphocytes resulted in enhanced IFN-γ and IDO expression (10).

CTLA-4 in Pregnancy Complications

In the case of spontaneous abortion/miscarriage peripheral and decidual Tregs fail to increase to the levels observed in normal pregnancy (10). Data about CTLA-4 expression in these conditions are conflicting. In one hand, the overall ratio of CTLA-4+ peripheral and decidual lymphocytes as wells as the ratio of CTLA-4+ Tregs was found to be significantly reduced. Moreover, the ratios of CTLA-4+/CD28+ in regulatory T cells from miscarriage were significantly lower than that of normal pregnancy (30, 31). On the other hand, there was no significant difference in intracellular and cell surface expression of CTLA-4 on both peripheral and decidual Tregs when compared to non-pregnant and healthy pregnant controls (10). These controversy data may result from different patient inclusion criteria. From the two possible CTLA-4 ligands, only CD86 expression was found to be affected in miscarriage: peripheral monocytes, decidual monocytes, and DC showed significantly lower expression rates compared to those in normal pregnancy (26, 30). Response levels of IDO expression by both peripheral and decidual monocytes and DCs in spontaneous abortion with CTLA-4 treatments were lower compared to a healthy pregnancy (26).

Extensive research focused on the role of *CTLA-4* gene polymorphism with different conclusions (32–37). The A/G polymorphism at position 49 in exon 1 of cytotoxic T lymphocyte antigen-4 (*CTLA-4*) gene may result in abnormal protein modification in the rough endoplasmic reticulum leading to reduced expression (38, 39). Further studies confirmed, that the 49 GG genotype was associated with a reduced inhibitory function of CTLA-4 whereas individuals with AA genotype had more expression of CTLA-4 both intracellular as on the cell surface of activated T cells (33, 40, 41). Further studies with larger sample sizes are needed to prove increased frequencies of G allele and GG genotype among patients with recurrent miscarriage.

Although preeclampsia is characterized by a diminished Treg frequency, a well-known alteration (42–46), little information is available about the possible role of CTLA-4 in the pathogenesis of the disease. CD80 and CD86 ligand expression levels on monocytes decrease in preeclampsia, while data about CTLA-4 expression of Treg are not conclusive, increased and unchanged expression patterns were reported as well. Therefore, it is difficult to determine the significance of the CTLA-4 pathway in preeclampsia (47–49). Two gene polymorphism studies of the exon-1 A49G region of the *CTLA-4* gene revealed an increased frequency of the heterozygosity and GG phenotype in pre-eclamptic women (38, 50).

In women with successful IVF treatment, there is an increase in the peripheral Treg population compared to failed IVF attempts. Investigating CTLA-4 expression at the mRNA level, no differences could be observed in the two IVF patient group (51).

Heterozygous mutations in the immune checkpoint protein CTLA-4 leading to CTLA-4 deficiency results in different autoimmune clinical features, but no further information is available about pregnancy proceeding in these patients (52, 53).

TIM-3

Extensive research has established that Tim-3/gal-9 pathway plays a significant role in the regulation of immune responses and induction of tolerance (54–58). TIM-3 was shown to be expressed by many types of immune cells, including Th1, Th17, NK and NKT-like cells, Tregs, and also on antigen-presenting immune cells (59). Interestingly, TIM-3 activity is thought to participate in both activation and inhibition of immune response (60, 61). In the case of a healthy pregnancy, expression of TIM-3 on Th1 cells may be a key element for reducing proinflammatory Th1-dependent T-cell response (57).

The ligand of TIM-3 receptor is galectin-9 (Gal-9), a β-galactose binding protein (62). Among other identified receptors of Gal-9, TIM-3 has been studied most intensively (54). Both in mice and humans, binding of TIM-3 to its ligand Gal-9 leads to the apoptosis of Th1 and Th17 cells and induce immunotolerance (63–65). Thus, the TIM-3/Gal-9 pathway may serve as a checkpoint regulator limiting the Th1- and Th17-driven immune response and modulating the Th1/Th2 cytokine balance (54).

TIM-3 in Murine Pregnancy

TIM-3 has been studied in detail in murine pregnancy models by several groups (66–71). First, immunofluorescence stainings revealed the presence of TIM-3 in midgestational uterus and flow cytometric analysis proved that this inhibitory molecule is expressed by a variety of immune cells residing locally in the uterus/decidua: uterine NK cells, γ/δ T cells, NKT-like cells, macrophages, dendritic cells (DC), and even by myeloid-derived (66–68). TIM-3 expression by these cells was shown to be dominant but variable throughout pregnancy, in the case of the most prevalent decidual immune cell type, NK cells upregulate TIM-3 during the first half of murine gestation (66, 67). Although TIM-3 expression of decidual NK cells and γ/δ T cells is similar to that in the periphery, their upregulated relative TIM-3 expression

locally suggest that these cells are more mature and entirely functional (68, 72). However, the cytotoxic capacity of TIM-3+ decidual NK cells and γ/δ T cells was shown to be reduced when compared to the periphery; this might be due to the special local microenvironment at the MFI (68). In contrast to these findings, there is a smaller TIM-3+ NKT-like cell subset in the decidua with stronger lytic capacity. Therefore, separate action of TIM-3 on different immune cell types with varying functional outcomes could be concluded (68).

The TIM-3 ligand, galectin-9 is also present at the MFI at different sites. Both murine placental spongiotrophoblast and decidual regulatory T cells express galectin-9 and decidual Gal-9+ Th cells are the main source of the secreted, soluble form of Gal-9 (68). Since the presence of both the ligand, Gal-9 and its receptor, TIM-3 side by side, their binding interaction could be hypothesized, and the inhibitory signal derived from TIM-3 might contribute to maternal immunotolerance observed in murine pregnancy. This hypothesis is supported by the observation that TIM-3 blockade of allogeneic murine pregnancy resulted in litter size reduction, reduced live births, and an increased rate of resorption *in vivo* (66, 71).

Blocking TIM-3 with monoclonal antibodies (mAbs) provided further information about the possible function of this molecule at the MFI. Following inhibition, both apoptotic cells and macrophages accumulate locally, suggesting a deficiency of phagocytic clearance via failed recognition of phosphatidylserine through TIM-3 and enhanced pro-inflammatory cytokine production (66). Uterine granulocytes were also shown to increase in number and to enhance Th1 cytokine expression. These observations are in line with previous studies of experimental autoimmune/ischemic murine models where increased inflammation was due to macrophage and granulocyte activation following TIM-3 blockade (73, 74). Blocking TIM-3 on uterinal NK (uNK) cells affect both physiologic phenotype and function of these dominant cell population at the MFI (67). Although local accumulation and cytotoxic capacity of TIM-3+ uNK cells did not change, uNK cells upregulated the activation marker CD69, and their expression pattern of activating and inhibitory cell surface receptors was notably altered. Secretion of both proangiogenic (VEGF, IFN-γ) and immunosuppressive (IL-10) cytokines by TIM-3+ uNK cells were decreased. Additionally, TIM-3 inhibition resulted in reduced placental expression of the cytokines IL-15 and IL-9, which are important factors for NK cell survival and development (67, 75).

In abortion-prone mouse models, a reduced number of TIM-3+ dNK and CD4+ Th cells can be observed with predominantly Th1 cytokine profiles (69, 70).

All these data from murine pregnancy models suggest a protective role of TIM-3 present at the MFI.

TIM-3 in Human Pregnancy (Figure 2)

TIM-3 at the Periphery

In pregnant women, upregulation of TIM-3 expression by peripheral leukocytes throughout pregnancy was mainly observed on monocytes and NK cells (59, 76). The percentage of TIM-3+ Th, Tc, and NKT-like cells remained relatively constant (57). In the third trimester of a healthy pregnancy, among lymphocytes, ∼80% of NK cells, 15% of CD8+ T cells express TIM-3, in the case of CD4+ T, and NKT-like cells, the ratio of TIM3+ cells was below 5% (77).

TIM-3+ CD8+ T and NK cells show increased cytotoxicity in the third trimester of pregnancy suggesting altered functional capacities toward the end of pregnancy. The increasing levels of soluble Gal-9 throughout pregnancy might have a counter-regulatory function to control enhanced cytotoxicity of TIM-3+ CD8+ T and NK cells (57, 78). These data are inconsistent with other findings where TIM-3+ NK cells were found to have a high capacity to secrete Th2 type cytokines and reduced cytotoxicity toward trophoblast cells as a possible consequence of galectin-9/TIM-3 interaction (76).

TIM-3 expression on monocytes is regulated by IL-4 (upregulation) and IFN-γ (downregulation) cytokines, and it is involved in the effective anti-microbial immune defense by synergizing with TLR signaling (59).

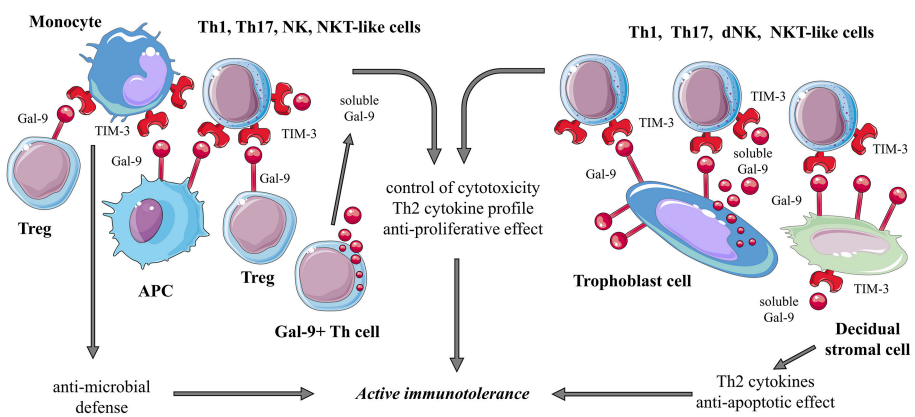


FIGURE 2 | Summarizing the possible role of the TIM-3/Gal-9 pathway during human pregnancy.

It has been demonstrated, that TGF-β1 can induce peripheral NK cells to form decidual NK-like phenotype (79, 80). TGF-β1 treatment upregulated TIM-3 expression on peripheral NK cells proposing an important function of this co-receptor at the MFI (81).

TIM-3 at the Maternal-Fetal Interface

Although TIM-3 expression at the MFI was shown on different decidual lymphocyte subsets, like CD8+, CD4+ T cells, NK cells (69–71, 81), little is known about their role in successful implantation and placentation. The majority of decidual NK (dNK) cells express TIM-3 (60–90%) (69, 81). According to CD117/CD94 expression, TIM-3+ dNK cells have a mature phenotype with Th2 cytokine profile (69, 81). Secretion of IL-4 could be further increased and secretion of TNF-α could be decreased by recombinant human Gal-9 treatment of LPS stimulated dNK cells suggesting regulatory function of TIM-3+ dNK cells on the exaggerated inflammatory response since trophoblast is capable of secreting a large amount of Gal-9, as well as decidual tissue showed high galectin-9 expression (69, 81). Interestingly, blocking TIM-3 signal with TIM-3 fusion protein resulted in the reduction of IFN-γ and TNF-α production of dNK cells (81). Immunohistochemical studies demonstrated, that the fetal part of the MFI, trophoblast cells of term placenta highly express galectin-9 as well (78).

Beside decidual immune cells, decidual stromal cells (DSCs) also express TIM-3, and TIM-3+ DSCs produce higher levels of Th2 cytokines suggesting immune activities of the decidual tissue itself (82). Furthermore, TIM-3 activation seems to be anti-apoptotic when DSCs were stressed through Toll-like receptor activation which is a new potential of this molecule since it acts pro-apoptotic on CD4+ T cells (64, 82, 83).

TIM-3 in Pregnancy Complications

The possible involvement of the TIM-3/Galectin-9 pathway in the pathogenesis of unexplained miscarriages, recurrent spontaneous abortion (RSA), and preeclampsia (PE) has been studied by several groups, both in the periphery as well as at the MFI. However, data should be interpreted cautiously since the inclusion and exclusion criteria for these clinical syndromes and recruitment of the patients involved may vary.

In RSA patient, reduced TIM-3 expression level of peripheral NK cells was observed which could be the result of lower serum TGF-β1 levels, a lack of stimulus for upregulation of TIM-3 (76, 81). Besides TIM-3 surface expression changes, there is an increase of soluble TIM-3 (sTIM-3) and a decrease of soluble galectin-9 in the sera of these patients assuming enhanced competitive binding of galectin-9 by sTIM-3 leading to failed inhibitory signals controlling inflammation (76, 84). Furthermore, TIM-3+ NK cells of RSA patients produce more pro-inflammatory and less anti-inflammatory cytokines suggesting functional deficiencies (76). The only genetic polymorphism analysis of the TIM-3 gene was carried out in RSA patients. TIM-3 polymorphism can affect ligand binding properties and may be involved in some immune-mediated diseases (85). However, analyzing polymorphism of the promoter region of the TIM-3 gene, no differences between the different genotype frequencies could be observed in healthy pregnant women and RSA patients (86). At the MFI, immunohistochemical studies revealed reduced expression of TIM-3 by decidual tissue of women with RSA. Furthermore, same findings were confirmed in the case of DSCs by flow cytometry (82). A decreased percentage of TIM-3 by dNK cells was also demonstrated, although in patients with unexplained miscarriage not with RSA (69). Conflicting data exist according to decidual TIM-3 expression, one study found upregulated TIM-3 and galectin-9 expression in decidua and chorionic villi, both at mRNA and at the protein level in patients with RSA. The authors interpret these findings as being reactive to downregulate Th1 responses observed in RSA (87).

There are few inconsistent data about the possible role of the TIM-3/Gal-9 pathway in the pathogenesis of preeclampsia. On the one hand, in preeclampsia, both TIM-3 and Gal-9 were found to be upregulated in decidual tissue, and TIM-3 expression of peripheral blood monocytes was shown to increase (4). On the other hand, a decreased ratio of TIM+3+ Th, NK, and Vdelta2+ T cells could be confirmed in the peripheral blood of preeclamptic patients (48, 77). Both findings suggest disturbed immune regulation of Th1 responses due to altered Gal-9 and TIM-3 interactions.

PD-1

PD-1 is a transmembrane receptor expressed by e.g., T cells, B cells, natural killer (NK) cells, antigen presenting cells (5, 88). PD-1 generates a strong inhibitory signal upon binding to its ligands PD-L1 and PD-L2, resulting in down-regulation of pro-inflammatory T-cell activity (89). PD-L1 can be found on several immune cells (resting T cells, B cells, dendritic cells, macrophages), in various tissues, like placenta, heart, spleen (5, 90, 91). In contrast to that, PD-L2 expression is limited to dendritic cells and macrophages (92). Furthermore, ligand expression of PD-1 can be regulated, e.g., through the local cytokine environment. PD-L1 expression is increased by many pro-inflammatory factors (LPS, GM-CSF, VEGF) and cytokines (IFN-γ, TNF-α) (5, 93, 94).

PD-1 in Murine Pregnancy

In the allogeneic murine pregnancy model, surface expression of PD-1 on peripheral CD4+ and CD8+ T cells was not altered after conception and during gestation (1). PD-1 blockade *in vivo* was shown to enhance the proliferation of CD4+ and CD8+ T cells in unmated and pregnant mice and to erase the protective effect of Treg cells in Treg treated abortion-prone animals (1, 22).

There are only a few but very informative data about the local presence of PD-1 in murine pregnancy showing PD-1 expression by a broad spectrum of decidual lymphocyte subsets including CD4+ T cells, CD8+ T cells, T follicular helper cells, γδ T cells, NK, and NKT-like cells (1, 68, 95, 96). Furthermore, increased PD-1 expression by decidual NK, NKT-like, and γδ T cells was associated with the reduced cytolytic activity of these cells when compared to the periphery suggesting PD-1 dependent regulation of innate effector functions at the MFI (68).

Concerning the tissue distribution profile of the two ligands for PD-1, PD-L1, and PD-L2 at the MFI, both fetal and maternal compartments are involved: PD-L2 is expressed throughout

the murine decidua, whereas PD-L1 expression is limited to the decidua basalis (97). Insufficient data exist about PD-L expression by the trophoblast suggesting PD-L1 expression by the syncytiotrophoblast but not by trophoblastic giant cells, which are next to the decidua basalis (97, 98). Therefore, PD-1 interaction with its ligands may occur in the decidua itself and is not affected by the fetal part. *In vivo* blockade of PD-1 ligands in allogenic murine pregnancy highlighted the functional role of the PD-1/PD-L1 pathway since anti-PD-L1 treatment resulted in increased fetal resorption rate and a reduction in the litter size, whereas PD-L2 blockade had no effect on fetal resorption (97). At the MFI, PD-L1 blockade resulted in infiltration of T cells, complement deposits, and higher levels of IFN-γ suggesting T cell-mediated rejection mechanisms locally. Another supportive report on the protective role of the PD-1/PD-L1 interaction in maternal-fetal tolerance was revealed by observations of the PD-L1 deficient pregnant mice, which showed similar results in fetal resorption rate, litter size, and a shift toward Th17 emphasizing the role of PD-L1 expressing regulatory T cells controlling fetal antigen-specific maternal T cell (99, 100). Yet, data are conflicting since in another experimental setting neither PD-L1 nor PD-1 deficient mice had significant alterations in gestational or in neonatal offspring parameters (101). These findings indicate doubt about the role of the PD-1/PD-L1 pathway in the survival of the fetal allograft in mice and further studies are needed to reconcile previous controversial results.

PD-1 in Human Pregnancy (Figure 3)

PD-1 at the Periphery

Although immunological acceptance of the fetus is primarily based on maternal tolerance mechanisms at the MFI locally, it exerts a significant impact on systemic immunity as well (102). Despite the fact that syncytiotrophoblast cells—which are bathed in maternal blood—express PD-L1 and PD-L2, data about possible changes in the PD-1 mediated systemic immune response during human pregnancy compared to healthy, non-pregnant controls are lacking (90, 103). The only information regarding this topic is that the frequency of PD-1 expressing T lymphocytes is elevated in the blood of healthy pregnant women compared to non-pregnant counterparts and soluble PD-L1 levels increase throughout gestation (78).

PD-1 at the Maternal-Fetal Interface

Immunofluorescent studies revealed a significantly higher PD-1 expression by decidual T lymphocytes similarly to non-pregnant endometrial T cells. This increase in PD-1 expression was demonstrated more in detail when compared to the periphery during pregnancy: decidual CD8+, CD4+, and regulatory T cells were shown to enhance PD-1 expression (104).

As already mentioned before, PD-L1 and PD-L2 are present in the placenta throughout pregnancy (90, 103, 105). In the first trimester, the major fetal source of PD-L1 is the villous syncytiotrophoblast and extravillous cytotrophoblast, while PD-L2 expression is much more restricted to villous cytotrophoblast (103, 105). On the maternal side, decidual stromal cells constitutively express both PD-L1 and PD-L2, but Th1 cytokines can further enhance their surface expression. PD-L1 expression by decidual macrophages is evident as well (104, 106).

PD-1 interaction with its ligand PD-L1 in different co-culture experiments resulted in reduced Th1 cytokine production by CD4+ T cells (104, 106). These findings suggest the contribution of the PD-1 mediated pathway to the establishment of a favorable Th2 type immune balance at the FMI in healthy human pregnancy.

PD-1 in Pregnancy Complications

Limited information is available about the involvement of the PD-1/PD-L pathway in pregnancy disorders. In preeclampsia, the percentage of PD-1 positive regulatory T cells was significantly higher than in healthy pregnancy with no difference in their PD-L1 expression. While the ratio of PD-1+ Th17 cells was not altered, the PDL1 expression by Th17 cells increased. PD-1 expression by CD3+, and CD4+ T cells did not significantly differ suggesting dysregulated PD-1/PD-L1 axis within the Treg/Th17 imbalance in the clinical phase of preeclampsia (47, 107).

In the case of RSA, decidual PD-L1 expression was significantly reduced on both mRNA as well as on protein

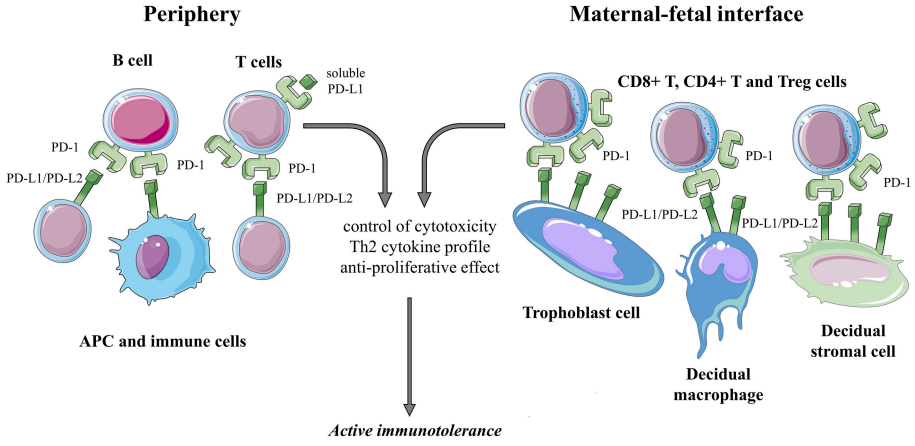


FIGURE 3 | Summarizing the possible role of the PD-1/PD-L1, PD-L2 pathway during human pregnancy.

TABLE 1 | Type of mouse models used in the experiments.

Type of mouse models used in the experiments	References
NORMAL ALLOGENEIC PREGNANCY MATINGS	
CBA/J × BALB/c	(16, 17, 20–22, 69–71)
CBA/J × C57BL/6	(66, 67, 97, 99, 100)
BALB/c × C57BL/6	(95, 96, 101)
NORMAL SYNGENEIC PREGNANCY MATINGS	
CBA/J × CBA/J	(67, 96)
BALB/c × BALB/c	(68)
C57BL/6 × C57BL/6	(1, 96, 101)
ABORTION-PRONE ALLOGENEIC MATING	
CBA/J × DBA/2	(15–17, 20–23, 69, 70)
NON-PREGNANT MICE	
BALB/c	(18, 19, 73, 98)
C57BL/6	(18, 19, 74, 75, 98)

level compared to healthy first-trimester decidua, while PD-1 expression by decidual lymphocytes showed no difference (108).

TIM-3 /PD-1 Co-expression

Effector cells of the immune system can be characterized by co-expression of different co-inhibitory molecules (109). In human pregnancy, TIM-3+PD-1+ CD8+ T cells preferentially accumulate in the decidua (71). Upregulation of both TIM-3 and PD-1 by decidual CD8+ T cells might be induced by embryonic trophoblast in an HLA-C dependent manner (71). These double positive cells display higher proliferative activity and produce more Th2 type cytokines than their TIM-3/PD-1 double negative CD8+ counterparts (71). Blocking both co-receptors increased cytotoxicity and decreased Th2 type cytokine production of TIM-3+PD-1+ CD8+ T cells suggesting a protective, anti-inflammatory role of TIM-3 and PD-1 co-expressing decidual CD8+ T lymphocytes at the MFI (71). This hypothesis is strengthened by further observations both in human as well as in mice. In murine pregnancy systemic blockade of both TIM-3 and PD-1 *in vivo* resulted in further reduction of fetal growth and litter size when compared to the blockade of either TIM-3 or PD-1 alone (71). In line with these findings, in patients with RSA, dual expression of TIM-3 and PD-1 by decidual CD8+ T cells was found to be significantly reduced and to be less proliferative in contrast to a healthy pregnancy (71).

SUMMARY

Immune checkpoint molecules have a major impact on cellular immunity by limiting inflammatory immune response and thereby maintaining physiologic tissue conditions. From this point of view, maternal-fetal immunotolerance represents a real immunological challenge for the immune system of the mother where accurate, tight, and dynamic immune control is required for healthy pregnancy proceeding from the time of implantation on.

