ADAPTIVE IMMUNITY IN PREGNANCY

EDITED BY: Marie-Pierre Piccinni, Shigeru Saito and Sarah Anne Robertson
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