As presented in this review in detail, the possible role of immune checkpoint molecules in the establishment of maternal-fetal immunotolerance has been extensively studied. In the case of CTLA-4, TIM-3, and PD-1, the participation and possible role of these molecules in maternal immune response have been confirmed by different approaches, e.g., animal and human experiments, *in vitro* and *in vivo* studies. **Table 1** shows the different mice strains used in animal experiments. However, data are sometimes conflicting and not comprehensive, which may be due to experimental setting differences, small sample size, and the highly complex and multilevel characteristics of immune cell activation. When considering the involvement of co-activatory molecules in maternofetal immune interactions, the co-signaling network is far more complicated (110).

Although there are no current clinical trials aiming at immune checkpoint molecules and interactions in pregnancy complications, some results of the studies discussed in this paper indicate the possible role of TIM-3 cell surface expression rate and serum levels of the soluble ligands PD-L1 and galectin-9 as potential biomarkers for screening during pregnancy (57, 76, 78).

Immune checkpoint inhibitors targeting the PD-1/PD-L1 and CTLA-4 pathways are revolutionary therapeutics in advanced malignancies and could be used in the treatment of chronic viral infections (HIV, HCV) as well in the future. Because of lacking a (68) adequate and well-controlled studies and based on the findings in mouse models, where blockade of the PD-1/PD-L1 pathway resulted in the adverse effect on pregnancy, checkpoint inhibitors are relatively contraindicated for the treatment of metastatic cancer in pregnant women requiring an individualized decision in each case (22, 97, 100, 111, 112).

Although intensive research and a large amount of information regarding the involvement of immune checkpoint molecules in reproductive immunology, the puzzle is not complete. Our current knowledge is quite deficient since there are several other immune checkpoint molecules described recently: Lymphocyte-activated gene-3 (LAG-3), T cell immunoreceptor with Ig and ITIM domains (TIGIT), B and T lymphocytes attenuator (BTLA), V-domain Ig suppressor of T cell activation (VISTA) are novel members of immune checkpoint molecules with proven immune regulatory activity (113). Until today, studies regarding this new generation of negative checkpoint regulators in the field of reproductive immunology are missing and urgently needed.

AUTHOR CONTRIBUTIONS

EM writing, original draft preparation. MM and AB original draft preparation. KD table and figure preparation and editing. LS writing, review, and editing.

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REVIEW
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Pregnancy Galectinology: Insights Into a Complex Network of Glycan Binding Proteins

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Galectins are a phylogenetically conserved family of soluble β-galactoside binding proteins, consisting of 15 different types, each with a specific function. Galectins contribute to placentation by regulating trophoblast development, migration, and invasion during early pregnancy. In addition, galectins are critical players regulating maternal immune tolerance to the embedded embryo. Recently, the role of galectins in angiogenesis during decidualization and in placenta formation has gained attention. Altered expression of galectins is associated with abnormal pregnancies and infertility. This review focuses on the role of galectins in pregnancy-associated processes and discusses the relevance of galectin-glycan interactions as potential therapeutic targets in pregnancy disorders.

Keywords: galectins, pregnancy, placentation, glycans, preeclampsia

INTRODUCTION

During pregnancy, a delicately regulated interplay of endocrine, immune and metabolic processes is established in order to sustain offspring development. The coordination of a series of simultaneous events occurring at both sides of the maternal-fetal interface, including multiple signaling pathways driving cell growth and differentiation, vascular development, and immune regulation, is critical for a successful pregnancy outcome. At the maternal site, complex immunoregulatory mechanisms support active tolerance of fetal alloantigens while also remaining competent to elicit an effective response toward pathogenic insults (1). Throughout pregnancy the uterine vascular bed experiences dramatical changes with extensive remodeling of existing vessels and formation of new networks through the process of angiogenesis (2), allowing for the proper delivery of oxygen and nutrients to the developing fetus. In parallel, at the fetal site, the process of placentation relies on a complex interaction between invasive trophoblasts and maternal immune cells involving developmentally regulated periods of branching angiogenesis, non-branching angiogenesis, trophoblast differentiation and syncytium formation. Disruption of this normal

pattern of placental development will directly impact placental function, with well-recognized consequences leading to suboptimal pregnancy outcomes (3).

The placenta sustains pregnancy by providing an immunological barrier between the mother and fetus, mediating the transfer of gases, nutrients and water and secreting a variety of hormones, cytokines, and signaling factors. As the active interface mediating maternal-fetal communication, the placenta plays a key role in sensing and modulating perturbations in the maternal environment and transmitting these stimuli to the developing fetus, with potential consequences in long-term offspring health. Indeed, it is now well-recognized than an adverse intrauterine environment during early development can modify disease predisposition in adult life as stated in the so-called "developmental origins of health and disease" or "fetal programming" paradigm. From the time of pioneer studies correlating birth weight and altered fetal growth with predisposition to chronic conditions as cardiovascular disease and diabetes (4), accumulating experimental evidence has contributed to the identification of multiple maternal constitutional and life style factors that can impact long-term individual health as well as the mechanisms involved in the transmission of these programming stimuli across the placenta (5–7).

Among the multiple mediators involved in pregnancy orchestration, the galectin family of β-galactoside binding proteins elicits great interest in the reproductive medicine field due to their unique ability to modulate diverse developmental processes and their potential use as biomarkers for gestational disorders. In this review we discuss the current knowledge on the role of galectins in supporting maternal adaptations to pregnancy and placental development, the impact of their dysregulation for development of disease and the potential application of galectinome profiling studies for diagnostic and therapeutic interventions in adverse pregnancy outcomes.

GENERAL ASPECTS OF GALECTINS

Complex carbohydrates on the cell surface and the extracellular matrix (ECM) encode abundant structural information that when decoded by specific carbohydrate-binding proteins (lectins) modulates interactions between cells, or cells and the ECM (8, 9). Based on their structural folds and canonical sequence motifs in the carbohydrate recognition domain (CRD), lectins have been organized into several families that include galectins (formerly S-type lectins), C-type, F-type, X-type, R-type, P-type, and several other families [Reviewed in (10)]. The taxonomic distribution of selected lectin families and their structural analysis have yielded critical information about their functional aspects and evolutionary history. While F- and C-type lectins—are largely heterogeneous and evolutionary diversified lectin families (11, 12), from a structural standpoint, galectins are relatively conserved (13, 14). Galectins are non-glycosylated soluble proteins characterized by a unique sequence motif in their CRDs and affinity for β-galactosides (13). Most galectins show preference for N-acetylated disaccharides such as N-acetyl-lactosamine (LacNAc; Galβ1,4GlcNAc) and related structures, whereas others have preference for blood group oligosaccharides (13–15).

Galectins are synthesized in the cytoplasm, and can be translocated into the nucleus where they can form part of the spliceosome (16, 17). Galectins can also be secreted to the extracellular space by non-classical mechanisms as they lack a typical signal peptide possibly by direct transport across the plasma membrane (18). Once secreted, galectins bind to carbohydrate ligands in the ECM and the cell surface, namely glycans that display LacNAc and polylactosamine chains [(Galβ1, 4GlcNAc)n] (13) (**Figure 1**). These include laminin and fibronectin, mucins, lysosome-associated membrane proteins, and numerous cell surface glycoproteins (19–22). Among the latter, galectins bind with high affinity to glycosylated cell surface signaling molecules such as α and β integrins (21) and the signaling mucin MUC1 (22). Integrins are the predominant laminin and fibronectin-binding proteins expressed on differentiating cells and represent important ligands for galectins involved in cell adhesion, motility and differentiation (21–24). For some galectins, the immediate binding to ligands in the oxidative extracellular environment is key to the stability of their carbohydrate-binding activity (25). The crystal structure of galectin-1 (Lgals1) revealed six key cysteine residues, some of which are located on the surface of the molecule on the face opposite to the CRD and are potentially susceptible to oxidation (26–28). Under non-reducing conditions, intramolecular disulfide bridges are formed resulting in conformational changes that preclude Lgals1 from forming a dimer (29). A critical interplay takes place between the oxidation state of cysteine sulfhydryl groups and the ligand binding and dimerization equilibrium, suggesting that specific binding to glycan ligands enhances dimerization and reduces sensitivity to oxidative inactivation (25). In addition, extracellular galectins can also recognize exogenous ligands, such as glycans on the surface of viruses, bacterial pathogens and parasites (30–33), a hallmark of other lectin types, such as C-type lectins (11). Furthermore, galectin secretion into the extracellular space upon stress has been proposed to constitute non-infectious "danger signals" that can initiate or exacerbate inflammatory responses (34).

STRUCTURAL ASPECTS OF GALECTINS

Galectins are characterized by their extensive taxonomic distribution and striking evolutionary conservation of primary structures, gene organization, and structural fold (11). The identification of galectin-like proteins in the fungus *Coprinopsis cinerea* (35) and in the sponge *Geodia cydonium* (36) revealed structural conservation of galectins in eukaryotic evolution. Furthermore, proteins sharing the galectin structural fold identified in the protozoan parasite *Toxoplasma gondii* (37, 38) and in rotaviruses (39–41) suggest either early emergence of the galectin fold or horizontal transfer from the vertebrate hosts, respectively. In general, galectin polypeptide subunits exhibit a relatively simple domain organization, housing one, two, or four galectin CRDs (11).

Although galectins have been evolutionarily conserved (42), the galectin repertoire in any given mammalian species is

Galectin	Classification	Glycan specificity	Source of expression	Function	Dysregulation in pregnancy disorders
gal-1	Prototype One CRD, which can dimerize	α2-3 sialylation Asn Ser/Thr	Endometrium/ decidua (stromal cells), uNK cells Trophectoderm/ICM Placenta (VT, EVT)	Immune modulation, embryo implantatiion, trophoblast invasion, angiogenesis	Placenta expression: ↑GDM, ↓ early onset PE, ↑ late onset PE, ↑ HELLP, ↓ SA Systemic levels: ↓GDM, ↑late onset PE, ↓SA
gal-3	Chimera One CRD fused with a non-lectin domain	Asn Ser/Thr	Endometrium/ decidua (epithelium), Placenta (VT, EVT)	Immune modulation, embryo implantation, uNK cell regulation?	Placenta expression: ↓ IUGR, ↑ PE, ↑ HELLP, ↑GTDs Systemic levels: unknown
gal-8	Tandem-repeat Two different CRD	α2-3 sialylation SO_4 GD1a GM3 Ser Ser	Decidua (stromal cells, glands) Placenta (VT, EVT, Syn)	ECM remodeling?, angiogenesis?, immune modulation?	Placenta expression: ↓ IUGR (gender specific) Systemic levels: unknown
gal-9	Tandem-repeat Two different CRD	Asn GM1 GA1 Ser Ser	Endothelium/decidua (Epithelium) Placenta (VT)	Immune modulation	Placenta expression: ↓ SA (Lgals9 D5/10), ↑/↓ IUGR (gender specific), ↑ PE Systemic levels: ↓SA
gal-13 (PP13)	Prototype One CRD, which can dimerize	GB-O BG-A BG-B	Placenta (Syn, endovascular trophoblasts)	Immune modulation, Arterial remodeling and vasodilation	Placenta expression: ↓PE, ↓GDM Systemic levels: ↑PE (↓ 1st trimester), ↑ HELLP, ↑GDM

FIGURE 1 | Summary of galectins' glycan specificity, functions and sources of expression in mammalian pregnancies. Question marks (?) denote putative functions (not yet experimentally confirmed in the context of pregnancy). BG, blood group; uNK, uterine natural killer; ICM, inner cell mass; VT, villous cytotrophoblast; EVT, extravillous trophoblast; Syn, syncytiotrophoblast; GDM, gestational *diabetes mellitus;* SA, spontaneous abortion; PE, preeclampsia; HELLP, hemolysis elevated liver enzymes and low platelet syndrome; GTD, gestational trophoblastic disease; IUGR, intrauterine growth restriction. ↑ and ↓ denote increased/peak and decreased expression, respectively. ● Mannose ● Galactose ■ N-acetylglucosamine ■ N-acetylgalactosamine ◆ Sialic Acid ▲ Fucose.

constituted by multiple galectin types, subtypes, and isoforms (13). Based on the CRD organization of the polypeptide monomer, mammalian galectins (and by extension, galectins in all vertebrate taxa) have been classified in three major types: "proto," "chimera," and "tandem-repeat" (TR) types (43) (**Figure 1**). Proto-type galectins contain one CRD per subunit and can form concentration-dependent non-covalently linked homodimers. Dimerization of proto-type galectins is key to their function in mediating cell-cell or cell-ECM interactions (44, 45). Two Lgals1 monomers interact via amino acid residues from a hydrophobic core that establish a dimerization equilibrium with a Kd of 7 μM (16). Both proto- and TR-type galectins comprise several distinct subtypes, all numbered in the order of their discovery, while chimera galectins include a single subtype (13). Lgals1,−2,−5,−7,−10,−11,−13,−14, and−15, are included in the proto-type. Chimera-type galectins, represented by Lgals3, have a C-terminal CRD and a proline- and glycine-rich N-terminal "tail." Ligand-driven interactions of Lgals3 subunits via the N-terminal domain mediate their oligomerization into trimers and pentamers (46). TR galectins display two similar—albeit not identical—CRDs connected by a functional linker peptide (47), and comprise the Lgals4, −6, −8, −9, and −12 subtypes. Galectin subtypes may be expressed as multiple isoforms in a single cell type or as tissue-specific variants generated by alternative splicing (13, 48), positive selection, and amino acid replacements in carbohydrate-recognition domains (49). Among the proto, chimera and TR galectin types, several subtypes, that include human Lgals1, Lgals2, Lgals3, Lgals9, and three galectins that cluster in the human chromosome 19 [Lgals13 (pp. 13), −14, and −16], have been recently investigated with regards to their potential roles in fertilization, embryo implantation, placentation, and the various stages of normal and pathological pregnancy (49–52).

The structural fold of Lgals1 and the amino acid residues of the CRD that directly or indirectly—via water molecules—interact with the hydroxyl groups on the carbohydrate ligands have been identified by the resolution of the crystal structure of the Lgals1/LacNAc complex (26, 27, 53). The Lgals1 subunit is a β-sandwich consisting of a 135 amino acid-long polypeptide that folds into two antiparallel β-sheets of five and six strands each (S1–S6 and F1–F5). This globular structure contains one carbohydrate binding cleft formed by three continuous concave strands (S4–S6) that includes all amino acid residues that interact with LacNAc and are responsible for the carbohydrate specificity of Lgals1: histidine 44, asparagine 46, arginine 48, histidine 52, asparagine 61, tryptophan 68, glutamic acid 71, and arginine 73 (27). Tryptophan 68 establishes a hydrophobic stacking interaction with the non-reducing terminal galactose ring. Additional water-mediated interactions between His52, Asp54, and Arg73 in the Lgals1 CRD with the nitrogen of the NAc group rationalize the higher affinity for LacNAc over lactose. The rigorous assessment of the galectins' carbohydrate-binding affinity has been enabled by biophysical approaches, such as

microcalorimetry measurements and surface plasmon resonance analysis. For example, the dissociation constants of bovine Lgals1 for Lac, LacNAc, and thiodigalactoside measured by microcalorimetry were in the range of 10^{-5} M, with two binding sites per Lgals1 dimer (54). The overall structure of the Lgals3 CRD is very similar to the Lgals1 CRD although in the former the carbohydrate-binding site is shaped as a cleft open at both ends, exposing the GlcNAc of the LacNAc to the solvent (55). This extended binding site in Lgals3 results in increased affinity for polylactosamines and for ABH blood group oligosaccharides [Fucα1, 2; GalNAcα1,3(Fucα1,2); and Galα1,3(Fucα1,2)] (55). The structures of the individual N- and C-terminal CRDs of TR galectins, such as galectins-4,- 8, and−9, have been resolved by either crystallization or NMR spectroscopy. Results have shown that the two CRDs in the same galectin molecule are structurally similar but exhibit either different affinities for the same ligand such as observed in Lgals4, or different fold and specificities altogether, such as reported for Lgals8 (56–58).

Based on analysis of the galectin primary structure and intron-exon position in various vertebrate species it has been proposed that along the vertebrate lineages leading to mammals, galectins evolved by duplication of a primordial single CRD galectin gene that produced a bi-CRD gene, with the N- and C-terminal CRDs later diverging into two subtypes (F4-CRD and F3-CRD) of distinct exon-intron organization. Single-CRD galectins display the F3- (e.g., Lgals1, −2, −3, −5) or F4- (e.g., Lgals7, −10, −13, −14) subtypes, while TR galectins display both F4 and F3 subtypes (Lgals4, −6, −8, −9, and −12) (13, 42). In invertebrate species, galectins exhibit one, two, or four tandem-arrayed CRDs (59–61). In those invertebrate galectins that carry multiple CRDs, these are structurally similar but not identical, suggesting that they differ in their fine carbohydrate specificity (61). How the multiple CRD galectins from invertebrates relate to the vertebrate TR galectins remains to be fully understood, but a preliminary phylogenetic analysis revealed that individual CRDs of a four-CRD galectin clusters with the mammalian single CRD galectins rather with the TR galectins, suggesting that this gene is the product of two consecutive duplications of a single-CRD galectin gene (61).

FUNCTIONAL ASPECTS OF GALECTINS

As discussed above, TR galectins display two CRDs in a single polypeptide, that can interact with and cross-link multivalent ligands, either soluble glycoproteins or glycolipids, or ECM and complex glycans on the cell surface. Although proto- and chimera-type galectin subunits possess a single CRD, they can organize as oligomeric structures that also bind multivalent ligands with increased avidity (44, 45). The density of the cell surface glycans and their scaffolding (as glycoproteins, glycolipids or polysaccharides) modulates affinity of the CRD-ligand interaction via negative co-operativity (45), and can lead to ligand cross-linking, and formation of lattices that cluster these ligands into lipid raft microdomains (44). These interactions can promote reorganization or association of cell surface components, regulate turnover of endocytic receptors, activate or attenuate signaling pathways, and in turn, modulate cell function (44). Further, because galectin types and subtypes exhibit notable differences in carbohydrate specificity and affinity and bind a broad range of glycans that display the requisite topologies, the galectin repertoire displays considerable diversity in recognition properties that together with their distinct and unique tissue distribution and local concentrations, supports extensive functional diversification (13, 30). Thus, the biological function of a particular galectin may vary among cells, tissues and fluids, depending on their concentration, the availability and multivalent presentation of suitable carbohydrate ligands, and the redox properties of any particular intra- or extracellular microenvironment (30).

EARLY DEVELOPMENT AND TISSUE REGENERATION

The initial description in the early "80s of developmentally-regulated galectins in chicken muscle suggested that their biological roles were related to embryogenesis and early development. Further, the finding that chicken galectins preferentially recognized the abundant polylactosamines present on the myoblast surface and the ECM, suggested that galectins mediate myoblast fusion [reviewed in (14)]. Later studies suggested roles of murine Lgals1 and Lgals3 in notochord development and somitogenesis, and in skeletal muscle and central nervous system development (62, 63). In recent years, the increasing availability of null mice for selected galectins enabled their developmental phenotypic analysis. Although the phenotypes identified have been in some cases rather subtle, which hindered a rigorous assignment of the galectins" biological roles, the use of galectin deficient models and tissue-specific knockouts is one of the most complete available tools for the analysis of the biological role of galectins. In addition, rodents express a complex galectin repertoire; this was attributed to functional redundancy of the multiple galectin types and subtypes. However, as the binding properties and natural ligands of each galectin have been rigorously characterized in recent years, it has become clear that this is not the case, and their unique biological roles are being elucidated in increasing detail. In the past few years, Drosophila, *C. elegans*, and zebrafish (*Danio rerio*) have become useful model systems to address the biological roles of galectins (64–68). For example, antisense knockdown approaches in zebrafish embryos for a Lgals1 isoform (Drgal1-L2) revealed a key role in differentiation and development of the myotome (69). The zebrafish model was also useful to assess the roles of galectins in tissue repair and regeneration (53, 70). Experimentally light-induced retinal injury in adult zebrafish was used in combination with an antisense knockdown approach to demonstrate that photoreceptor cell death upregulates expression and secretion of DrGal1-L2 by stem cells and neuronal progenitors in the Müller glia, and selectively regulates the regeneration of rod photoreceptors (70).

GENOME ORGANIZATION OF GALECTIN FAMILY MEMBERS

While galectins are usually grouped based on their architecture, a potential relationship between gene location and function has been investigated (42, 50). Genes encoding Lgals1 and Lgals2, named *LGALS* in humans and *Lgals* in mice and other chordates, map to syntenic regions of chromosome 22 and chromosome 15, in humans and mice, respectively. Studies of the promoter sequences and expression of murine *Lgals1*, *-2* and *-7* revealed significant differences in the proximal promoter regions for putative transcription factor binding sites in these genes, which is believed to correlate with the ubiquitous gene expression of *Lgals*1 and a more restricted expression of *Lgals*2 and *Lgals*7 (71).

Members of the human galectin gene family are found in different chromosomes, including chromosome 1, 11, 14, 17, 19, and 22. Than et al. proposed that some of the human galectin genes clustered in chromosome 19 and expressed in villous trophoblasts, including *LGALS13, LGALS14, LGALS16*, are developmentally regulated by DNA methylation and induced by transcription factors that drive villous trophoblast differentiation and trophoblast-specific gene expression (49). In addition, dysregulation of these galectin genes with a potential role in immune tolerance to the semi-allogeneic fetus was proposed to be associated with preeclampsia (49).

Interestingly, the *Lgals*3 gene is different from other galectin genes in which gene duplication and inversion within a cluster has been reported. A single member has been identified per species hinting at a conserved function of Lgals3 during evolution (42). In addition, *LGALS3* contains an internal gene, which is much less abundant than *LGALS3* transcripts and is expressed mostly in peripheral blood leukocytes producing an entirely distinct protein from Lgals3 (72).

ROLE OF GALECTINS IN PREGNANCY ASSOCIATED PROCESSES

Expression profiling studies in reproductive tissues have shed important insights on the biological roles played by galectins in pregnancy orchestration, highlighting the importance of a delicate interplay between maternal and fetal sources of galectin expression for healthy outcomes (**Figure 1**). The following section provides a brief overview of the role of individual galectins expressed at the maternal-fetal interface in the establishment and maintenance of pregnancy.

GALECTIN-1 (LGALS1)

The functions of Lgals1 in the context of pregnancy are the best characterized when compared to other members of the galectin family (**Figure 1**), likely due to its high level of expression by decidual stromal cells and trophoblast cell populations which suggested an important function (73). Indeed, Lgals1 has been shown to play a role in a variety of biological processes highly relevant for pregnancy orchestration including angiogenesis, immune response regulation, cell adhesion, invasion, and cell cycle progression through intracellular or extracellular mechanisms (23, 74–76).

Lgals1 expression is observed in 3–5 days human embryos potentially increasing trophoblast attachment to the uterine epithelium (77). After embryo attachment as the trophoblast layer differentiates, Lgals1 localizes to villous cytotrophoblast where it may play a role in promoting syncytium formation, although this function has only been studied *in vitro* using the BeWo trophoblast tumor cell line (78, 79). More recently, Lgals1 has been demonstrated to drive the differentiation of mouse trophoblast stem (TS) cells *in vitro*, by enhancing cell migration and invasiveness associated with a shift in the expression of matrix metalloproteinases, epithelial-mesenchymal transition markers and the TGF-β signaling pathway (80). Circulating levels of Lgals1 increase significantly during pregnancy and several studies indicate the potential use of Lgals1 as a biomarker for miscarriage, recurrent fetal loss and preeclampsia (PE) (77, 81–84). Whether circulating Lgals1 retains carbohydrate-binding activity within the oxidative nature of the extracellular environment remains unknown as Lgals1 exhibits exquisite sensitivity to oxidative inactivation (25, 85). In addition, a further question regarding concentration of galectins in serum or plasma is whether high picomolar concentrations are sufficient for galectins to act at a distance similar to circulating hormones (86). In this regard, some galectin-mediated cellular activities (e.g., Lgals3 and Lgals7) (87, 88) might be sufficiently sensitive to be elicited by serum levels of galectins.

Lgals1 is highly expressed in the most invasive trophoblast cells of the placenta and membrane bound Lgals1 has been proposed to regulate migration of primary trophoblasts and of an extravillous trophoblast (EVT) cell line (77, 89–91). Modulation of EVT migration by Lgals1 could be related to its interaction with the β1 integrin chain on the EVT membrane (90, 92–94, 94). Another reported ligand for Lgals1 on the EVT membrane is the mucin MUC1 (95). Expression of *MUC1* is increased during placental development and was found to be elevated in severe pre-eclamptic placentas (96) although the significance of this finding is unclear as MUC1 has been shown to have adhesive and anti-adhesive properties (97). Interestingly, adhesion and invasion of the HTR-8 SV/neo EVT cell line to ECM components is negatively affected by *MUC1* overexpression (98). In endothelial cells, the membrane protein neuropilin-1 was identified as a ligand for Lgals1 and the expression of neuropilin-1 in decidual cells, intermediate trophoblasts, and syncytiotrophoblasts has been recently reported (99, 100). The potential interaction of Lgals1 with neuropilin-1 in these placental cells could potentially have functional consequences for placentation. As stated above, besides interacting with glycoproteins on the cell membrane, Lgals1 interacts with glycoproteins deposited in the ECM and has been shown to have both anti-adhesive as well as pro-adhesive extracellular functions (23). In the placental ECM, Lgals1 ligands include fibronectin, laminin, and osteopontin, which are also integrin ligands (19, 101–104).

The importance of Lgals1 as a contributor to feto-maternal tolerance has been described by many investigators and has been extensively reviewed (51). Several immune cells with essential

roles in the establishment and maintenance of pregnancy synthesize and respond to Lgals1, e.g., CD4+ CD25+ regulatory T-cells, which play a very important role in tolerating the immunogenic paternal alloantigens (83, 105–110). In addition, *in vitro* studies showed that Lgals1 regulates the expression of human leucocyte antigen (HLA-G) in EVTs demonstrating that Lgals1 contributes to tolerance via its interaction with immune and trophoblast cells (77).

GALECTIN-2 (LGALS2)

Lgals2 is predominantly expressed in the gastrointestinal tract and has been identified as one of the main gastric mucosal proteins proposed to have a protective role in the stomach by interacting with mucins (111). In addition, immune functions of Lgals2 have been proposed including its ability to induce apoptosis in activated $CD8^+$ T-cells and its effects on monocytes (112, 113). Lgals2 was shown to polarize monocytes and macrophages to a pro-inflammatory, non-arteriogenic M1 phenotype, and reduce monocyte motility. Interestingly, Lgals2 regulation of monocyte/macrophage phenotype were attributed to its interaction with the lipopolysaccharide-binding protein CD14 in a non-carbohydrate dependent manner (112, 113). Lgals2 expression in the placenta was reported in both VT and EVT cells and was shown to be expressed at higher levels in VT and EVT of male compared to female placentas. Interestingly, in cases of intrauterine growth restriction (IUGR), there was no change in expression in female placentas compared to controls; however, expression of Lgals2 in male IUGR placentas was reported to be decreased compared to controls (114). While these studies should be repeated with a larger sample number, they may serve to caution investigators for the need to take the gender of the fetus into account as an important variable when analyzing possible changes in galectin expression when comparing normal to pathologic pregnancies. In addition, *Lgals2* expression was decreased in third-trimester EVT trophoblast cells in cases of PE on the protein and mRNA level (115) and also significantly down-regulated in the VT and EVT trophoblast of spontaneous and recurrent abortion placentas (116).

GALECTIN-3 (LGALS3)

Lgals3 has been implicated in the regulation of innate and adaptive immune responses, where it participates in the activation or differentiation of immune cells and contributes to phagocytic clearance of microorganisms and apoptotic cells by macrophages (117, 118). Lgals3 has been reported to promote but also to inhibit T-cell apoptosis depending on whether it binds to glycoproteins on the cell surface (CD45 and CD71) or to intracellular ligands (Bcl-2) (119, 120). In the placenta, Lgals3 was detected in all trophoblastic lineages including villous cytotrophoblasts (CTB) and EVT with a reduction of Lgals3 expression observed from the VT to the trophoblastic cell columns (121). This pattern of Lgals3 expression correlates with the switch from a proliferative to a migratory trophoblast phenotype and while placental Lgals3 dysregulation has been associated with some obstetric complications including spontaneous or recurrent miscarriages, further studies are needed to understand its contribution to trophoblast biology (81, 122). In addition to trophoblasts, Lgals3 is expressed by maternal decidual cells (73). While showing a different expression pattern, both Lgals1 and Lgals3 have been proposed to play a role in cell-cell and cell-matrix interactions of trophoblast during placentation (121). Studies of the importance of Lgals3 in murine pregnancy by Yang et al. indicate that Lgals3 is expressed in the luminal and glandular epithelium and that an increase in Lgals3 is required for proper embryo implantation (123). In addition, Lgals3 affects chemotaxis and morphology of endothelial cells and stimulates capillary tube formation and angiogenesis *in vivo* (124). Therefore, besides its proposed roles in embryo implantation, immune regulation and trophoblast-matrix interactions, Lgals3 has a potential role in placental angiogenesis. It must be noted, however, that despite considerable research efforts over the past years, the precise physiological relevance of this lectin during pregnancy remains ill-defined. Comprehensive analysis of the placental phenotype, the regulation of vascular development and maternal adaptations in *Lgals3* deficient models could greatly aid our understanding of this lectin's role in pregnancy orchestration.

GALECTIN-7 (LGALS7)

Lgals7 is produced by the premenstrual and menstrual endometrial luminal and glandular epithelium, where it accumulates in menstrual fluid and has been proposed to act as a paracrine factor to facilitate post-menstrual endometrial re-epithelialization (125). While *Lgals7* mRNA was not detected in term placenta by real time-PCR, using immunohistochemistry, expression of Lgals7 was reported in the syncytiotrophoblast (STB), EVT and glandular epithelium in first trimester placenta, decidua and in the STB and endothelial cells of normal term placenta (50, 126). Menkhorst et al. suggested that Lgals7 may facilitate adhesion of the embryo to the endometrium and reported that the serum concentration of Lgals7 was significantly elevated in women (weeks 10–12 and 17–20) who subsequently developed PE compared to women with healthy pregnancies (126, 127). Another study, also explored the potential value of Lgals7 measurement as a biomarker and indicated that maternal serum Lgals7 levels had no value to predict the risk of spontaneous abortion (128). Clearly, further studies are required to confirm the expression of Lgals7 in placental cells and the potential usefulness of Lgals7 measurements in maternal serum as a biomarker for pregnancy pathologies should be evaluated with larger patient cohorts.

GALECTIN-8 (LGALS8)

Lgals8 is ubiquitously expressed and analysis of its expression in normal first trimester placentas indicated that *Lgals8* is expressed by VT and EVT, and is highly expressed in decidual stromal cells (129). Lgals8 has been referred to as an "angiogenesis regulator" in vascular and lymphatic endothelium by binding to

podoplanin in lymphatic vessels and CD166 (ALCAM, activated leukocyte cell adhesion molecule) in vascular endothelial cells (130). The role of this galectin in placental angiogenesis has not been explored but an initial report indicates that Lgals8 is not expressed in the endothelium of the placenta (130). The human gene (*LGALS8*) encodes seven different isoforms resulting from alternative splicing but the functional consequences of *Lgals8* splicing are poorly understood (130). Pro-inflammatory and immunosuppressive functions have been both attributed to this galectin in different experimental systems but so far, no studies on its possible role as an immunomodulator during pregnancy have been reported (131–135). Potentially, Lgals8 could play a role in trophoblast cell adhesion and migration as was reported in other cell types, but experimental evidence for the regulation of trophoblast function by this lectin is also lacking (136).

GALECTIN-9 (LGALS9)

The tandem-repeat Lgals9 has been implicated in immune regulation through binding to TIM-3, CD44 and the cell surface protein disulfide isomerase (PDI) (137–139). This galectin is expressed by many cell types including epithelial cells of the endometrium, trophoblasts, stromal cells of the decidua, endothelial cells including those in the placenta, and several types of immune cells (140–143). Splice variants of *Lgals9* have been reported with six of them expressed in human decidua, which may differ in their biological functions (143–145).

Compared to non-pregnant individuals, regulatory T cells show higher level of *Lgals9* expression as pregnancy proceeds and the level of Lgals9 in serum is significantly higher in women with normal pregnancies compared to post-partum and non-pregnant female controls (146). Interestingly, the serum levels of Lgals9 in pregnancy varied with the gender of the fetus as was also reported for some inflammatory cytokines and pro-angiogenic factors; Lgals9 is further increased in the serum of women carrying a male compared to a female fetus (147, 148). Li et al. proposed that Lgals9 contributes to the generation of $CD25^{+}$ $FoxP3^{+}$ T regulatory cells in -circulation and in the spleen and that engagement of Tim-3 by Lgals9 in peripheral NK cells facilitates the immunosuppressive activity of these cells during the first trimester of pregnancy (149). Additionally, they report that the concentration of Lgals9 in the plasma of women with normal pregnancies is significantly higher from that in women suffering from recurrent miscarriages but caution should be taken as the sample size was small and fetal gender was not considered in these studies (149).

Recently, lower levels of Lgals9 expression analyzed by immunohistochemistry were described in trophoblasts of the DBA/2-mated CBA/J mouse model of spontaneous abortion/PE when compared to normal CBA/J × BALB/c matings, further showing that Lgals9 blockade promoted a significant imbalance of Th1/Th2 immunity in this model (150). Additionally, altered placental Lgals9 expression together with dysregulated Tim-3 signaling in distinct NK and T cell subsets have been suggested to mediate the abortifacient effects of mifepristone in mouse pregnancies (151). Furthermore, activation of Tim-3/Lgals9 signaling pathway promotes decidual macrophages polarization to M2 subtype, alleviating the PE-like syndrome induced by LPS in a rat model (152). In conclusion, while a role for Lgals9 in immune tolerance during pregnancy has been reported by a handful of investigators, more studies considering splice variants, fetal gender, and Lgals9 receptors on target cells are required to better understand the potential role for this galectin as a contributor of the systemic and local immune regulation during pregnancy.

GALECTIN-10 (LGALS10)

Prototype Lgals10, also known as eosinophil Charcot-Leyden crystal protein, appears to play an important role in the differentiation of neutrophils and the functional properties of CD25+Treg cells (153, 154). Subsequently, expression of this lectin at the maternal-fetal interface has been described mainly in the STB and to a smaller extent in the decidua during the first trimester, showing decreased levels in spontaneous abortion patients (116, 155). The precise physiological role played by this lectin in pregnancy is still unknown but interestingly, its expression is driven from a chromosome 19 gene cluster comprising also galectins −13, −14, −16, and −17, which emerged during primate evolution as a result of duplication and rearrangement of genes via a birth-and-death process (49, 50). Galectins in the chromosome 19 cluster show primarily placental expression and may be involved in the regulation of unique pregnancy associated processes, including maternal immune tolerance and villous trophoblast differentiation (49, 50, 156).

GALECTIN 13 (LGALS13)

Lgals13 is also known as placental protein 13 (PP13) and was first isolated from human placenta (157). This galectin is predominantly expressed by STB cells of the placenta, in which nuclear staining and strong labeling of the brush border membrane is observed (158, 159). Although originally reported to be absent in serum of pregnant women, Lgals13 is detected in increasing concentration in maternal serum as pregnancy progresses becoming undetectable 2–5 weeks post-partum (158, 160, 161). Besides being found in a soluble form in circulation, Lgals13 is also located inside and on all types of STB-derived extracellular vesicles (162).

Lgals13 has been proposed to have immune regulatory functions, and in studies in rodents it has been shown to reduce blood pressure associated with activation of endothelial prostaglandin and nitric oxide signaling pathways (163–166). The potential for Lgals13 as a useful biomarker for PE has been suggested by Burger and co-workers. They reported that in the 1st trimester, lower than normal Lgals13 levels were found in IUGR and PE, particularly in the early-onset form. In the 2nd and 3rd trimesters, higher than normal concentrations were found in PE, IUGR and in preterm delivery (PTD) (160). On the other hand, lower placental *Lgals13* mRNA and protein expression were found in preterm PE and HELLP syndrome, although the immunoreactivity of the STB microvillous membrane was

reported to be stronger in these pregnancies than in age-matched controls suggesting increased membrane shedding (167). The usefulness of Lgals13 as a biomarker for PE has been questioned as late second-trimester Lgals13 alone does not increase the ability to predict PE when compared to second-trimester Doppler pulsatility index and other potential biochemical markers (168), highlighting the need to consider the interactions between different signaling pathways in disease pathogenesis when in search for sensitive, reliable biomarkers. In this regard, recent studies suggest that the kinetics of Lgals13 expression in PE would result from the concerted actions of this protein and antiangiogenic factors as sFlt-1 on the maternal vascular system, with a dual role for Lgals13: first in low levels acting as a priming insult promoting endothelial activation and angiogenic imbalance, and increasing later in the third trimester as a natural rescue response promoting maternal vasodilation to lower blood pressure (169). In the context of gestational diabetes mellitus (GDM), increased Lgals13 serum levels during the early second trimester and lower expression in trophoblast cells of the term placenta have been reported (170, 171). Dysregulation of *Lgals13* was suggested to contribute to an imbalance in inflammatory processes in the placenta during pregnancy and therefore possibly lead to GDM.

Interestingly, while Lgals13 has hemagglutination activity when tested with chicken erythrocytes, a recent report suggested that contrary to what was observed for other galectins, Lgals13 may not bind carbohydrates (172). Prior studies, however, had reported that not only the binding of Lgals13 to erythrocytes or T cells is carbohydrate-dependent (50, 173), but also that N-acetyllactosamine is the preferred disaccharide ligand for Lgals13 (50, 159). This inconsistency in the results from the aforementioned laboratories may reside in that Su et al. (172) had tested lactose as an inhibitor for Lgals13, as well as other carbohydrates, such as xylose and arabinose, that are unrelated to the structures recognized by most galectins. Nevertheless, the identification of natural ligand(s) for Lgals13 at the cell surface and extracellular matrix will be of great importance to better understand its role(s) during pregnancy (165).

GALECTIN-GLYCAN INTERACTIONS AS REGULATORS OF THE FETAL-MATERNAL DIALOGUE

Glycosylation is the most common and structurally diverse type of post-translational modification, affecting proteins, lipids and the extracellular matrix. Glycans play fundamental roles in most biological processes, thus it is not surprising that glycans are profusely expressed in the mammalian uterus (174). During implantation, the uterine epithelium and the outer trophoblast cell layer of the implanting embryo interact in a glyco-specific manner, such that perturbations of the system generally result in failure of implantation or poor placentation and compromised pregnancy outcomes. Glycans are essential functional groups that facilitate and influence the reproduction process. The synthesis of glycans relies on specific modification enzymes (glycosyltransferases and glycosidases) (**Figure 2**), and the glycocode expressed in a particular tissue is highly dependent on the cell type and its developmental, nutritional, and pathological state. The glycans within the glycome can have multiple functions during pregnancy. For example, N-linked glycans (attached to the nitrogen of an asparagine side-chain) play an important role in trophoblast cell invasion in early pregnancy (175, 176) and maternal-fetal tolerance (177, 178). O-Linked glycans (attached to the hydroxyl oxygen of serine, threonine, tyrosine, hydroxylysine or hydroxyproline side-chains) can influence recognition events during fertilization (e.g., sperm-egg interactions) (179). As a detailed discussion about the role of glycosylation—in pregnancy outcome is beyond the scope of this review, we will focus on modifications that affect binding and function of members of the galectin family during gestation.

Extracellularly galectins act by cross-linking *N*- and *O*-glycans on the surface glycoproteins of maternal immune cells, trophoblasts and endothelial cells at the fetal-maternal interface. As glycosylation often represent highly regulated post-translational modifications related to the physiological cellular status, alterations in glycan composition can fundamentally impact galectin activity (180–182). Given the prominent expression of galectins (e.g., Lgals1 and Lgals3) at the fetal-maternal interface, studies designed to examine the relevance of cell surface glycans on maternal/ placental compartments are of key importance. In a physiological context, enhanced expression of N-acetylglucosaminyl transferase V [GnTV, encoded by the *Mgat5* gene (**Figure 2**)] was found in placentas from the first trimester compared with those from full-term pregnancies (175). GnTV generates β1-6-N-acetylglucosamine branches in complex N-glycans which are recognized by Lgals1. LacNAc motives are a glycan signature of EVT (91, 183) as their presence was detected not only on their surface but also on their secretion product HLA-G (91, 184). Since Lgals1 promotes trophoblast invasion and EVT differentiation during early pregnancy (90), it is possible that the increased activity of GnTV results in enhanced Lgals1 signaling (176); particularly, by promoting the interaction of Lgals1 with β1 integrin at the trophoblast cell membrane (94, 176, 185–190) (**Figure 3**). Furthermore, the presence of (β-6) branches and the expression of the glycosyltransferase GnTV involved in the generation of these glycan structures were reduced in villous tissues from early spontaneous miscarriages in comparison with healthy pregnancy villous tissues (191). Thus, differences in the glycan composition of trophoblast related-proteins at the same gestational age could be important disease biomarkers that should be further explored with newly available mass spectroscopy techniques. Indeed, the expression of GnTV was reported to be elevated in PE placentas compared to normal placentas (192). We have shown that *Lgals1* expression is increased in late onset PE and could represent a compensatory mechanism of the trophoblast to overcome the severe inflammation microenvironment that characterizes PE disease (74) (**Figure 3**). This is an interesting link to the metabolic status of trophoblast cells, which is mediated by the intracellular levels of GnTV that affect quality and branching of complex N-glycans and therefore regulate galectin binding.

During pregnancy the STB layer of the placenta releases extracellular vesicles (STBEV) containing a complex cargo of RNAs, proteins, lipids, and also glycans into the maternal

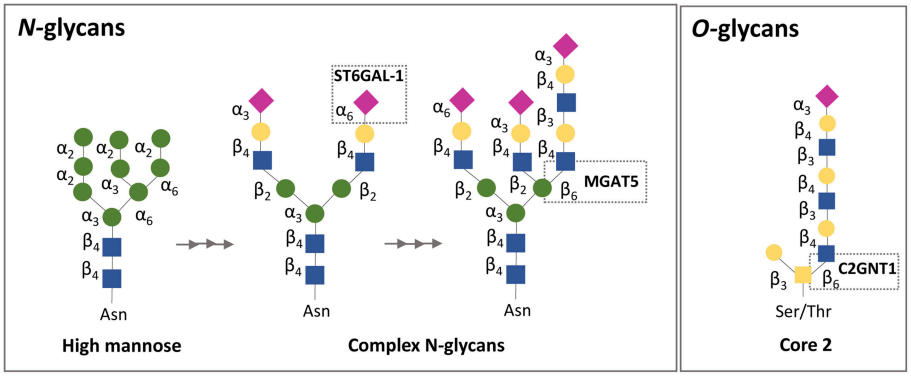


FIGURE 2 | Simplified schematic representation of *N*-and *O*-glycan biosynthesis focusing on galectin-1 binding affinity. N-glycans are attached to asparagine (Asn) residues, whereas O-glycans are attached to either serine (Ser) or threonine (Thr) residues. Gal-1 recognizes galactose on complex *N*-glycans and sialylation on the terminal galactose in the α2,6-linkage, but not in the α2,3-linkage, prevents the binding of gal-l. Regarding *O*-glycans, gal-1 binds to the N-acetyllactosamine (LacNAc) motif in core 2 *O*-glycans. ST6GAL-1, β-galactoside α2,6-sialyltransferase 1; *MGATS*, α1,6-mannosylglycoprotein 6β-N-acetylglucosaminyltransferase; *C2GnT*, core 2 β1,6 N-acetylglucosaminyltransferase.

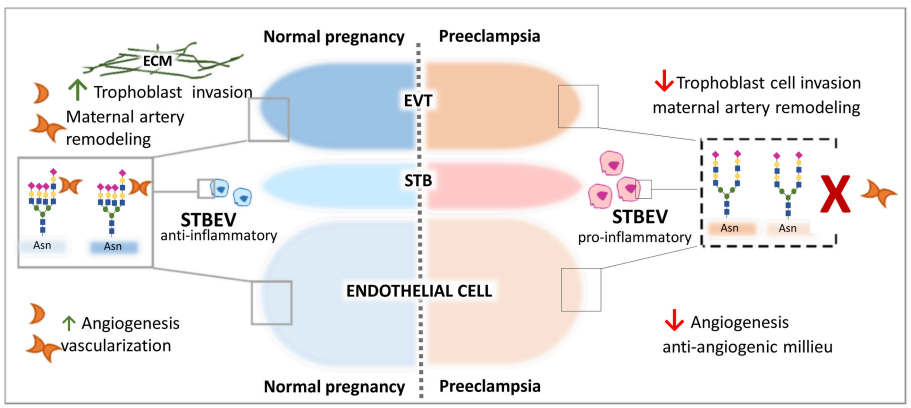


FIGURE 3 | Galectin-1-Giycan axis in the control of pregnancy protective programs. Galectin-1 regulates key process during the course of normal gestation including trophoblast invasion, maternal immune regulation, and angiogenesis. Relevant examples are illustrated. During Preeclampsia an aberrant α2-6 sialylation decorates α5β1integrin on the cell surface of EVT trophoblast, cell surface of STVEV released from the STB trophoblast and on endothelial cells. The high expression of α2-6 sialylated *N*-glycans impairs gal-1-mediated trophoblast ETV cell migration process interfering with the binding to the ECM and subsequently invasion. High α2-6 sialylation on STBEV and impaired gal-l binding might contribute to the pro-inflammatory milieu in maternal circulation and endothelial dysfunction. On vascular endothelial cells, the aberrant α2-6 sialylation may disrupt gal-1-mediated angiogenesis and early vascularization promoting the anti-angiogenesis status typical of the syndrome.

circulation potentially to induce maternal immune adaption. Under pathological conditions such as preeclampsia, however, STBEV exhibit a differential glycan composition compared to uneventful pregnancies. In particular, STBEV derived from PE placentas depict an increased content α2-6-linked sialic acid (193). The presence of α2-6-linked sialic acid on cell surface glycoproteins—is mainly determined—by the activity of the sialyltransferase gene *ST6GAL1* and results in blocking of Lgals1 signaling (194, 195). The selective glycosyltransferase expression (e.g., ST6Gal-1) on trophoblast cells may be an early pathological mechanism of masking Lgals1 activity in modulating the maternal immune response to the developing embryo. Moreover, an increased α2-6 sialylation was observed on the STB layer and also in placenta vessels derived from pregnancies complicated with hypertensive disorders including superimposed PE, PE, and PE + HELLP (196). This is important since high α2-6 sialylation on endothelial cells can reduce Lgals1 mediated angiogenesis (195), which is in line with our *in vivo* experiment showing that blocking Lgals1 mediated angiogenesis with anginex during early gestation in mice induced spontaneously PE development (74) (**Figure 3**). Moreover, the inhibition of Lgals1 binding by sialylation at the position 6 of galactose has been suggested to make Th1 cells resistant to apoptosis (197) and might contribute to uncontrolled maternal inflammation during preeclampsia. Thus, analysis of the glycosylation signature of trophoblast and placental vessels constitute a valuable approach to unravel the importance of galectin signaling through VEGFR2 during gestation.

CONCLUSIONS AND FUTURE DIRECTIONS

There is ample evidence showing that galectins are expressed widely at the feto-maternal interface. Their expression is regulated during pregnancy and galectins are highly specific to certain trophoblast and maternal cell types. Multiple galectin functions have been described in the orchestration of healthy pregnancy, which include maternal immune adaptation, placental development, and angiogenesis. Studies on the association of pregnancy pathologies with dysregulated galectin expression are still at an early stage, with most of our knowledge on the biological role of galectins in pregnancy being inferred from *in vitro* models and clinical correlations. However, sufficient evidence is already available to suggest galectins, especially Lgals1 and Lgals13, are promising candidates for further investigation aimed at understanding the pathogenesis of pregnancy complications including life threatening pregnancy related diseases such as PE. Because galectins are unique proteins with ability to recognize and decode a complex array of glycan motifs, future research could include: (1) a systemic study of the trophoblast cell-type glycome and galectin expression at the maternal-fetal interface in health and disease to determine whether glycomodifications on trophoblast cells that prevent galectin binding are responsible for the development of some pregnancy disorders and what is the galectin distribution in the maternal and placental compartments in health and disease; (2) a comprehensive analysis of the role of galectins in maternal circulation during pregnancy to establish whether galectins act at distance and if the presence of galectins in maternal circulation is a consequence of leakage from placenta tissue; (3) a deep analysis of galectin-glycan interactions either at the maternal or placental compartments with the goal to reveal the critical contribution of the physiological and pathophysiological galectin functions during gestation. In the years ahead, the development of novel *in vivo* strategies to test hypotheses related to the biology of galectin-glycan interactions during pregnancy represents a worthwhile pursuit, which will greatly advance reproductive medicine

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Placental Galectins Are Key Players in Regulating the Maternal Adaptive Immune Response

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Galectins are potent immunomodulators that regulate maternal immune responses in pregnancy and prevent the rejection of the semi-allogeneic fetus that also occurs in miscarriages. We previously identified a gene cluster on Chromosome 19 that expresses a subfamily of galectins, including galectin-13 (Gal-13) and galectin-14 (Gal-14), which emerged in anthropoid primates. These galectins are expressed only by the placenta and induce the apoptosis of activated T lymphocytes, possibly contributing to a shifted maternal immune balance in pregnancy. The placental expression of Gal-13 and Gal-14 is decreased in preeclampsia, a life-threatening obstetrical syndrome partly attributed to maternal anti-fetal rejection. This study is aimed at revealing the effects of Gal-13 and Gal-14 on T cell functions and comparing the expression of these galectins in placentas from healthy pregnancies and miscarriages. First-trimester placentas were collected from miscarriages and elective termination of pregnancies, tissue microarrays were constructed, and then the expression of Gal-13 and Gal-14 was analyzed by immunohistochemistry and immunoscoring. Recombinant Gal-13 and Gal-14 were expressed and purified, and their effects were investigated on primary peripheral blood T cells. The binding of Gal-13 and Gal-14 to T cells and the effects of these galectins on apoptosis, activation marker (CD25, CD71, CD95, HLA-DR) expression and cytokine (IL-1β, IL-6, IL-8, IL-10, IFNγ) production of T cells were examined by flow cytometry.

Gal-13 and Gal-14 are primarily expressed by the syncytiotrophoblast at the maternal-fetal interface in the first trimester, and their placental expression is decreased in miscarriages compared to first-trimester controls. Recombinant Gal-13 and Gal-14 bind to T cells in a population- and activation-dependent manner. Gal-13 and Gal-14 induce apoptosis of Th and Tc cell populations, regardless of their activation status. Out of the investigated activation markers, Gal-14 decreases the cell surface expression of CD71, Gal-13 increases the expression of CD25, and both galectins increase the expression of CD95 on T cells. Non-activated T cells produce larger amounts of IL-8 in the presence of Gal-13 or Gal-14. In conclusion, these results show that Gal-13 and Gal-14 already provide an immunoprivileged environment at the maternal-fetal interface during early pregnancy, and their reduced expression is related to miscarriages.

Keywords: angiogenesis, glycomics, immune privilege, PP13, trophoblast differentiation, trophoblast invasion

INTRODUCTION

The mechanisms sustaining maternal immune tolerance to the semi-allogeneic fetus while shielding against microbial infections during pregnancy as well as the changes and interplay of maternal, fetal, and placental immune responses during pregnancy are of major interest in reproductive research (1–41). These immune tolerance mechanisms are complex and dynamic given that implantation involves decidual inflammation; the second trimester of pregnancy is characterized by a predominantly anti-inflammatory milieu in the womb, while at the end of the third trimester, the initiation of parturition requires a transition toward physiologic pro-inflammatory responses (42–44). Recent evolutionary evidence has shown that the pro-inflammatory implantation reaction in humans, as in all eutherian mammals, is derived from an inflammatory attachment reaction in the uterus of the ancestral therian mammals that directly leads to parturition, and that a key innovation in eutherian mammals was the shift from this inflammatory attachment reaction to the non-inflammatory mid-pregnancy period, which allowed an extended period of intimate placentation (45, 46). Although the molecular changes of this evolutionary shift in uterine immune responses are not yet explored in detail, these may include the placental expression of molecules that down-regulate maternal immune responses (47–67). This is substantiated by the fact that the dysregulated expression of immunoregulatory molecules at the maternal-fetal interface and the consequent disturbances in maternal-fetal immune regulation and pro-inflammatory processes are associated with the development of the great obstetrical syndromes, including miscarriage (68–71), preterm labor (72–80), or preeclampsia (81–87).

Regulation of the immune system is mediated by a complex network of cellular and molecular interactions, including glycan recognition by endogenous lectins (61, 88–90). Galectins, a subfamily of lectins specifically bind β-galactoside-containing glycoconjugates, also on immune cell surfaces where they modify immune responses by cross-linking receptors (61, 89, 91–93). Galectins have pleiotropic functions given their binding to a diverse set of cell surface ligands on immune and other cells including trophoblasts (94, 95). In mammals, 19 galectins have been identified, of which 13 are expressed in human tissues (56, 61, 92, 93). Studies of past decades began the exploration of the diverse functions of human galectins, primarily galectins-1, -3, and -9, in innate and adaptive immune responses including the regulation of leukocyte homing, adhesion, apoptosis, pathogen sensing, and immune signaling, also observed in reproductive processes (52, 96–102). Of major interest, several human galectins have an abundant expression at the maternal-fetal interface (31, 52, 53, 56, 58, 97, 103–109), and galectins-13, -14, and -16 are solely expressed by the human placenta (53, 56, 58, 61). These three galectins are expressed from a gene cluster on Chromosome 19 that had emerged in anthropoid primates (53, 56, 61, 110).

We recently started to explore the biological functions of Chromosome 19 galectins in pregnancy (53). Galectin-13 and galectin-14 (Gal-13 and Gal-14), originally described as placental protein 13 (PP13) (111) and placental protein 13-like (PPL13) (112), respectively, are strongly expressed in the syncytiotrophoblast at the lining of the maternal-fetal interface (53, 106, 110, 113, 114). The expression of these galectins is dependent on trophoblast differentiation, and this developmentally regulated process in the trophoblast emerged during primate evolution (110). Of importance, Gal-13 is secreted from the syncytiotrophoblast into the maternal circulation, and low Gal-13 concentration in the maternal circulation in the first trimester was found in women who subsequently developed preterm preeclampsia (115–122), a severe obstetrical syndrome with a strong systemic immune dysregulation (51, 82, 86, 123–128) that already exists in the first trimester (129). Our studies have also shown that the placental expression of Gal-13 and Gal-14 is down-regulated in preterm preeclampsia (81, 110, 113, 129), where the placental pathology and the pro-inflammatory changes are similar to that of miscarriage (130–135).

Since we and our collaborators have shown that Gal-13 and Gal-14 induce the apoptosis of pre-activated T lymphocytes (53) and that Gal-13 increased IL-1α and IL-6 secretion from peripheral blood mononuclear cells (PBMCs) in pregnant women (114), an unanswered question remained: are Gal-13

TABLE 1 | Demographic and clinical data of the first-trimester placental study groups.

Groups	Control	Miscarriage
Number of cases[a]	30	10
Maternal age (years)[b]	30 (19–41)	36 (27–42)*
Gestational age at surgery (weeks)[b]	8.7 (5.0–11.9)	9.4 (7.0–13.0)
Gravidity[b,d]	2 (1–6)	2 (1–7)
Parity[b,d]	0 (0–4)	0 (0–1)
Habitual abortion[c,d]	3	30**

All women were Caucasian
[a]Values are presented as numbers.
[b]Values are presented as medians (interquartile (IQR) range).
[c]Values are presented as a percentages.
[d]Data were available for 29 cases in the control group.
**$p < 0.05$ compared to gestational age-matched controls.*
***$p < 0.01$ compared to gestational age-matched controls.*

and Gal-14 critical regulators of immune processes at the early maternal-fetal interface that can be considerably dysregulated in miscarriage? Therefore, we investigated the placental expression of Gal-13 and Gal-14 in miscarriage and also the effects of Gal-13 and Gal-14 on human T lymphocyte functions, which may play a critical role in immune tolerance and rejection. Indeed, we show herein that these placenta-specific galectins moderate adaptive immune responses and are down-regulated in miscarriages, suggesting that their reduced expression is related to the immunopathology of miscarriage.

MATERIALS AND METHODS

Study Groups, Clinical Definitions, and Sample Collection

Placental tissue samples, collected from Caucasian women, were processed immediately after sample collection as previously described (81, 136), fixed in 10% neutral-buffered formalin, and were then embedded in paraffin (FFPE). First- ($n = 40$) and third- ($n = 2$) trimester placentas were collected prospectively at the Maternity Private Department, Semmelweis University (Budapest, Hungary). Pregnancies were dated according to ultrasound scans collected between 5 and 13 weeks of gestation. Patients with a twin gestation were excluded. Women were enrolled in two groups: those who underwent elective termination of pregnancy (control, $n = 30$) and those who miscarried their pregnancy (cases, $n = 10$) (**Table 1**). Miscarriage was defined according to the American College of Obstetricians and Gynecologists Practice Bulletin, as a non-viable, intrauterine pregnancy with a gestational sac containing an embryo or fetus without fetal heart activity within the first 12 6/7 weeks of gestation (137).

Clinical samples and data collection were approved by the Health Science Board of Hungary (ETT-TUKEB 4834-0/2011-1018EKU). Written informed consent was obtained from women prior to sample collection and the experiments conformed to the principles set out in the World Medical Association Declaration of Helsinki. Specimens and data were stored anonymously.

Histopathologic Evaluation of the Placentas

Five-micrometers-thick sections were cut from FFPE tissue blocks and stained with hematoxylin and eosin for histopathological evaluation at the 1st Department of Pathology and Experimental Cancer Research, Semmelweis University. The sections were examined using light microscopy by a perinatal pathologist blinded to the clinical information. Histopathologic changes were defined according to published criteria (136, 138, 139).

Tissue Microarray Construction, and Galectin-13 and Galectin-14 Immunostainings

As previously described (140–143), representative areas were selected for the construction of tissue microarrays (TMAs), which contained 2 mm cores in diameter. To investigate protein expressions, two TMAs were created, using an automated tissue arrayer (TMA Master II, 3DHISTECH Ltd.), to contain one block of each first-trimester ($n = 40$) placenta as well as a positive control (third-trimester healthy placenta) and a negative control (liver) in triplicate.

Five-micrometers-thick sections were cut from TMAs and placed on silanized slides. After deparaffinization and rehydration, antigen retrieval was performed using citrate buffer (10 mM Sodium citrate, 0.05% Tween 20, pH = 6) for 5 min at 100°C in a pressure cooker. Endogen peroxidase blocking was performed using 10% H_2O_2 for 20 min. Immunostaining was carried out using the Novolink Polymer Detection System (Novocastra Laboratories), according to the manufacturer's protocol, as detailed in **Supplementary Table 1**. Slides were blocked for 10 min with Protein Block. To evaluate Gal-13 expression, slides were incubated with anti-galectin-13 mouse monoclonal antibody (clone 215-28-3) in 1% BSA-TBS for 60 min at 37°C. To evaluate Gal-14 expression, slides were incubated with anti-galectin-14 recombinant human antibody in 1% BSA-TBS for 60 min at room temperature. In the case of Gal-14 staining, after three washes with Tris buffer saline with 0.05% Tween 20 (TBST), slides were incubated with anti-His_6 mouse monoclonal antibody for 30 min at room temperature. In both circumstances, subsequent steps were the same. Briefly, after three washes with TBST and Post Primary treatment (30 min, at room temperature), Novolink Polymer was used as the secondary antibody for 30 min at room temperature. This was followed by three washes with TBST, and then the sections were developed using 3,3′-diaminobenzidine (DAB, Novolink) in 1:20 dilution. Finally, sections were counterstained with hematoxylin, and these were mounted with DPX Mountant (Sigma-Aldrich) after dehydration.

Evaluation of Immunostainings

Gal-13 or Gal-14 immunostained placental TMAs were digitally scanned by a high-resolution bright field slide scanner (Pannoramic Scan, 3DHISTECH Ltd.), and cytoplasmic staining in the syncytiotrophoblast was evaluated on virtual slides using Pannoramic Viewer 1.15.4 (3DHISTECH Ltd.) by two examiners

blinded to the clinical information. All villi were scored semi-quantitatively. The intensity of immunostaining was graded from 0 to 3. The average intensity was determined for each core as the representative data for that core. By averaging immunoscores of the cores, the overall intensity score was assigned to each placenta and then to each patient group.

Expression and Purification of Recombinant Galectin-13 and Galectin-14

Recombinant Gal-13/Gal-14 was expressed as previously described (53) with modifications. Expression plasmids used earlier (53) were modified by the N-terminal insertion of maltose-binding protein (MBP) tag on these galectins. These modified plasmids, containing either full-length Gal-13 or Gal-14 as well as N-terminal maltose-binding protein (MBP)- and C-terminal His_6-tags, were transformed into ClearColi BL21 (DE3) (Lucigen). For protein expression, cells were grown in LB-Miller broth to $OD_{600} = 0.6$ at 37°C, induced with 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), and further grown for 4 h at 30°C. The following purification steps were applied: affinity purification on MBPTrap HP column (GE Healthcare Life Sciences); size exclusion chromatography (Superdex 200 Increase SEC column, GE Healthcare Life Sciences) for elimination of aggregates (only for Gal-14); MBP cleavage by Tobacco Etch virus (TEV) protease [expressed and purified according to Kapust et al. (144)]; affinity chromatography on HisTrap HP columns (GE Healthcare Life Sciences); desalting and buffer exchange on Bio-Gel P-6 Desalting Cartridge (Bio-Scale Mini, Bio-Rad). All steps were carried out in the presence of 1 mM dithiothreitol (DTT). Finally, Gal-13 and Gal-14 in PBS, supplemented with 1 mM DTT, were aliquoted and stored at −80°C.

Checking the Purity and Carbohydrate Binding Properties of Recombinant Gal-13 and Gal-14

The purity of the recombinant galectins was verified by heating the samples in Laemmli buffer for 10 min at 70°C, followed by 15% SDS polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad). After gel electrophoresis, recombinant galectins were either subjected to Coomassie blue staining (**Supplementary Figure 1A**) or transferred to nitrocellulose membranes. Membranes were blocked with 5% non-fat dry milk in TBST for 1 h, and then these were incubated overnight at 4°C with primary antibodies to Gal-13 (clone 27-3-2) or Gal-14 in TBST with 5% BSA. After repeated washing with TBST, blots were incubated for 1 h with HRP-goat anti-mouse IgG antibody (ThermoFisher Scientific) for Gal-13 or with HRP-goat anti-human IgG F(ab')2 antibody (Bio-Rad) for Gal-14 (**Supplementary Table 2**). After repeated washing with TBST, protein bands were visualized by enhanced chemiluminescence (ECL; Amersham International) (**Supplementary Figure 1B**).

To determine their functional activity, the binding of purified galectins to asialofetuin (ASF), a naturally occurring multivalent glycoprotein serving as a ligand for several galectins, was assayed by ELISA (145, 146). Briefly, ASF (50 μL of 10 μg/mL bovine ASF in sodium carbonate buffer pH = 9.6) was immobilized in microtiter plates overnight. After blocking residual binding sites with BSA (5% in PBS-Tween, PBST) for 2 h, different amounts of Gal-13 or Gal-14 were incubated for 1 h in PBST with 0.5% BSA. Washing with PBS was done three times between the incubation steps. Bound galectins were detected by incubation with anti-His_6-HRP antibody in PBST with 0.5% BSA (Biolegend, 1:1,000) and by the subsequent conversion of 3,3′5,5′-tetramethylbenzidine (TMB; Sigma-Aldrich) with a readout at 450 nm (reference filter: 620 nm). The reaction was stopped by 4N H_2SO_4 (**Supplementary Figure 1C**). Additionally, 50 μg/mL recombinant galectins were pre-incubated with gentle rotation on lactose-agarose beads (Sigma-Aldrich) for 1 h at room temperature prior to performing ELISA to also check for lactose inhibition. The inhibition was moderate for Gal-13 and weak for Gal-14, as we found differential binding of Gal-13 and Gal-14 to lactose and other carbohydrates in a previous study (53).

Isolation of Primary Immune Cells

Blood samples were obtained from a donor pool of non-pregnant, healthy, human females ($n = 18$ in total, $n = 4$–8 per assay, median age: 29.5) who were in the pre-ovulatory phase. PBMCs were isolated by Ficoll-Hypaque (Sigma-Aldrich) density-gradient centrifugation and washed in RPMI 1640 medium (ThermoFisher Scientific) before experimentations. T cells were isolated from PBMCs with the Dynabeads untouched human T cell kit (ThermoFisher Scientific) according to the manufacturer's protocol. PBMCs or T lymphocytes were kept in RPMI 1640 medium supplemented with 10% FBS and gentamycin or were activated for 48/72 h with the Dynabeads human T-Activator CD3/CD28 (ThermoFisher Scientific), according to the manufacturer's instructions, before treatment with Gal-13 or Gal-14 for 24 h.

Binding of Gal-13 and Gal-14 to Peripheral Blood T Cells

Fresh PBMCs from three donors, or PBMCs activated or not with human T-Activator for 72 h, were used for the Gal-13/Gal-14 binding study. To measure the binding of recombinant Gal-13 or Gal-14 to the surface of T cells, 2×10^5 PBMCs were initially washed in PBS containing 1% BSA. Recombinant Gal-13 or Gal-14 (4 μM), which we conjugated with CF488 fluorophore using the Mix-n-Stain CF488 kit (Sigma-Aldrich) according to the technical bulletin, was added to the cells, and samples were incubated for 45 min on ice. After washing, Fc receptors were blocked with human FcR blocking reagent (Miltenyi Biotec) for 5 min on ice. Anti-CD3-APC, anti-CD4-PerCP, and anti-CD8-APC/Fire750 antibodies (Biolegend) were applied to discriminate between T lymphocyte populations. All antibodies and reagents are listed in **Supplementary Table 3**. Flow cytofluorimetric measurements were carried out on a CytoFLEX device (Beckman Coulter) by collecting data from 50,000 cells. Data were analyzed using FlowJo v10 software (FlowJo, LLC).

Apoptosis Assay

PBMCs (5×10^5), previously activated or not with human T-Activator for 48 h, were incubated for 24 h on tissue culture plates with 0.25 or 4 μM recombinant Gal-13 or Gal-14 in RPMI 1640 medium supplemented with 10% FBS. The 10^5 cells were stained with anti-CD3-APC and anti-CD8-FITC antibodies, as described above. Cells were then incubated in 100 μL of annexin-binding buffer containing phycoerythrin-conjugated Annexin V (Annexin V-PE) and 7-amino-actinomycin D (7-AAD) (Annexin-V Apoptosis Detection Kit, ThermoFisher Scientific; **Supplementary Table 3**) for 15 min at room temperature in the dark. After incubation, 400 μL annexin binding buffer was added, and samples were measured immediately on a FACSCalibur cytofluorimeter using Cell Quest software (BD Biosciences). The Annexin V-PE^-/7-AAD^- population was regarded as normal, while the Annexin V-PE^+/7-AAD^- and Annexin V-PE^+/7-AAD^+ populations were taken as measurements of early and late apoptotic cells, respectively. Data were analyzed using FlowJo v10 software.

Flow Cytometry Measurement of Activation Markers

The PBMCs (5×10^5), previously activated or not with human T-Activator for 72 h, were incubated for 24 h on tissue culture plates with 4 μM recombinant Gal-13 or Gal-14 in RPMI 1640 medium supplemented with 10% FBS. To examine cell surface markers, 2×10^5 PBMCs were initially washed in PBS containing 1% FBS. Fc receptors were blocked with human FcR blocking reagent for 5 min on ice; then, specific antibodies to mid-late and late activation markers CD25 (Interleukin-2 Receptor alpha, IL-2Rα), CD71 (Transferrin Receptor, TfR), CD95 (Fas Cell Surface Death Receptor, Fas), and HLA-DR (Human Leukocyte Antigen, DR isotype; member of MHC-II) were added to the cells. Anti-CD3-APC and anti-CD8-FITC antibodies were added simultaneously and samples were incubated for 20 min on ice. All antibodies are listed in **Supplementary Table 3**. After washing, cells were measured in a CytoFLEX flow cytofluorimeter. A total of 20,000 cells were collected and data were analyzed using FlowJo v10 software.

Measurement of Cytokine Production by Bead Array

The T lymphocytes were isolated as described above. The 5×10^5 cells, previously activated or not with human T-Activator for 72 h, were incubated for 24 h on tissue culture plates with 4 μM recombinant Gal-13 or Gal-14 in RPMI 1640 medium supplemented with 10% FBS. Supernatants were collected in all cases, centrifuged at 400 g for 10 min, aliquoted and stored at −80°C until use. LEGENDplex bead-based immunoassays (Biolegend) were applied to measure the concentration of IL-8, IL-10, IFNγ, IL-1β, and IL-6 cytokines in cell culture supernatants of T cells, according to the manufacturer's instruction. Beads were measured in a FACSCalibur flow cytofluorimeter and data were analyzed using FlowJo v10 software.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software). An unpaired *t*-test with or without Welch's correction was used to analyze demographic data. An unpaired *t*-test was also used to analyze Gal-13 and Gal-14 immunostainings when comparing first-trimester control and miscarriage groups. The Fisher's exact test was performed to test the distribution of Gal-13 or Gal-14 immunoscores between the control and miscarriage groups. Repeated ANOVA tests with Tukey's *post-hoc* test were used for the analysis of galectin binding and CD4:CD8 ratio upon different treatments. One sample *t*-test was used to compare apoptosis of the Gal-13- and Gal-14-treated groups to the PBS-DTT-treated group, and to analyze the binding of Gal-13 and Gal-14 to ASF with or without lactose pre-treatment. Repeated ANOVA tests with Dunnett's *post-hoc* test were used to compare the non-treated group with Gal-13/Gal-14-treated groups in activation marker expression and cytokine production studies. Results were considered statistically significant at $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

RESULTS

Gal-13 and Gal-14 Are Expressed in First-Trimester Placentas and Their Expression Is Decreased in Miscarriage

Immunostainings of TMAs revealed that Gal-13 (**Figures 1A,B**) and Gal-14 (**Figures 1E,F**) are predominantly expressed in the cytoplasm of the syncytiotrophoblast of chorionic villi in the first trimester, and there were no stainings in the cytotrophoblasts and villous stroma, similar to later stages of pregnancy (53, 81, 106, 110, 113). Moreover, chorionic villi exhibited more intense syncytiotrophoblast cytoplasmic staining in the first trimester than in the third trimester (**Supplementary Figure 2**). The specificity of the galectin antibodies was confirmed by previous studies and by the lack of Gal-13 and Gal-14 immunostaining of human livers on our TMAs.

Next, we examined whether the expression of Gal-13 and Gal-14 is dysregulated in first-trimester placentas obtained from women who miscarried, as a potential sign of fetal rejection. There was no significant change in Gal-13 or Gal-14 immunoscores with gestational age in control placentas ($R^2 = 0.0078$ for Gal-14; $R^2 = 10^{-5}$ for Gal-13). However, the average immunoscore of syncytiotrophoblast decreased by 11.5% for Gal-13 ($p = 0.027$, **Figures 1A–D**) and by 20% ($p = 0.001$) for Gal-14 (**Figures 1E–H**) in miscarriages compared to gestational age-matched controls. Also, there was a significant difference in the distribution of Gal-13 and Gal-14 immunoscores ($p = 0.002$ and $p < 0.001$, respectively) between the disease and control groups (**Figures 1D,H**).

Gal-13 and Gal-14 Bind to Peripheral Blood T Cells

As Gal-13 and Gal-14 are released from the placenta into the maternal circulation, where they may regulate maternal T lymphocytes (53), we further characterized their effects on T cell populations. First, we examined the binding of fluorescent

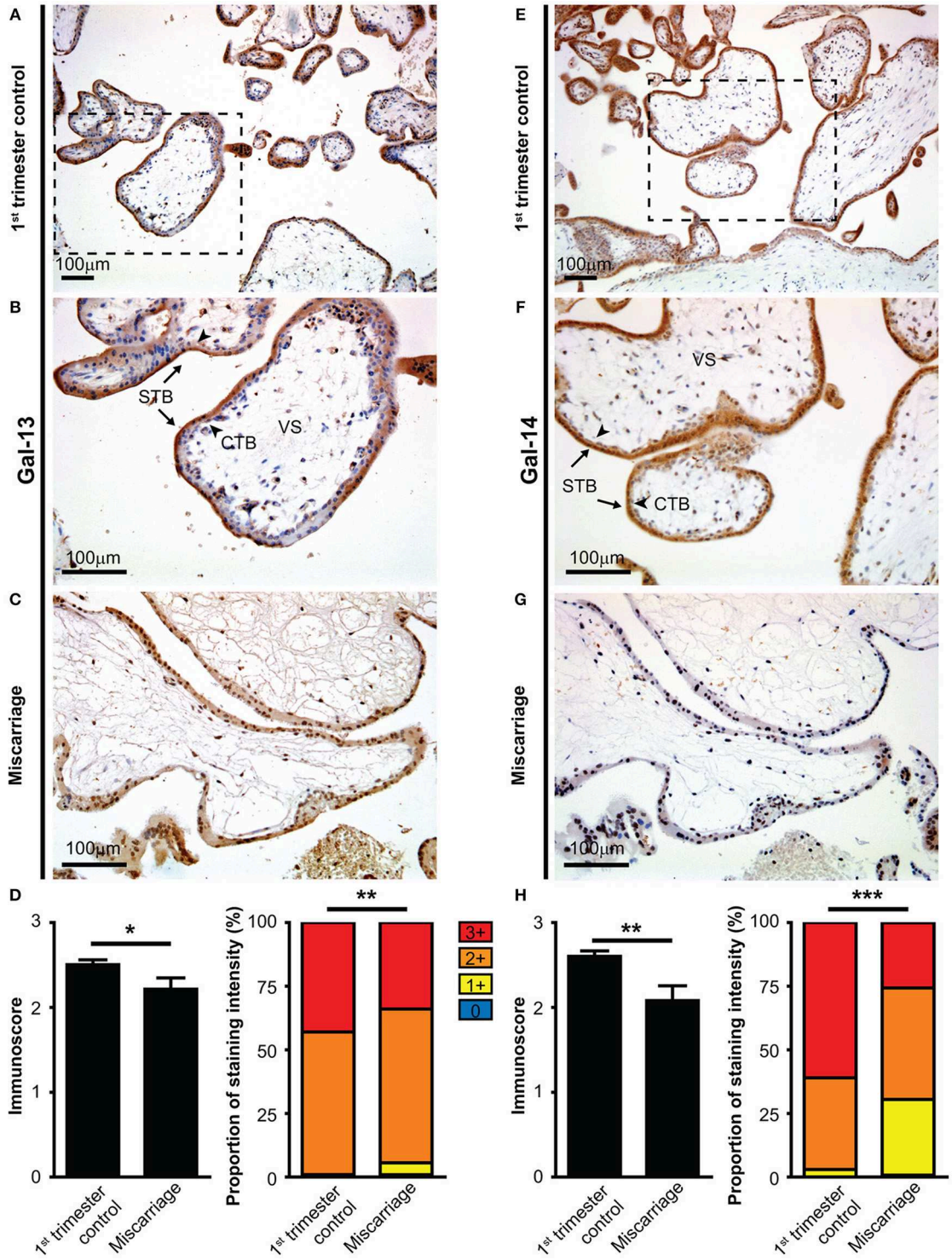


FIGURE 1 | The syncytiotrophoblast expresses galectin-13 and galectin-14 in the first-trimester placenta, which is decreased in miscarriage. Five-micrometers-thick first-trimester placental sections from normal pregnancy **(A,B,E,F)** or from miscarriage **(C,G)** were stained for Gal-13 **(A–C)** or Gal-14 **(E–G)** by specific monoclonal antibodies. Chorionic villi exhibited intense syncytiotrophoblast cytoplasmic staining (arrows, STB), while the villus stroma (VS) and cytotrophoblasts were negative (arrowheads, CTB). Representative images, hematoxylin counterstain, 100x **(A,E)** and 200x **(B,C,F,G)** magnifications. Gal-13 **(D)** and Gal-14 **(H)** immunoscores (mean ± SEM) and proportion of staining intensities in control placentas ($n = 30$) and placentas with miscarriage ($n = 10$) are displayed on left and right graphs, respectively (Gal-13: $n_{total\ villus} = 775$ and $n_{total\ villus} = 106$, respectively; Gal-14: $n_{total\ villus} = 797$ and $n_{total\ villus} = 121$, respectively). Unpaired *t*-test was used for the comparison of the mean immunoscores of the two groups. Fisher's exact test was performed to test the frequency difference of Gal-13 or Gal-14 immunostaining between control and miscarriage groups (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Gal-13 or Gal-14 to primary T cells. We found that Gal-13 and Gal-14 bound to freshly isolated T lymphocytes, either to helper (Th: 3.4 ± 0.9% and 5.1 ± 1%, respectively) or cytotoxic T (Tc: 3.9 ± 0.8% and 7.7 ± 2.2%, respectively) cells (**Figures 2A,B, Supplementary Figure 3**). Upon activation, binding of Gal-13 to Th and Tc cells was increased by 21% ($p < 0.001$) and 42% ($p < 0.001$), respectively (**Figure 2B, Supplementary Figure 3B**). Binding of Gal-14 was increased by 20% ($p < 0.01$) and by 30% ($p < 0.001$) to activated Th and Tc cells, respectively, compared to non-activated ones (**Figure 2B, Supplementary Figure 3B**). Of note, both galectins tended to bind more to Tc over Th lymphocytes, a difference that reached statistical significance in the case of activated cells ($p < 0.001$ for Gal-13 and $p < 0.05$ for Gal-14).

Gal-13 and Gal-14 Increase Apoptosis of Non-activated and Activated T Cells

Since certain galectins can induce the apoptosis of T cells depending on T cell subsets and their activation status (147–149), we investigated the effects of Gal-13 and Gal-14 on various T cell populations, either in an activated or a non-activated state. Flow cytometry results, overall, show that 4 μM, but not 0.25 μM, of Gal-13 or Gal-14 increased apoptosis of T lymphocytes either pre-activated or not (**Figures 3A,B**, **Supplementary Figure 4A**). Gal-13 increased apoptosis of both non-activated and pre-activated T cells by 5.3% ($p = 0.010$) and 9%, ($p = 0.011$), respectively. Gal-14 increased apoptosis of pre-activated T cells by 8.9% ($p = 0.040$) compared to PBS-DTT treated cells. We further analyzed Th and Tc lymphocytes separately, based on CD3 and CD8 expression. Tc cell apoptosis was increased for both galectins regardless of the activation state (Gal-13, non-activated: 3.9%, $p = 0.002$; Gal-13, pre-activated: 8.2%, $p = 0.022$; Gal-14, non-activated: 11%, $p = 0.001$; Gal-14, pre-activated: 11.2%, $p = 0.032$), while Gal-13 increased apoptosis rate (8.3%, $p = 0.031$) of non-activated Th cells (**Figures 3A,B**). The proportion of early apoptotic (Annexin V^+ 7-AAD^-) T lymphocytes, Th cells, and Tc cells as well did not change upon Gal-13 or Gal-14 treatment (**Supplementary Figure 4B**).

Gal-13 and Gal-14 Treatment Alters Cell Surface Expression of T Cell Activation Markers

Next, we investigated the impact of Gal-13 and Gal-14 on the expression of well-known activation markers—CD25 (IL-2Rα), CD71 (TfR), CD95 (Fas), and HLA-DR (MHC-II)—of T lymphocytes. Interestingly, Gal-13 treatment increased both the percentage of CD95 positive cells and the cell surface expression of CD95 on Th (% control: 11.3 ± 5.2%, Gal-13: 19.6 ± 7%, $p < 0.05$; RMFI control: 1.7 ± 0.2, Gal-13: 2.2 ± 0.2, $p < 0.01$) and Tc (% control: 6.1 ± 1.7%, Gal-13: 13.7 ± 3.7%, $p < 0.05$; RMFI control: 1.4 ± 0.1, Gal-13: 1.7 ± 0.2, $p < 0.01$) lymphocytes. Gal-14 treatment increased the percentage of CD95 positive cells (18.7 ± 8.5%, $p < 0.05$) and the cell surface expression of CD95 on Th (2.1 ± 0.3, $p < 0.05$) but not on Tc lymphocytes (**Figure 4A**, **Supplementary Figure 5B**).

The percentage of cells expressing CD71 and the cell surface expression of CD71 decreased upon Gal-14, but not Gal-13 treatment on both Th (% control: 96.9 ± 0.8%, Gal-14: 91 ± 1.3%, $p < 0.05$; RMFI control: 175.1 ± 17, Gal-14: 129 ± 6.1, $p < 0.05$) and Tc (% control: 97 ± 0.5%, Gal-14: 95 ± 1.1%, $p < 0.05$; RMFI control: 191.4 ± 26, Gal-14: 129.3 ± 33.2, $p < 0.01$) lymphocytes (**Figure 4B**, **Supplementary Figure 5B**).

Neither the percentage of CD25 nor of HLA-DR positive cells changed upon galectin treatment (**Figures 4C,D**, **Supplementary Figure 5B**). However, cell surface expression of CD25 increased upon Gal-13 treatment (Th RMFI control: 59.1 ± 8.1, Gal-13: 161 ± 30, $p < 0.05$; Tc RMFI control: 42.7 ± 9.6, Gal-13: 103.5 ± 24, $p < 0.05$), and tended to increase upon Gal-14 (RMFI Th: 110.6 ± 23.7; Tc: 64.4 ± 9.1) treatment in both T cell populations (**Figure 4C**). Of note, treatment of non-activated cells with Gal-13 or Gal-14 did not change the expression of these activation markers (**Supplementary Figure 5A**).

Gal-13 and Gal-14 Induce Il-8 Secretion of T Cells

Next, we sought to explore whether T lymphocytes contribute to the altered cytokine production, previously measured in PBMCs (114). IL-1β and IL-6 concentrations were below the detection limit in cell culture supernatants (data not shown). Surprisingly, IL-8 production was increased upon treatment with either Gal-13 (260.7 ± 78 pg/mL, $p < 0.01$) or Gal-14 (237.4 ± 73.5 pg/mL, $p < 0.05$) compared to the control (10.6 ± 9.2 pg/mL), when T cells were not activated. In the case of activation through CD3 and CD28, galectins could not further increase IL-8 production (**Figure 5**). Neither IL-10 nor IFNγ production changed upon galectin treatment (**Figure 5**).

DISCUSSION

Principal Findings of the Study

(1) Gal-13 and Gal-14 are mainly expressed by the syncytiotrophoblast at the maternal-fetal interface in the first trimester, stronger than in the third trimester of pregnancy; (2) the syncytiotrophoblastic expression of both Gal-13 and Gal-14 is down-regulated in miscarriages compared to first trimester control placentas; (3) recombinant Gal-13 and Gal-14 differentially bind to peripheral blood T cell populations, predominantly to Tc over Th cells; (4) Gal-13 and Gal-14 induce the apoptosis of both T cell populations regardless of their activation status; (5) Gal-14 decreases the cell surface expression of CD71, Gal-13 and Gal-14 increase the cell surface expression of CD95 and Gal-13 increases the cell surface expression of CD25 on T cells; and (6) non-activated T cells produce larger amounts of IL-8 in the presence of Gal-13 or Gal-14.

Placental Galectin-13 and Galectin-14 Expression Is Decreased in Miscarriage

This is the first study to characterize the simultaneous expression of Gal-13 and Gal-14 in first-trimester placentas in healthy

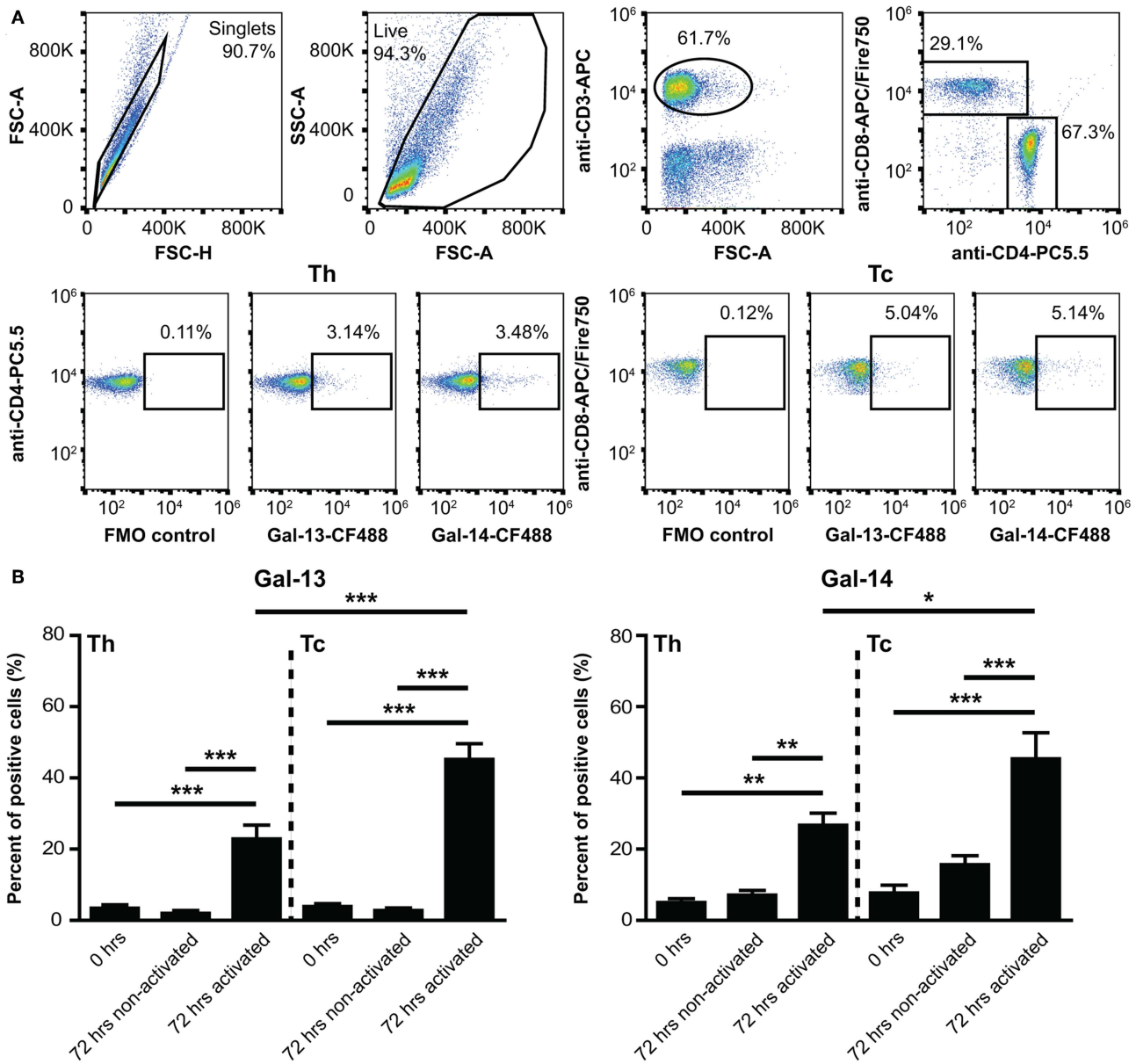


FIGURE 2 | Binding of galectin-13 and galectin-14 to T lymphocytes is population- and activation-dependent**. (A)** Detection of Gal-13 or Gal-14 binding to human T lymphocytes was achieved by flow cytometry with the following cells: freshly isolated PBMCs (0 h) or PBMCs, kept in culture for 72 h in the presence (72 h activated) or absence (72 h non-activated) of anti-CD3/CD28 microbeads. Cells were incubated with 4 μM Gal-13-CF488 or Gal-14-CF488 for 45 min on ice. PBMCs were also stained for CD3 (anti-CD3-APC), CD4 (anti-CD4-PC5.5), and CD8 (anti-CD8-APC/Fire750), in order to distinguish between helper (Th) and cytotoxic (Tc) T lymphocytes. The gating strategy is shown in **(A)**. **(B)** Graphs show the percentage of cells, to which Gal-13 (left) or Gal-14 (right) were bound, as mean ± SEM. Repeated ANOVA with Tukey's *post-hoc* test was used for the comparison of groups (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Four non-pregnant female donors were included in each group. FMO, Fluorescence minus one; PBMCs, peripheral blood mononuclear cells.

and complicated pregnancies. We found that these placenta-specific galectins are mainly expressed by the syncytiotrophoblast at the lining of the maternal-fetal interface, similar to these galectins that are expressed in the placenta in the third trimester (53, 106, 110, 113). The expression of these galectins in the syncytiotrophoblast is developmentally regulated during trophoblast differentiation by transcription factors binding to non-coding elements upfront of these galectin genes on Chromosome 19, a process that emerged in anthropoid primates (110). This is particularly interesting from an immunological point of view given that these anthropoids had a long gestation, which necessitated additional immune tolerance mechanisms at their maternal-fetal interface to prevent fetal rejection (53). In this latter evolutionary and immune functional study, we proposed that the emergence of these galectins in anthropoid primates provided additional immune tolerance

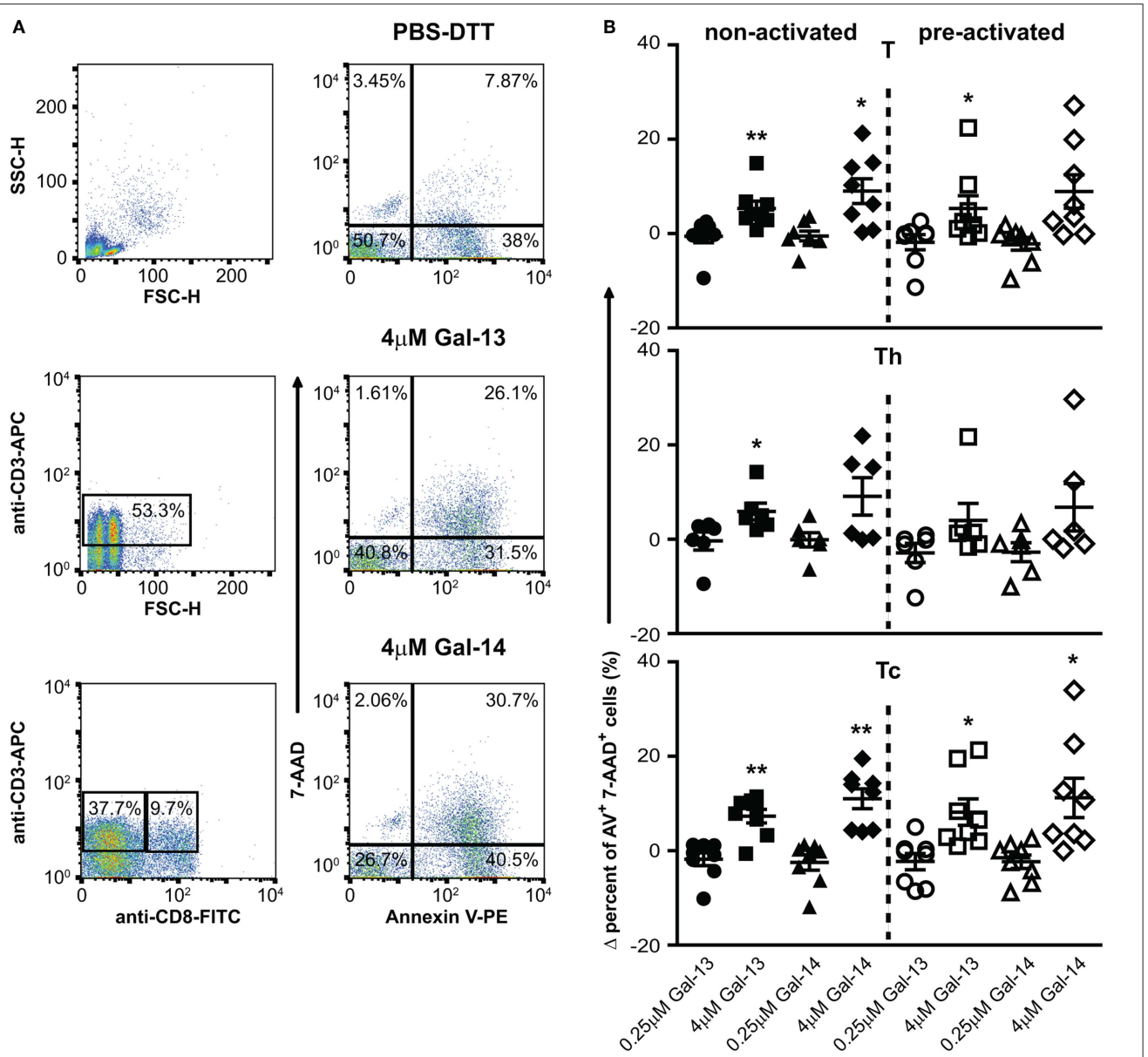


FIGURE 3 | Galectin-13 and galectin-14 increase apoptosis of T lymphocytes**. (A)** PBMCs were kept in culture for 48 h in the presence (activated) or absence (non-activated) of anti-CD3/CD28 microbeads, then treated with Gal-13 or Gal-14 for 24 h. To detect apoptosis of T lymphocytes by flow cytometry, cells were stained with Annexin V-PE and 7-AAD and were also stained for CD3 (anti-CD3-APC), and CD8 (anti-CD8-FITC), in order to distinguish between helper (Th) and cytotoxic (Tc) T cells, respectively. **(A)** The gating strategy (left: $CD3^+$ T gate, $CD3^+CD8^+$ Tc gate, $CD3^+CD8^-$ Th gate) and representative Annexin V and 7-AAD dot plots of vehicle- (PBS-DTT), Gal-13- or Gal-14-treated T cells ($CD3^+$) are displayed. **(B)** Graphs show the Δ percentage of double positive (Annexin V-PE^+ 7-AAD^+) cells as mean ± SEM. One sample t-test was used for the comparison of galectin-treated groups with the PBS-DTT-treated group of non-activated or pre-activated cells (*$p < 0.05$, **$p < 0.01$). Six-eight non-pregnant female donors were included in each group. PBMCs, Peripheral blood mononuclear cells.

toward the fetus. Previous studies on placentas delivered by women with preeclampsia, a severe obstetrical syndrome with an immune rejection component, revealed the down-regulation of Gal-13 and Gal-14 placental expression, suggesting that this phenomenon may be linked to altered immune tolerance (81, 110, 113, 129).

Herein, we report for the first time that the placental expression of Gal-13 and Gal-14 is also decreased in first-trimester miscarriages. As most of these cases have a normal karyotype, our finding suggests that altered maternal-fetal immune tolerance is not closely associated with chromosomal abnormalities but can be a separate underlying mechanism for miscarriages. Indeed, recent publications presented that miscarriages are associated with immune etiologies (150), where the development of the fetus and placenta is affected by either auto- or alloimmune rejection-type activity (151).

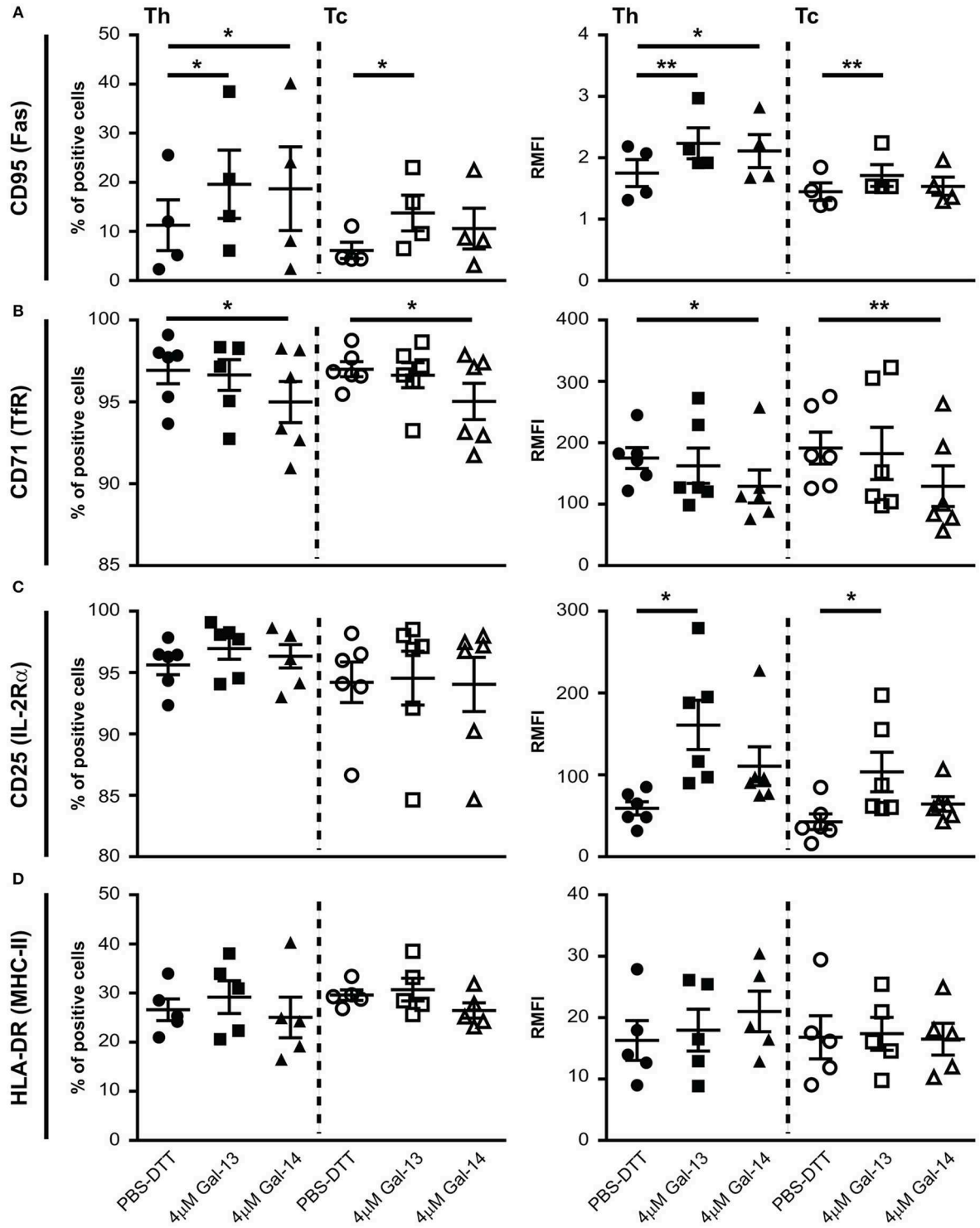


FIGURE 4 | Galectin-13 and galectin-14 affect cell surface expression of activation markers on T lymphocytes**.** PBMCs were kept in culture for 72 h in the presence of anti-CD3/CD28 microbeads, then treated with Gal-13 or Gal-14 for 24 h. To detect cell surface expression of CD95 **(A)**, CD71 **(B)**, CD25 **(C)**, HLA-DR **(D)** activation markers on T lymphocytes by flow cytometry, cells were stained with anti-CD25-PE and anti-CD71-PerCP/5.5, or anti-CD95-PE and anti-HLA-DR-PerCP/5.5. Cells were also stained for CD3 (anti-CD3-APC), and CD8 (anti-CD8-FITC) in order to distinguish between helper (Th) and cytotoxic (Tc) T cells. Left graphs show the percentage of positive cells and right graphs show relative median fluorescence intensity (RMFI) values (mean ± SEM). RMFI was calculated by dividing specific median fluorescence intensity with the median fluorescence intensity of the isotype control. Repeated ANOVA with Dunnett's *post-hoc* test was used for comparison of the non-treated group with Gal-13/Gal-14-treated groups (*$p < 0.05$, **$p < 0.01$). Four-six non-pregnant female donors were included in each group. HLA-DR, Human leukocyte antigen DR isotype; PBMCs, peripheral blood mononuclear cells.

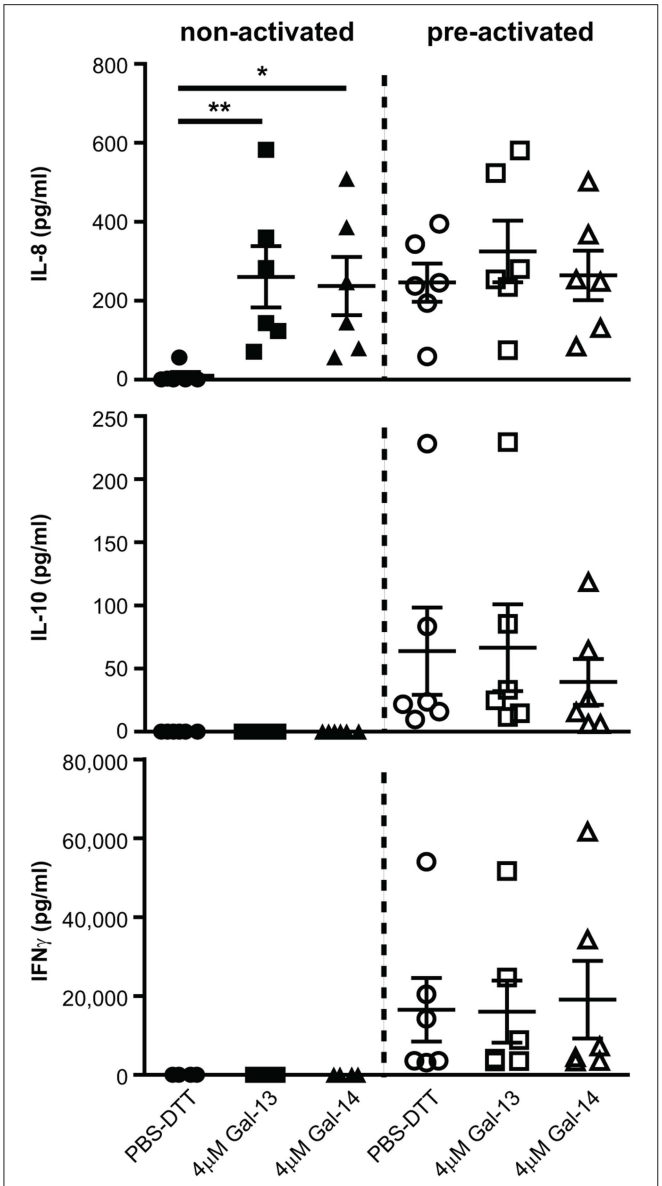


FIGURE 5 | Galectin-13 and galectin-14 induce IL-8 production in resting T lymphocytes**.** T lymphocytes after negative selection were kept in culture for 72 h in the presence (pre-activated) or absence (non-activated) of anti-CD3/CD28 microbeads, then treated with 4 μM Gal-13 or Gal-14 for 24 h. Supernatants were collected and IL-8, IL-10, and IFNγ cytokines were measured by flow cytometry using LEGENDplex assays. Graphs show calculated cytokine concentrations as mean ± SEM. Repeated ANOVA with Dunnett's *post-hoc* test was used for the comparison of the non-treated group with Gal-13/Gal-14-treated groups (*$p < 0.05$, **$p < 0.01$). Six non-pregnant female donors were included in each group.

Of interest, the expression of other galectins, although in modest extent, is also decreased at the maternal-fetal interface in spontaneous and recurrent miscarriages, including Gal-1, Gal-2, Gal-7, Gal-9, and Gal-10 (152–154). Furthermore, in good accordance, serum concentrations of Gal-1 and Gal-9 were also found to be decreased in miscarriage (99, 154, 155). An elegant study revealed that Gal-1 has pivotal functions supporting maternal-fetal immune tolerance and its decreased expression leads to fetal loss in a mouse model. Gal-1 prevents fetal loss and restores tolerance through multiple mechanisms, including the induction of tolerogenic dendritic cells, which, in turn, promotes the expansion of IL-10-secreting regulatory T cells *in vivo* (107). On the other hand, Gal-9 was found to exert its functions in non-pregnant and pregnant states on NK cells, T cells, and B cells (101, 102, 147, 156–159). In addition, Gal-9 promotes trophoblast invasion in a Tim-3 dependent manner (154). Consistently, a higher proportion of decidual T cells that express activation markers (CD25, and CD69) was found in spontaneous abortion than in elective termination of pregnancy, and decidual lymphocytes from spontaneous abortion increased the apoptosis of trophoblast cells (160). Since Gal-13 and Gal-14 are only expressed in anthropoid primates, it is not possible to investigate the role of these proteins in knock-out mammalian models *in vivo*. Nevertheless, we can conclude that several galectins, including Gal-13 and Gal-14, potentially act in concert and play a role in maintaining pregnancy and that their lower expression at the maternal-fetal interface in early pregnancy may lead to an immune imbalance that interferes with implantation, trophoblast invasion, and placentation, leading to fetal rejection and miscarriages.

Galectin-13 and Galectin-14 Promote Apoptosis of T Cells

The majority of the galectin family regulates adaptive immune responses through the induction of T cell apoptosis, which then leads to a shift in the innate/adaptive, Th1/Th2, and Th17/Treg immune balances (161–163). Similarly, we reported that exogenously added Gal-13 and Gal-14 are able to induce the apoptosis of activated T cells to a similar extent as Gal-1 (53). In accord with these findings, the interesting study from Kliman et al. (114) showed that Gal-13 is secreted from the syncytiotrophoblast and forms perivenous aggregates in the decidual extracellular matrix in the first trimester. These Gal-13 aggregates, found around decidual veins, were associated with T cell-, neutrophil-, and macrophage-containing "decidual zones of necrosis" (ZONEs), in which apoptotic T cells were also found. Based on these findings in normal pregnancies, and also based on cases in which fewer ZONEs and apoptotic T cells were found in association with very low serum Gal-13 levels, the authors hypothesized that Gal-13 is a key placental protein that downregulates maternal immune responses in the first-trimester decidua to avoid rejection of invasive trophoblasts at the maternal-fetal interface, and that low Gal-13 expression leads to heightened immune responses and impaired trophoblast invasion. It is possible that in cases where this mechanism is very defective (e.g., due to the concerted downregulation of Gal-13, Gal-14, Gal-1, and other immunoregulatory molecules in the placenta or decidua), pregnancies will be miscarried.

To better elucidate the role of Gal-13/Gal-14 in the regulation of T cells, we further characterized the pro-apoptotic effect of these galectins on T lymphocytes. We found that both Gal-13 and Gal-14 increased the rate of late-apoptotic T lymphocytes

with ∼5–10%, which was not affected by the activation status of the cells, while other galectins promote the apoptosis of only activated leukocytes (164, 165). Our result suggests that Gal-13 and Gal-14 have a basic pro-apoptotic activity on T cells. Of note, Tc cells bound more galectins than Th cells and Tc cells were more susceptible to Gal-13/Gal-14 induced apoptosis than Th cells, which may be related to the differential glycosylation pattern on these two T cell populations. This phenomenon has not been deeply explored in other galectins, which, however, were studied for their effects on different Th subsets. For example, Gal-1 selectively induced the apoptosis of pro-inflammatory Th1 and Th17 cell subsets, but not of naïve, Th2, or Treg cells (166). Moreover, Gal-9 induced the apoptosis of Th1 cells (147) in a Tim-3 dependent manner. Our results warrant further characterization of the pro-apoptotic effects of Gal-13 and Gal-14 on different Th subsets and determination of glycophenotype on T cell populations.

Apoptotic cell death in activated T cells is mediated by signaling through the activation marker CD95 (Fas), following binding to its ligand CD95L/FasL (167). In addition, another T cell activation marker CD25 (Il-2Rα), important for T cell proliferation, is also involved in this process by increasing the expression of CD95 (168). Herein, we found that the cell surface expression of CD95 and CD25 are increased upon Gal-14 and/or Gal-13 treatment. This is important since activated T cells are more prone to apoptosis (169); thus, Gal-13 and Gal-14 may increase the sensitivity of activated T cells to die by the activation-induced cell death. This is concordant with an earlier study in which Gal-1 increased the percentage of Th1 cells expressing CD95, although the Gal-1-mediated apoptosis of these cells was independent of CD95 (149). Interestingly, we found decreased expression of another activation marker (CD71) on T cells treated with Gal-14. This is seemingly contradictory, however, CD71 transiently associates with the TCR in response to TCR engagement (170) and is an essential factor for proliferating T cells (171, 172). Thus, galectins may inhibit T cell proliferation, which still needs to be tested in later studies.

Galectin-13 and Galectin-14 Regulate Cytokine Production of T Cells

Several galectins have been shown to alter cytokine production of immune cells. For example, Gal-1 induces IL-10 production in Treg cells (107, 173) and Gal-9 promotes IL-2 and IFNγ production in T cells (164). Herein, we found in the applied experimental settings that Gal-13 and Gal-14 did not alter most pro-inflammatory (IFNγ, IL-1β, and IL-6) or anti-inflammatory (IL-10) cytokine production of T cells; however, both of these galectins induced IL-8 production in non-activated T cells. This is particularly interesting since IL-8 exerts a pro-angiogenic effect on endothelial cells by decreasing the apoptosis of endothelial cells and increasing their proliferation and capillary formation (174). In addition, a novel neutrophil population was identified by recent studies in second-trimester human deciduas, which promoted *in vitro* angiogenesis in an IL-8-dependent manner (175, 176). Furthermore, decidual NK cell subsets release significant amounts of pro-angiogenic factors, such as VEGF and IL-8, necessary for spiral artery formation during decidualization (177–179). Of note, the pro-angiogenic effects of other galectins during gestation have been discovered, as reviewed recently (180). Therefore, it is tempting to speculate that Gal-13 and Gal-14 may induce angiogenesis at the maternal-fetal interface through increasing IL-8 production of T cells. This finding is related to the *in vivo* vasodilator effect of Gal-13 (181–184). In this context, reduced Gal-13 and Gal-14 expression may play a role in the disturbed vascular changes in preterm preeclampsia (58, 185–194). All our data discussed above support the idea that Gal-13 and Gal-14 also have immunoregulatory and vascular effects, as found for galectin-1 or galectin-3 (195, 196). Since the immunomodulatory effects of Gal-13 and Gal-14 could be observed on a broad scale, changing the habit of adaptive immune cells may affect innate immune cells, as well. More experiments are warranted in this direction to comprehensively elucidate the effects of these galectins at the maternal-fetal interface.

Strengths and Limitations of the Study

The strengths of the study are as follows: (1) strict clinical definitions and homogenous patient groups; (2) standardized, quick placental sample collection during pregnancy terminations; (3) standardized histopathological examination of the placentas based on international criteria; (4) protein expression profiling on placentas with tissue microarray and immunostaining followed by semiquantitative immunoscorings and statistical analysis; (5) expression and purification of large amounts of recombinant galectins with standardized methods; and (6) an array of functional experiments with primary cells and recombinant proteins.

Limitations of the study are as follows: (1) the relatively modest number of cases in each patient group due to the strict clinical and histopathological inclusion criteria used for patient enrollment. On the other hand, this was one of the most important strengths of our study; (2) for *in vitro* experiments, only non-pregnant donors were included in the study given the conditions in our patient recruitment. However, this was also a value of our study, since we used a "naïve" population of immune cells to test the effects of Gal-13 and Gal-14, while experiments with PBMCs isolated from the peripheral circulation or decidua of pregnant women pre-exposed to placental Gal-13/Gal-14 might not have revealed the true effects of these molecules. Nevertheless, our results warrant further characterization of the effects of Gal-13 and Gal-14 on peripheral blood and decidual leukocytes isolated from pregnant women, as pregnancy hormones, especially estrogen and progesterone, may impact glycosylation pattern and galectin-biding capacity of these cells; (3) Gal-13 and Gal-14 concentrations applied in our *in vitro* experiments were supraphysiologic, similar to experimental settings in previous studies on the functional effects of galectins (7, 165). The use of higher galectin concentrations is due to the fact that blood concentrations of galectins do not reflect their effective local/cell surface concentrations. In fact, these studies usually applied recombinant galectins between 10 and 100 μg/mL, representing

a reasonable range of the local galectin concentration expected in the tissues (197). Another technical reason to use higher galectin concentrations is to prevent their subunit dissociation in the solvent that contains DTT (198); and (4) the evaluation of blood concentrations of Gal-13/Gal-14, which may change in parallel with their placental dysregulation in miscarriages, as also seen in the case of Gal-1 (99, 153, 155, 184), was beyond the scope of this study, but our results warrant further investigation.

CONCLUDING REMARKS

The causes and consequences of the down-regulation of placental Gal-13 and Gal-14 expression in miscarriages still have to be uncovered by later functional studies. This work suggests that Gal-13 and Gal-14 down-regulate adaptive immune responses at the maternal-fetal interface through T cell apoptosis, and that their impaired expression leads to fetal rejection in miscarriages. Another process in which these galectins may function is angiogenesis, which is altered in both miscarriage and preeclampsia, in which Gal-13 and Gal-14 expression is decreased. Since galectins have pleiotropic functions on various immune and non-immune cells given their promiscuous binding to various cell surface receptors via glycan binding, we envision that both actions may be functional in human pregnancy. In conclusion, our results suggest that Gal-13 and Gal-14 provide an immunoprivileged environment at the maternal-fetal interface, already in early pregnancy, either through down-regulating maternal immune responses or via the support of placental development, and their reduced expression is related to the immune pathology of miscarriages.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the **Supplementary Files**.

ETHICS STATEMENT

Clinical sample and data collection were approved by the Health Science Board of Hungary (ETT-TUKEB 4834-0/2011-1018EKU). Written informed consent was obtained from women prior to sample collection and the experiments conformed to the principles set out in the World Medical Association Declaration of Helsinki. Specimens and data were stored anonymously.

AUTHOR CONTRIBUTIONS

AB, IH, SH, JM, SR, EP, and NT conceptualized study and designed research. AB, DC, KJ, AK, EP, KP, NS, and ET performed research. SH, PH, HM, EP, ZP, RR, NT, and PZ contributed new reagents, analytic tools, and clinical specimens. AB, DC, OE, JM, EP, SR, AT, and NT analyzed and interpreted data. All authors contributed to the writing of the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01240/full#supplementary-material

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Human Miscarriage Is Associated With Dysregulations in Peripheral Blood-Derived Myeloid Dendritic Cell Subsets

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Dendritic cells (DC) are critically involved in decisions related to the acceptance or rejection of the foreign fetal antigens by the maternal immune system. However, particularly for human peripheral blood DCs (PBDC), available literature is rather inconsistent and the factors regulating these cells are ill-defined. Here, we investigated the phenotype and functionality of different human PBDC subsets during normal and pathologic pregnancies and studied an involvement of human chorionic gonadotropin (hCG) in PBDC regulation. Peripheral blood samples were obtained from normal pregnant women in all three trimesters, from first trimester miscarriage patients and from healthy non-pregnant women. Samples were analyzed for plasma hCG levels, for regulatory T (Treg) cell numbers, for frequencies of total and mature plasmacytoid (PDC) and myeloid (MDC1 and MDC2) PBDC subsets and for their cytokine secretion. *In vitro* assays, culturing PDC, MDC1 or MDC2 in the presence of two trophoblast cell lines, placenta explant supernatants or two hCG preparations were performed. The Treg-inducing capability of hCG- or non-hCG-treated stimulated MDC1 was assessed. Total and mature MDC1 and MDC2 frequencies increased during the first and second trimester of normal pregnancy, respectively. Miscarriage was associated with a reduced MDC1 and an increased MDC2 activation profile. PDC were not altered neither during normal pregnancy progression nor during miscarriage. *In vitro*, the culture of isolated PBDC subsets in the presence of placenta-derived factors impaired the maturation of MDC1 and differentially affected PDC maturation. An inhibitory effect on MDC1 and PDC maturation was also proven for the urine-derived hCG preparation. Finally, we observed a Treg cell elevation during early normal pregnancy that was not present in miscarriages. Stimulated MDC1 induced Treg cells *in vitro*, however, hCG was not involved in this process. Our findings suggest that during normal pregnancy PBDC subsets are differentially regulated dependent on gestational age. Miscarriage seems to be associated with dysregulations

in the myeloid PBDC subsets and with disturbances in Treg cell frequencies. Moreover, our results propose an interdependency between MDC1 and Treg cells during early pregnancy. hCG, although shown to impair MDC1 maturation, does not seem to be a key regulator of PBDC alterations during pregnancy.

Keywords: peripheral blood dendritic cells, plasmacytoid dendritic cells, myeloid dendritic cells, regulatory T cells, human chorionic gonadotropin, placenta factors, fetal tolerance, pregnancy

INTRODUCTION

Pregnancy is characterized by finely regulated immunological adaptions that allow the persistence of the foreign fetal antigens in the maternal womb. Notably, since Schmorl and colleagues in 1893 identified for the first time fetal cells at various maternal tissue sites (1), it became obvious that not only local immune cell populations residing directly at the fetal-maternal interface but also immune cell types circulating in the periphery get in direct contact to fetal structures and are able to react toward them. Thus, pregnancy-driven immunological adjustments take place locally and peripherally and dysregulations in uterine as well as circulating immune responses may interfere with normal pregnancy progression. Several studies implicated immune modulatory properties of placenta-derived factors suggesting that the fetal tissue itself regulates its surrounding environment (2). These factors include among others cytokines, growth factors and hormones and were reported to educate local and peripheral immune cells in such a way that they contribute to fetal tolerance and growth (3–9). Thereby, peripheral immune cell populations reportedly adapt their phenotype and functionality so to enter the fetal-maternal interface as "fetal-friendly" cells. However, the precise mechanisms how this is realized during pregnancy are still under investigation.

As one of the first immune cell types encountering fetal antigens, dendritic cells (DCs) possess the capacity to induce either immunity or tolerance toward the fetus (10). Their behavior highly depends on the maturation state and cytokine secretion pattern (10). In the human decidua, the presence of predominantly myeloid DCs possessing an immature phenotype and secreting anti-inflammatory cytokines was associated with fetal tolerance (11–14), while a mature DC phenotype could be associated with various pregnancy complications (15–18). By contrast, data referring to the distribution and the phenotype of peripheral blood DC (PBDC) subsets during normal and pathologic pregnancies are rather inconsistent which might be due to the different DC characterization strategies applied in the past (19–22). Moreover, most of the studies divided the total PBDC pool into two major subsets, one lymphoid, and one myeloid subset. However, in 2000 Dzionek and colleagues described the existence of three clearly defined PBDC subsets. Based on the expression of specific blood DC antigens (BDCA), the authors classified the total PBDC pool into one plasmacytoid (PDC) and two myeloid (MDC1 and MDC2) subsets (23). Later on, the Nomenclature Committee of the International Union of Immunological Societies approved this classification (24). Interestingly, the presence of the BDCA-defined DC subsets seems to be not restricted to the peripheral blood as Ban and colleagues were able to detect all three subsets in the human decidua during early normal pregnancy. Decidual BDCA-1^+ MDC1 and BDCA-3^+ MDC2 expressed low levels of the maturation markers CD86 and CD80 confirming an immature phenotype of local DCs during normal pregnancy (25). This finding gives rise to speculations about the origin of these cells. On the one hand, it can be assumed that BDCA-1^+ MDC1 and BDCA-3^+ MDC2 are part of the DC pool resident in the uterus and regulated locally once pregnancy arised. On the other hand, it can be hypothesized that both DC subsets immigrate from the peripheral blood stream into the fetal-maternal interface. In both cases, placenta-derived factors may contribute to the local regulation as well as to DC immigration (26–28). Zhao and colleagues suggested a participation of human placenta-produced factors in the differentiation process from blood-derived monocytes into decidual DCs. The authors further confirmed a tolerogenic phenotype of these differentiated DCs (29). Additionally, placental factors reaching the circulation may provoke alterations of PBDCs directly in the periphery. The pregnancy hormone human chorionic gonadotropin (hCG) may represent a good candidate to fulfill this function as it represents one, if not the first signal, provided by the fetal tissue to the mother and can be detected already 6–8 days following fertilization in the blood stream (30). Furthermore, there is evidence that hCG has the capability to modulate human PBDCs (20, 31, 32).

Here, we aimed to investigate the phenotype and functionality of PDC, MDC1, and MDC2 derived from human peripheral blood during normal and pathologic pregnancies. Moreover, we addressed a potential regulation of all PBDC subsets by placenta-derived factors with a specific focus on hCG.

METHODS

Sampling and Ethical Approval

Peripheral blood samples were obtained from healthy non-pregnant woman within the luteal phase of their menstrual cycle as well as from normal pregnant woman in all three trimesters and from first trimester miscarriage patients. Placental tissue from normal pregnant women and miscarriage patients was obtained by curettage during surgery. Sampling of blood and placental tissue was realized by physicians of the University Women's Clinic in Magdeburg, Germany after all subjects were informed in detail and gave their written consent. This study was carried out in accordance with the recommendations of ethic guidelines defined by the ethics board at the University of Magdeburg with written informed consent from all subjects.

TABLE 1 | Patient characteristics.

	Total number	Week of pregnancy	Age
***Ex vivo* analyses**			
Non-pregnant women	$n = 12$	N/A	24.82 ± 4.41
Normal pregnant women (I. Trimester)	$n = 20$	10.30 ± 1.39	25.95 ± 5.06
Normal pregnant women (II. Trimester)	$n = 14$	19.21 ± 3.97	28.36 ± 5.45
Normal pregnant women (III. Trimester)	$n = 18$	34.28 ± 3.38	30.06 ± 3.22
Miscarriage patients	$n = 20$	8.65 ± 1.11	30.70 ± 6.01
***In vitro* Assays**			
Non-pregnant women	$n = 6$	N/A	28.50 ± 4.15
Normal pregnant women (I. Trimester)	$n = 16$	9.81 ± 1.70	30.00 ± 5.97
Miscarriage patients (I. Trimester)	$n = 16$	8.69 ± 0.98	31.50 ± 4.85

All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the ethics board at the University of Magdeburg (study 28/08). Characteristics of all study subjects are displayed in **Table 1**.

Determination of hCG Isoforms in Plasma and Placenta Supernatants by ELISA Analysis

After tissue collection, 500 mg of placental tissue (explants) was cultured in 1 ml of RPMI 1640 (Thermo Fisher Scientific, Germany) supplemented with 3% of charcolized fetal bovine serum (FBS, PAN-Biotech, Germany) and 1% penicillin/streptomycin (Thermo Fisher Scientific, Germany) for 24 h. Afterwards, placenta explant supernatants were collected and analyzed for the concentration of either regular hCG, free β-hCG or hyperglycosylated hCG (H-hCG) by enzyme-linked immunosorbent assay (ELISA). The concentrations of all hCG isoforms were evaluated in the plasma fraction of all blood samples. Regular and free β-hCG were determined using kits from DRG systems, Germany whereas H-hCG concentrations were evaluated using a kit from My Biosource, USA. All steps were performed according to the manufacturer's instructions.

Isolation of MDC1, MDC2, or PDC From PBMCs

The cellular fraction from all blood samples was used to isolate peripheral blood mononuclear cells (PBMCs) by density gradient centrifugation using Ficoll-Paque^{TM} (GE Healthcare, Sweden) under sterile conditions. Afterwards, MDC1, MDC2, or PDC were separately isolated from PBMCs of non-pregnant and normal pregnant women (I. trimester) as well as from miscarriage patients (I. trimester) by magnetic activated cell sorting. The following isolations kits from Miltenyi Biotec, Germany were applied: MDC1 (CD1c Dendritic Cell Isolation Kit, human); MDC2 (CD141 MicroBead Kit, human); and PDC (CD304 MicroBead Kit, human). All steps were performed under sterile conditions following the instructions given. Purities of isolated MDC1, MDC2 and PDC were above 95, 45, and 85%, respectively. After isolation, all PBDC subsets were cultured for 24 h in RPMI 1640 supplemented with 50 μM β-mercaptoethanol (Sigma Aldrich, Germany), 10% FBS (Biochrom, Germany) and 1% penicillin/streptomycin (dendritic cell medium; DCM).

Cytometric Bead Array (CBA) Analysis of Cytokine Secretion by MDC1, MDC2, and PDC

5×10^4 isolated MDC1, MDC2, or PDC from either normal pregnant women or miscarriage patients in their first trimester of pregnancy were cultured in DCM for 48 h. Following, cell supernatants were collected and analyzed for the levels of IL-1β, IL-6, IL-8, IL-10, and TNF by CBA using the TH1/TH2 Cytokine Kit from BD Biosciences, Germany. All steps were performed according to the instructions provided by the manufacturer. Measurements were conducted by using a 4-color FACSCalibur^{TM} flow cytometer (BD Biosciences, Germany) and analyses were performed using FCAPArray software (BD Biosciences, Germany).

Assessment of PBDC Maturation Under Different Culture Conditions Involving Trophoblast Cell Lines, Placental Explant Supernatants or Purified hCG Preparations

For the following experiments, MDC1, MDC2, or PDC were isolated from PBMCs derived from healthy non-pregnant women in the luteal phase of their menstrual cycle. The maturation of all three PBDC subsets was induced by the addition of 10 ng/ml lipopolysaccharide (LPS) to the DCM. In each experimental setting, the maturation state was assessed after 24 h by determining the numbers of CD83-, CD86-, or HLA-DR-expressing MDC1, MDC2, or PDC. The appropriate gating strategy is displayed in **Supplementary Figure 1**.

In the first set of experiments, 1×10^4 MDC1, MDC2, or PDC were co-cultured with 3×10^4 JEG-3 cells (hCG-secreting human choriocarcinoma cell line) or SWAN-71 cells (non hCG-secreting human immortalized extravillious cytotrophoblast cell line). Therefore, both trophoblast cell lines were plated 1 day before starting the co-cultures to allow adherence to the culture plate. JEG-3 and SWAN-71 cells were cultured in Dulbecco's modified eagle medium (DMEM, Invitrogen, Germany) supplemented with 10% FBS, 1% penicillin/streptomycin, 100 nM MEM non-essential amino acids (Invitrogen, Germany), 1 mM sodium pyruvate (Sigma-Aldrich, Germany), and 10 mM hepes (Biochrom, Germany). Both cell lines were cultured at 37°C and 5% CO_2.

In the second set of experiments, 1×10^4 MDC1, MDC2, or PDC were cultured in placental explant supernatants enriched with DCM in a ratio 1:1. Placental explants were derived from normal pregnant women or miscarriage patients in their first trimester of pregnancy.

In the third set of experiments, 1×10^4 MDC1, MDC2, or PDC were treated with either 100IU/ml recombinant hCG (rhCG, Ovitrelle, Merck, Germany) or 250 IU/ml urine-derived hCG (uhCG, Sigma, Germany). The concentration of uhCG was chosen according to physiological hCG levels found in normal pregnant women during the first trimester (25-288IU/ml during gestation weeks 9–12 according to the *American Pregnancy*

Association) and rhCG concentration was chosen according to concentrations used for rhCG and other recombinant gonadotropins in previous studies (33, 34).

Assessment of Treg Cell Generation After Co-culture With hCG- or Non hCG-Treated MDC1

MDC1 were isolated from non-pregnant women, stimulated with 10 ng/ml LPS and cultured in the absence or presence of hCG (100 mIU/ml rhCG or 250IU/ml uhCG) for 24 h as described above. Afterwards, 1×10^4 MDC1 were co-cultured with 1×10^4 $CD4^+CD25^-$ T cells in DCM containing 10 ng/ml recombinant IL-2, 1 μg/ml anti-CD3 and 5 μg/ml anti-CD28 for another 24 h. $CD4^+CD25^-$ T cells were obtained from the same donors who provided the MDC1 by using the human $CD4^+CD25^+$ Regulatory T Cell Isolation Kit (Miltenyi Biotec, Germany). Frequencies of $CD4^+CD25^{high}Foxp3^+$ Treg cells were determined by flow cytometry using a 4-color FACSCalibur™ flow cytometer. The appropriate gating strategy is displayed in **Supplementary Figure 2**.

Flow Cytometry Analysis

To estimate the frequencies of total and mature MDC1, MDC2, and PDC within PBMCs from non-pregnant and normal pregnant women as well as from miscarriage patients, PBMCs were stained for distinct surface markers defining the different DC subsets and for the maturation markers CD83, CD86, and HLA-DR. Additionally, Treg cell frequencies were determined in all samples. Therefore, PBMCs were stained for the extracellular markers CD4 and CD25 as well as for the intracellular marker Foxp3. Briefly, PBMCs were washed in PBS containing 1% bovine serum albumin (BSA) and 0.1% sodium azide (flow cytometry (FC) buffer). Afterwards, staining for extracellular markers (1:100 antibody dilutions) was performed for 30 min at 4°C in the dark. Following another washing step in FC buffer, cells were fixed overnight using the fixation/permeabilization set from Thermo Fisher Scientific, Germany. For intracellular staining, PBMCs were washed in permeabilization buffer and then incubated for 30 min at 4°C in the dark in the staining solution (1:200 antibody dilution). After washing in permeabilization buffer, cells were resuspended in FC buffer and measured using a 4-color FACSCalibur flow cytometer. The appropriate gating strategy is displayed in **Supplementary Figure 3**. MDC1, MDC2, PDC, and T cells obtained from *in vitro* culture experiments were stained accordingly. The following antibodies were used: APC-conjugated anti-human CD303 (PDC, clone: AC144), APC-conjugated anti-human CD1c (MDC1, clone: AD5-8E7) or APC-conjugated anti-human CD141 (MDC2, clone: AD5-14H12); all purchased from Miltenyi Biotec, Germany. PE-Cy7-conjugated anti-human CD83 (clone: HB15e), FITC-conjugated anti-human CD86 (clone: FUN-1), PE-conjugated anti-human HLA-DR (clone: G46-6), FITC-conjugated anti-human CD4 (clone: OKT4), PerCp-Cy5.5-conjugated anti-human CD25 (clone: BC96), and AF647-conjugated anti-human Foxp3 (clone: 259D/C7); purchased from BioLegend, eBiosciences and BD Biosciences, Germany, respectively.

Data Analysis and Statistics

Data analysis and presentation were performed with GraphPad Prism 7.0 software (Statcon, Germany). Data are displayed as means plus standard deviation (S.D.) or standard error of the mean (S.E.M.). The total number of replicates is indicated in the figure legends for each specific data set. All data sets were tested for normality by using the Shapiro-Wilk test and parametric or non-parametric tests were used accordingly. Normal distributed data sets were analyzed by using One- or Two-Way-ANOVA followed by Tukey's or Dunn's multiple comparison test. Not normal distributed data sets were analyzed by applying the Mann-Whitney-*U*-test. For *in vitro* culture experiments, controls (LPS alone) were set as "1" and experimental groups (trophoblast co-cultures, placenta explant supernatants and hCG preparations) were calculated as fold change to controls.

RESULTS

Pregnancy Provokes Alterations in the Number of Total and Mature Myeloid PBDC Subsets

First, we wondered whether PBDC subsets would change depending on gestational age during normal pregnancy progression. All subsets represented minor populations within total PBMCs (PDC: 0.25–2.64%; MDC1: 0.07–3.87%; MDC2: 0.03–1.06%). Frequencies of total and mature PDC from normal pregnant women of all trimesters were not altered when compared to non-pregnant women (**Figures 1A,D**). However, we observed an increase in the frequencies of total MDC1 and MDC2 during the first and second trimester in normal pregnant women, respectively, as compared to non-pregnant individuals (**Figures 1B,C**). Accordingly, HLA-DR-expressing MDC1 as well as CD83 and CD86-expressing MDC2 were elevated at the same gestational ages (**Figures 1E,F**).

Miscarriage Is Associated With an Altered Profile of Both Myeloid PBDC Subsets

Next, we sought to determine whether similar alterations in both myeloid PBDC subsets could be observed in miscarriage patients. We found no augmentation of total MDC1 frequencies in patients suffering from abortions when compared to non-pregnant women and a significant diminution as compared to normal pregnant women (**Figure 2B**). Moreover, frequencies of HLA-DR-expressing MDC1 were reduced in miscarriage patients when compared to normal pregnant women (**Figure 2E**). However, this did not reach statistical significance. Notably, frequencies of total and mature MDC2 were significantly elevated in miscarriage patients as compared to non-pregnant and normal pregnant women (**Figures 2C,F**). This included CD83-, CD86- and HLA-DR-expressing MDC2 (**Figure 2F**). As for PDC, no significant changes could be detected in miscarriage patients, neither to normal pregnant women nor to non-pregnant individuals (**Figures 2A,D**).

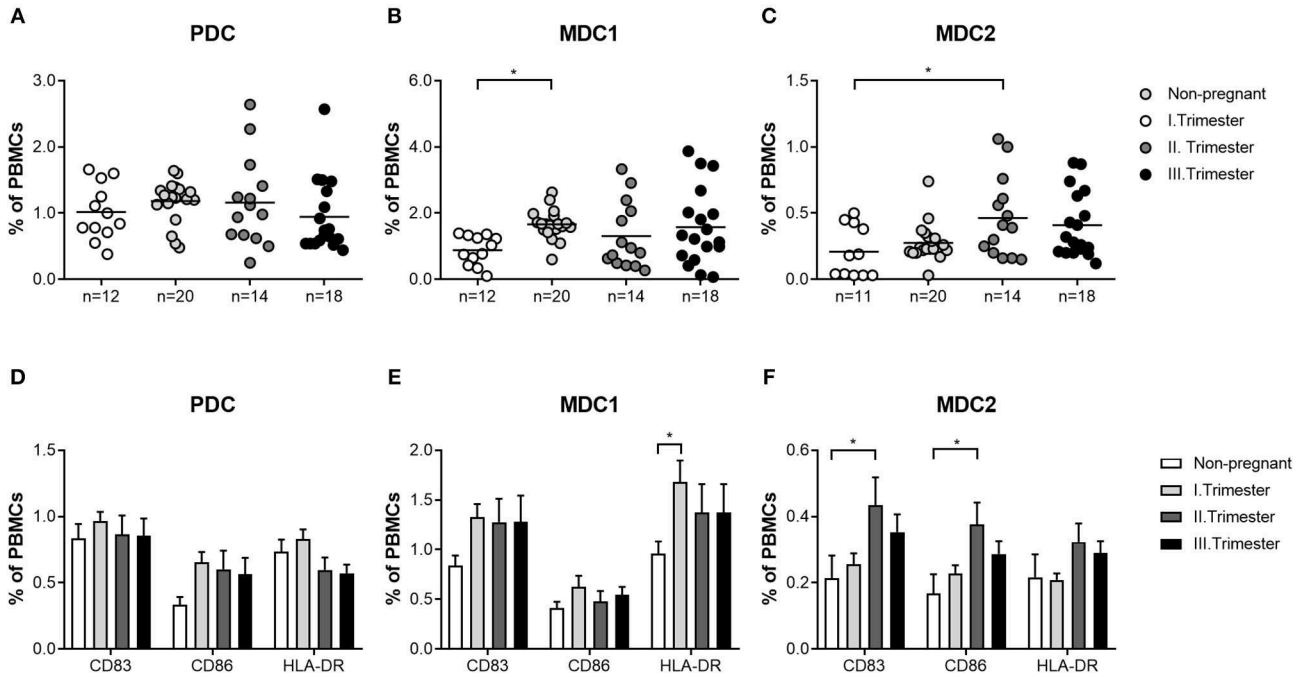


FIGURE 1 | The frequencies of total and mature MDC1 and MDC2 are altered depending on gestational age. PBMCs were isolated from normal pregnant women in all trimesters ($n = 14$–20) as well as from healthy non-pregnant women ($n = 12$) and stained for markers defining the distinct PBDC subsets and for the maturation markers CD83, CD86, and HLA-DR. The total frequencies of **(A)** PDC, **(B)** MDC1, and **(C)** MDC2 as well as the frequencies of mature **(D)** PDC, **(E)** MDC1, and **(F)** MDC2 were determined by flow cytometry. Statistical analysis was performed using One- or Two-Way-ANOVA followed by Tukey's multiple comparison test. $^*p \leq 0.05$.

Miscarriage Is Associated With a Disturbed Cytokine Secretion Capacity of the MDC1 Subset

Based on our findings, showing altered frequencies of total and mature MDC1 and MDC2 in miscarriage patients, we were curious whether both myeloid subsets would also possess a disturbed cytokine secretion profile. Therefore, we separately assessed the capacity to secrete cytokines of all PBDC subsets after their isolation from normal pregnant women or miscarriage patients. In agreement with our previous results, PDC from miscarriage patients secreted equal amounts of pro- and anti-inflammatory cytokines as the ones from normal pregnant women (**Figure 3A**). MDC1 from miscarriage patients had a significant reduced capacity to secrete IL-1β, IL-6, IL-10, and TNF (**Figure 3B**), while MDC2 from miscarriage patients did not behave differentially from MDC2 of normal pregnant women (**Figure 3C**). However, we have to admit that the purity of the MDC2 isolation was rather poor.

Miscarriage Is Associated With Reduced Peripheral and Local Levels of Different hCG Isoforms

As our results revealed changes during early normal pregnancy and predominantly in the MDC1 subset that could not be observed in pathologic pregnancies, we wondered whether placenta-derived factors might be responsible for the observed alterations. Here, we particularly focused on the pregnancy hormone hCG, known to be highly elevated during the first trimester. Analyses of plasma samples as well as placental explant supernatants exhibited significant diminished levels of regular hCG in plasma samples from miscarriage patients when compared to normal pregnant women but not in placental explant supernatants (**Table 2**). Even more interestingly, we found significant reduced levels of two other hCG isoforms, namely free β-hCG and H-hCG, in the plasma and the placenta supernatants from miscarriage patients (**Table 2**). Correlation analyses between all hCG isoforms and all PBDC subsets demonstrated a negative correlation between regular hCG or H-hCG and MDC2 but no correlation between free β-hCG and MDC2 (data not shown). Moreover, no correlations could be proven for all hCG isoforms and MDC1 or PDC (data not shown).

Placenta-Derived Factors Differentially Regulate the Maturation of PBDC Subsets

To determine whether placenta-derived factors and particularly hCG might be involved in PBDC alterations, we first co-cultured PDC, MDC1 or MDC2 with two different trophoblast cell lines and evaluated the effect on the maturation process. We chose one hCG-secreting trophoblast cell line (JEG-3) and one non hCG-secreting trophoblast cell line (SWAN-71). The presence of hCG-secreting JEG-3 cells contributed to an increase of CD83-expressing PDC but impaired an elevation

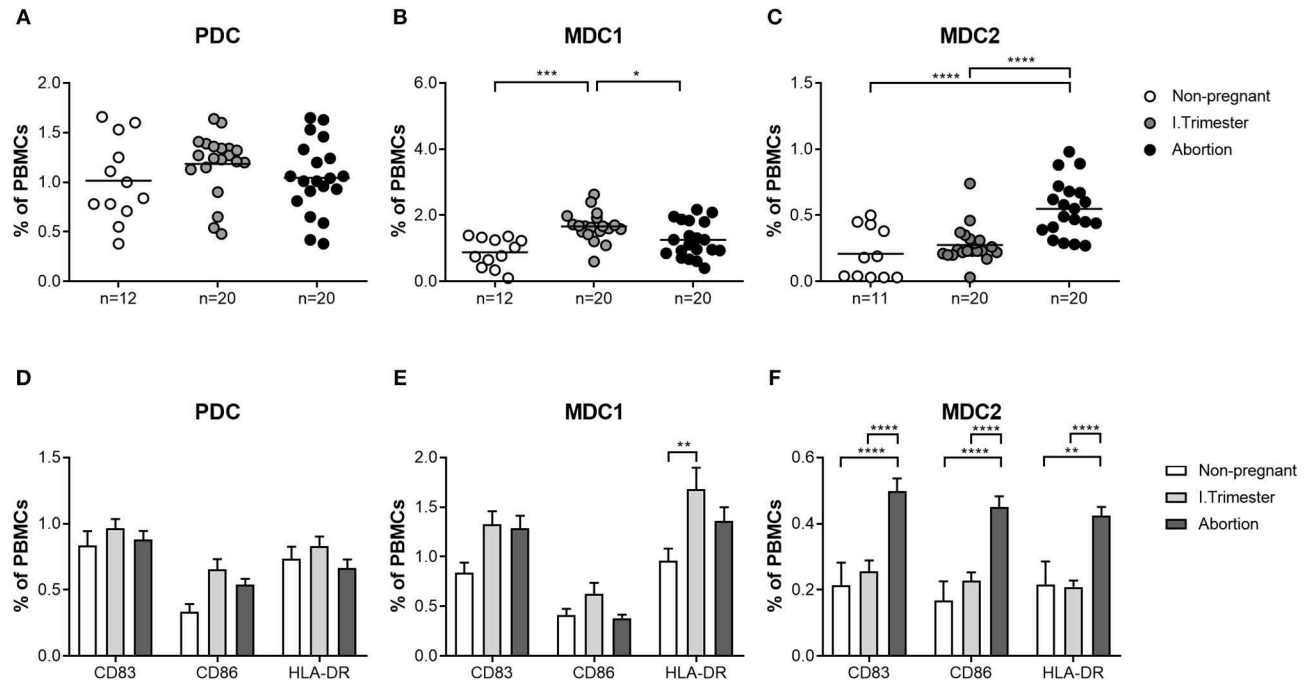


FIGURE 2 | The frequencies of total and mature MDC1 and MDC2 are altered in miscarriages. PBMCs were isolated from normal pregnant women and miscarriage patients in the first trimester ($n = 20$) as well as from healthy non-pregnant women ($n = 12$) and stained for markers defining the distinct PBDC subsets and for the maturation markers CD83, CD86, and HLA-DR. The total frequencies of **(A)** PDC, **(B)** MDC1, and **(C)** MDC2 as well as the frequencies of mature **(D)** PDC, **(E)** MDC1, and **(F)** MDC2 were determined by flow cytometry. Statistical analysis was performed using One- or Two-Way-ANOVA followed by Tukey's multiple comparison test. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$.

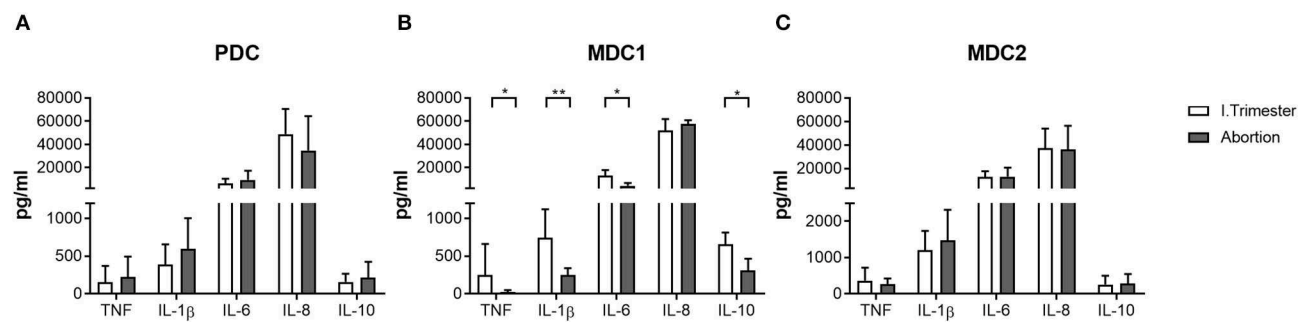


FIGURE 3 | MDC1 from miscarriages showed an altered cytokine profile. PDC, MDC1, or MDC2 were isolated from PBMCs of normal pregnant women and miscarriage patients in the first trimester ($n = 4$–5). All PBDC subsets were cultured for 48 h. Afterwards, cell culture supernatants were analyzed for the levels of different cytokines by CBArray. The concentration of TNF, IL-1β, IL-6, IL-8, and IL-10 produced by **(A)** PDC, **(B)** MDC1, and **(C)** MDC2 is displayed. Statistical analysis was performed using the Mann-Whitney-*U*-test. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$.

of CD86- and HLA-DR-expressing MDC1 (**Figures 4A,B**). Non hCG-secreting SWAN-71 cells favored an augmentation of CD83- and CD86-expressing PDC and hampered an increase of HLA-DR-expressing MDC1 (**Figures 4A,B**). None of the cell lines significantly affected the maturation process of MDC2 (**Figure 4C**).

Secondly, we decided to culture each PBDC subsets with supernatants from placental explants derived from either normal pregnant women or miscarriage patients. Here, in addition to our co-cultures with the trophoblast cell lines, we studied a potential influence of soluble factors derived from primary trophoblast cells mimicking a more physiologic scenario. Besides, it is important to mention that placental explant supernatants contain a mixture of all hCG isoforms whereas JEG-3 cells almost exclusively secrete H-hCG. Unfortunately, most of our primary trophoblast cultures lose the ability to secrete hCG in culture and were therefore not suitable for these kind of experiments. Supernatants from miscarriage patients significantly hampered an augmentation of CD86-expressing PDC and HLA-DR-expressing MDC1 (**Figures 4D,E**), whereas

supernatants from normal pregnant women had no effect on PDC and MDC1 (**Figures 4D,E**). Moreover, maturation of MDC2 was not influenced by the addition of placental explant supernatants (**Figure 4F**).

TABLE 2 | Miscarriage is associated with reduced levels of hCG isoforms.

	regular hCG (mIU/ml)	free β-hCG (pg/ml)	H-hCG (nmol/L)
Plasma			
I. Trimester	112855 ± 106610	43.63 ± 37.91	38.21 ± 43.43
Abortion	43840 ± 46906*	23.57 ± 22.56*	12.70 ± 5.43**
Placenta explants			
I. Trimester	175.60 ± 216.10	97.99 ± 42.48	198.40 ± 261.40
Abortion	252.30 ± 261.20	53.67 ± 54.53*	64.69 ± 92.57*

*Plasma and placenta explants from normal pregnant women and miscarriage patients in the first trimester (n = 15–20) were analyzed for the levels of different hCG isoforms by ELISA. Statistical analysis was performed using the Mann-Whitney-U-test. *p < 0.05 vs. I. Trimester; **p < 0.01 vs. I. Trimester.*

Thirdly, as our data were rather inconsistent with regard to a potential effect of hCG, we cultured PDC, MDC1, or MDC2 in the presence of two hCG preparations. We chose an rhCG preparation containing only intact regular hCG molecules and an uhCG preparation containing the broad variety of all hCG isoforms. We observed an impaired elevation of CD86-expressing PDC as well as CD83-, CD86-, and HLA-DR-expressing MDC1 when uhCG was added to the cultures (**Figures 4G,H**), whereas the presence of rhCG did not affect the maturation of PDC and MDC1 (**Figures 4G,H**). In line with our previous results, MDC2 maturation was not affected at all (**Figure 4I**). However, again we would like to point out that our MDC2 isolations suffered from a poor purity.

hCG Does Not Enhance MDC1-Induced Treg Generation

Own previous studies identified hCG as a factor driving Treg induction during early pregnancy (35). However, it is still a matter of debate through which pathways hCG

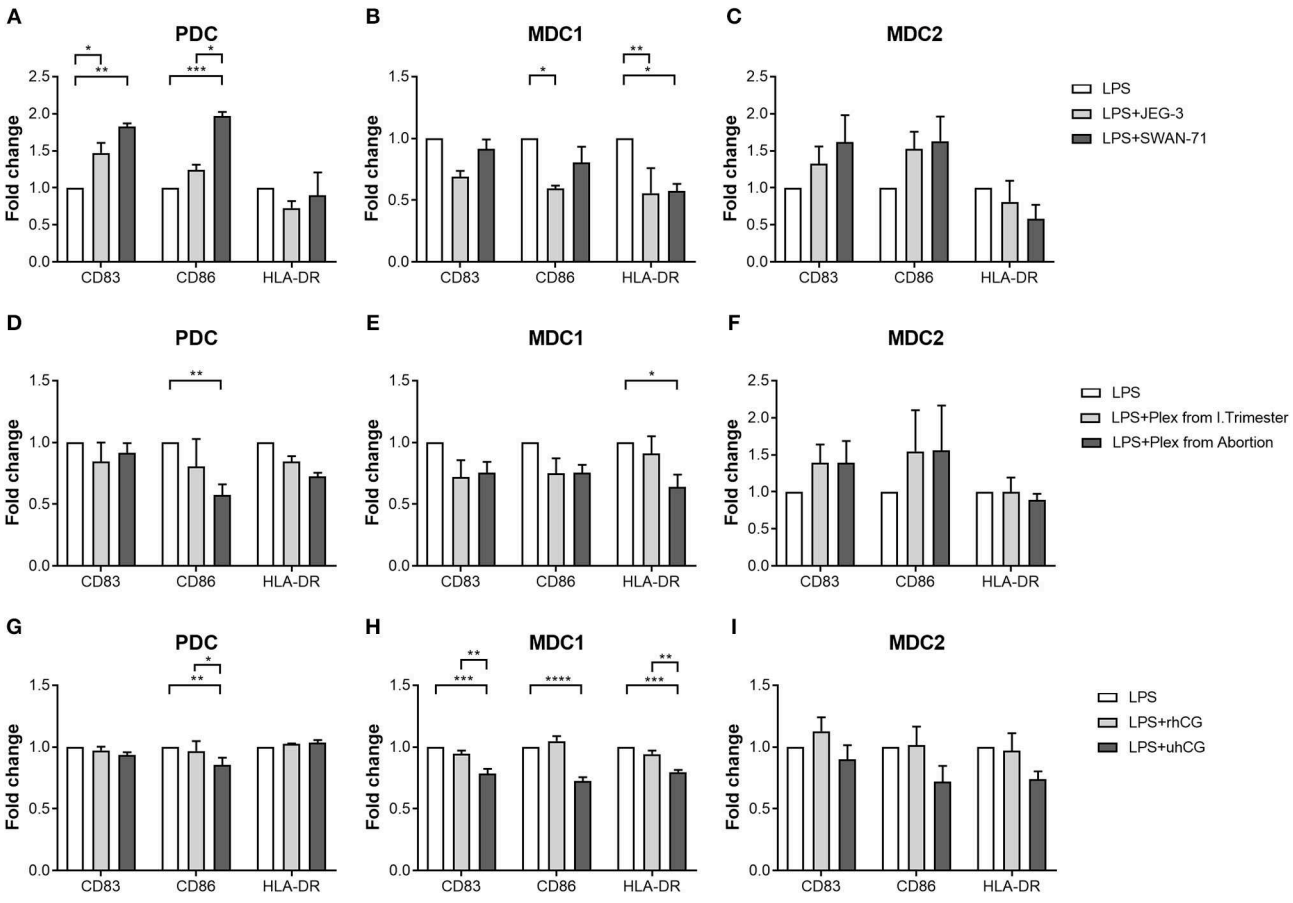


FIGURE 4 | PDC, MDC1 and MDC2 are differentially affected by placenta-derived factors. PDC, MDC1, or MDC2 were isolated from PBMCs of healthy non-pregnant women and cultured in the presence of **(A–C)** JEG-3 or SWAN-71 trophoblast cell lines ($n = 3$) **(D–F)**, supernatants of placental explants (Plex; $n = 4$) or **(G–I)** two hCG preparations [100 mIU/ml recombinant hCG (rhCG); 250IU/ml urine-derived (uhCG)]. The frequencies of mature **(A,D,G)** PDC, **(B,E,H)** MDC1, and **(C,F,I)** MDC2 were determined by flow cytometry. Statistical analysis was performed using Two-Way-ANOVA followed by Tukey's multiple comparison test. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$.

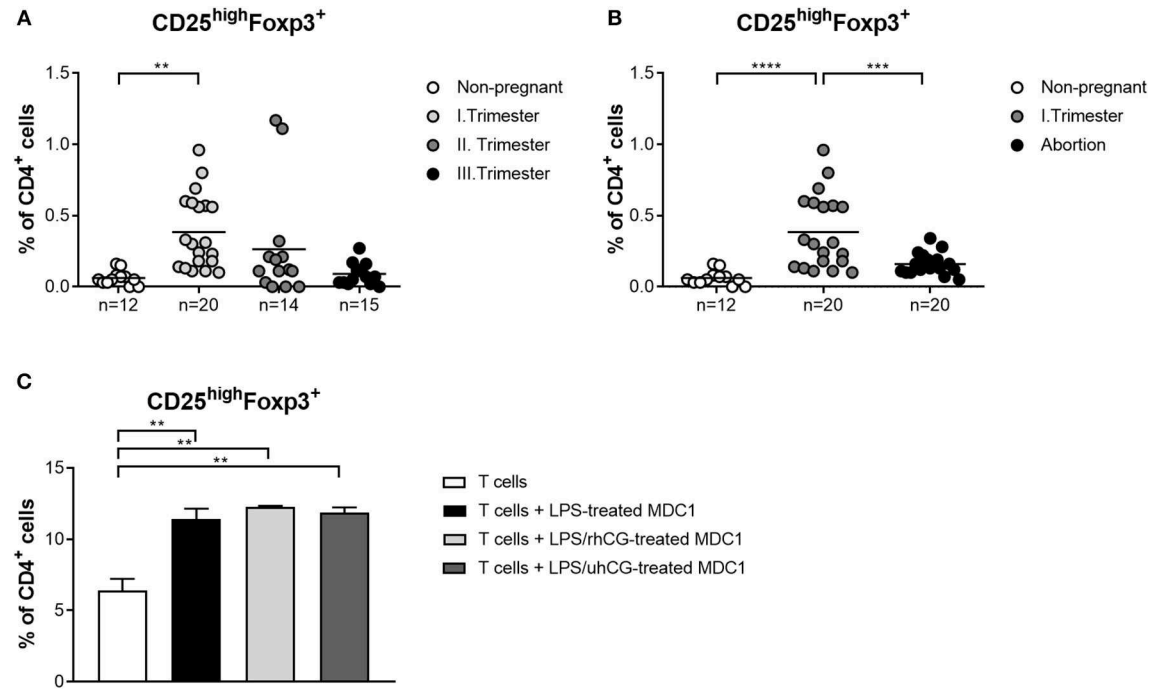


FIGURE 5 | MDC1 cells are able to induce Treg cells that are reduced in miscarriages. PBMCs were isolated from normal pregnant women in all trimesters ($n = 14$–20), from miscarriage patients in the first trimester ($n = 20$) as well as from healthy non-pregnant women ($n = 12$) and stained for Treg cell markers. Treg frequencies are shown for **(A)** normal pregnancy progression and for **(B)** miscarriage patients compared to normal and non-pregnant women. Moreover, MDC1 were isolated from non-pregnant women ($n = 3$), stimulated with 10 ng/ml LPS in the presence or absence of 100 mIU/ml recombinant hCG (rhCG) or 250IU/ml urine-derived hCG (uhCG) for 24 h and then co-cultured with $CD4^+CD25^-$ T cells derived from the same donors for another 24 h. **(C)** Treg frequencies were determined by flow cytometry. Statistical analysis was performed using One-Way-ANOVA followed by Dunn's multiple comparison test. $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$.

mediates its Treg-inducing properties. Here, we wondered whether an interdependency between Treg and PBDCs during early pregnancy exists and if hCG might represent the intermediate factor between both immune cell types. Therefore, we determined the frequencies of Treg during normal pregnancy progression as well as during miscarriage. $CD4^+CD25^{high}Foxp3^+$ Treg frequencies were significantly elevated in first trimester normal pregnant women when compared to non-pregnant women and declined during the second and third trimester (**Figure 5A**). Miscarriage patients had significantly reduced Treg frequencies as compared to normal pregnant women (**Figure 5B**). Notably, normal pregnancy-associated Treg alterations are in line with changes observed for MDC1 (**Figures 1B**, **5A**). Moreover, miscarriage was characterized by lower Treg and MDC1 frequencies suggesting an interrelation between these two cell populations. To follow up this idea, we tested whether MDC1 possessed the ability to induce Treg *in vitro*. We pre-treated MDC1 with rhCG or uhCG to study a potential involvement of hCG in MDC1-mediated Treg induction. We confirmed the ability of MDC1 to induce Treg generation. However, pre-treatment with either rhCG or uhCG did not further enhance Treg numbers (**Figure 5C**).

DISCUSSION

Pregnancy can be considered as a remarkable challenge for the maternal immune system where a variety of immunological adaptions has to go hand in hand to guarantee the survival of the semi-allogeneic fetus. Hereby, not only local immune cell populations residing at the fetal-maternal interface but also immune cell types circulating through lymphoid tissues and the peripheral blood are aware of the foreign fetal antigens. Thus, pregnancy-driven immunological regulations take place in the proximity as well as in the distance of the fetal tissues and it is suggested that the fetus itself contributes to these regulatory processes by expressing and secreting immune modulating factors.

DCs are one of the major immune cell populations that are affected by pregnancy-driven alterations and on their part function as key regulators for other immune cells types. There is accumulating evidence that during normal human pregnancy progression myeloid DCs are the predominant DC population, locally as well as peripherally (11, 19, 20). Moreover, human decidual DCs adopt a tolerogenic profile by expressing an exclusive set of markers and possessing a reduced T cell stimulatory capacity (11, 12). PBDCs also undergo changes in their phenotype and functionality throughout normal human pregnancy. However, several studies presented different outcomes (19–22, 36) that might be partly explained by the combination of markers used to characterize the various PBDC subsets. Furthermore, the majority of the previous studies did not separately examine the frequencies of MDC1 and MDC2 but rather focused on one myeloid PBDC population. This kind of analysis may

mask potential gestational-dependent alterations within distinct myeloid PBDC subsets.

Our current findings propose significant augmentations of total MDC1 and MDC2 during the first and second trimester of normal pregnancy, respectively, while total PDC remained unchanged at all trimesters. Likewise, we found elevations of mature myeloid PBDCs during the first and second trimester while the number of mature PDC was not altered. These observed gestational age-related changes in the number and functionality of PBDCs seem to be relevant to finely regulate the degree of alloreactive immune reactions. On the one hand, PBDCs have to be alert to systemic infections at any time while on the other hand ensure tolerance toward the fetal alloantigens. To overcome this paradoxical situation, it was proposed that PBDCs pass through an incomplete activation process during normal pregnancy enabling them to present fetal alloantigens without provoking overwhelming anti-fetal immune reactions. Moreover, there is evidence that PBDCs are involved in the induction of acquired thymic tolerance and it was suggested that incompletely activated PBDCs promote tolerance to fetal alloantigens by educating thymic T cells (19). Notably, the extent of DC activation seems to be decisive for pregnancy outcome as an over- or under-activation of PBDCs was associated with pregnancy complications such as pre-eclampsia, intrauterine growth retardation or miscarriage (18, 22). Our own findings support this idea, as we were able to demonstrate a significant lower number of mature MDC1 in miscarriage patients compared to normal pregnant women. Moreover, these cells had a reduced capacity to secrete cytokines suggesting an overall diminished potential to stimulate T cells. Our data suggest that also an underactivation of specific PBDC subsets may provoke fetal demise. Limited activation of MDC1 may result in a reduced potential to induce Treg cells that at first glance seems to be contradictory, as it is believed that predominantly DCs being in an immature or semi-mature state force Treg generation (37). However, Banerjee and colleagues proved that human myeloid DCs matured with a cocktail of inflammatory cytokines had the highest capability to induce Treg cells. Even myeloid DCs matured in the presence of LPS induced Treg cells to a greater extent than immature DCs (38). Our own data revealed that LPS-stimulated MDC1 supported the generation of Treg cells *in vitro*. Hence, we propose that during early stages of normal pregnancy, activated MDC1 enhance Treg cells in the periphery whereas underactivated MDC1 may lack the capacity for an adequate Treg induction. Our findings provide another explanation for the observed early Treg increment that is fundamental for fetal tolerance induction as reported earlier (39, 40) and again confirmed in our current study. Interestingly, MDC2 showed a completely different pattern when compared to MDC1 underlying the need to separately study the different myeloid PBDC subsets during pregnancy. Mature MDC2 were significantly augmented in miscarriage patients as compared to normal pregnant women. We assume that MDC1 and MDC2 overtake different functions at different gestational ages and that an over or underrepresentation of each subset at other pregnancy time points may be deleterious for pregnancy. According to a study by Hayashi and colleagues, MDC1 can be seen as TH1-promoting cells while MDC2 are rather TH2-promoting cells (41). As normal pregnancy begins with TH1-related immune responses (peri-implantation phase), changes to TH2-related immune responses (mid-gestation) and finally shifts back to TH1-related immune responses at the end of pregnancy (labor induction) (42), our data suggest a critical role for MDC1 during the first trimester and for MDC2 during the second trimester. Here, at specific pregnancy periods both myeloid PBDC subsets may keep the delicate balance between pro- and anti-inflammatory immune reactions that are required for maintenance of a normal pregnancy.

After confirming pregnancy-associated alterations in the PBDCs and particularly in the MDC1 subset, we wondered whether placental-derived factors might provoke these changes. Previous studies proposed various effects of placental factors on DCs from different origins (26, 43–46). Our own data revealed no remarkable effect of soluble factors derived from normal primary trophoblast cells on all PBDC subsets. In contrast, supernatants from miscarriage trophoblast cells and co-culture of PBDCs with the trophoblast cell lines JEG-3 and SWAN-71 profoundly affected the number of mature PDC and MDC1. Among the molecules suggested to participate in placenta-driven PBDC modulation are: HLA-G, TGF-β, and indoleamine-2,3 dioxygenase (46) as well as pregnancy hormones. The latter ones, due to their autocrine and paracrine modes of action, can affect resident cells at the fetal-maternal interface and distant cells in the periphery (47, 48). Segerer and colleagues confirmed an inhibitory effect of hCG on DC maturation while estrogen and progesterone did not have any impact (32). Interestingly, Della Bella and colleagues, although not concentrating on hCG-mediated effects, suggested this hormone to be involved in the incomplete activation of PBDC during human pregnancy (19). In our current study, the presence of uhCG impaired the maturation of MDC1 and affected the frequency of mature PDC while rhCG did not. This only partly reflects our previous results where we found an inhibitory effect of hCG on the MDC1 subset that was mainly mediated by rhCG. However, in this study, we did not separately examine each PBDC subset and interdependencies between PDC, MDC1 and MDC2 could therefore not be excluded (31). A limiting factor in the present study was the purity of the isolated MDC2 that could be achieved by magnetic cell isolation. According to the manufacturer's instructions in the manual of this specific MDC2 isolation kit, some MDC1, PDC and monocytes are co-enriched with MDC2. Thus, we cannot completely exclude an influence of these "contaminating" immune cell populations on a potential hCG-mediated effect on MDC2 and conclusions derived from our MDC2 data have to be considered with caution.

In contrast to our data, Yoshimura and colleagues exhibited a stimulatory effect of hCG on myeloid and plasmacytoid PBDCs including the maturation, the cytokine secretion and the T cell stimulatory capacity (20). This shows that data referring to an hCG-mediated function on PBDCs during pregnancy are still inconsistent and further studies are needed to finally clarify this issue. Particularly, the PBDC characterization strategy has to be standardized to make studies comparable. Moreover, equivalent hCG preparations should be applied or

at least a clear definition of the preparation used in each study should be provided. Our own investigations let assume that the immunological effects of rhCG or uhCG differ in some aspects and we suggest that this might be due to the different composition of both preparations. While rhCG preparations only contain intact molecules of regular hCG, uhCG preparations comprise intact and nicked variants of all hCG isoforms that can be physiologically found during pregnancy. These hCG isoforms have been shown to possess distinct functions. Regular hCG primarily promotes *corpus luteal* progesterone production, induces cytotrophoblast differentiation and contributes to uterine angiogenesis whereas free-β hCG exerts growth-promoting activity and blocks apoptosis (49). H-hCG, together with regular hCG promotes angiogenesis of the uterine vasculature and represents a key factor for trophoblast invasion (50). Notably, H-hCG was reported to activate the TGF-β receptor (51) implicating a potential pathway through which this isoform may regulate immune cell activity. Although our analysis of the different plasma hCG isoforms revealed that miscarriage patients suffer from reduced levels of regular hCG; free-β hCG and H-hCG as well as from altered MDC1 and MDC2 frequencies, based on our *in vitro* assays we do not believe that hCG is causative for the PBDC changes associated with miscarriage. However, we suggest that hCG has the potential to affect the phenotype of the distinct PBDC subsets.

In conclusion, our findings suggest that during normal pregnancy progression PBDC subsets are differentially regulated depending on gestational age. Miscarriage seems to be associated with dysregulations in the frequencies and functionality of the myeloid PBDC subsets as well as with disturbances in Treg frequencies. In the light of our results, we further propose the existence of an interdependency between MDC1 and Treg cells during early pregnancy. hCG, although shown to correlate negatively with MDC2 frequencies and to impair MDC1 maturation, does not seem to be a key regulator of PBDC alterations during pregnancy.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the manuscript/**Supplementary Files**.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of ethic guidelines defined by the ethics board at the University of Magdeburg with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the ethics board at the University of Magdeburg (study 28/08).

AUTHOR CONTRIBUTIONS

SE and KS performed and analyzed experiments. AS designed and supervised experiments. SE and AS prepared figures, interpreted data, and wrote the manuscript. S-DC, RN, LL, AO, and FF provided human sample material. SL and AZ critically revised the manuscript and provided financial support.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02440/full#supplementary-material

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

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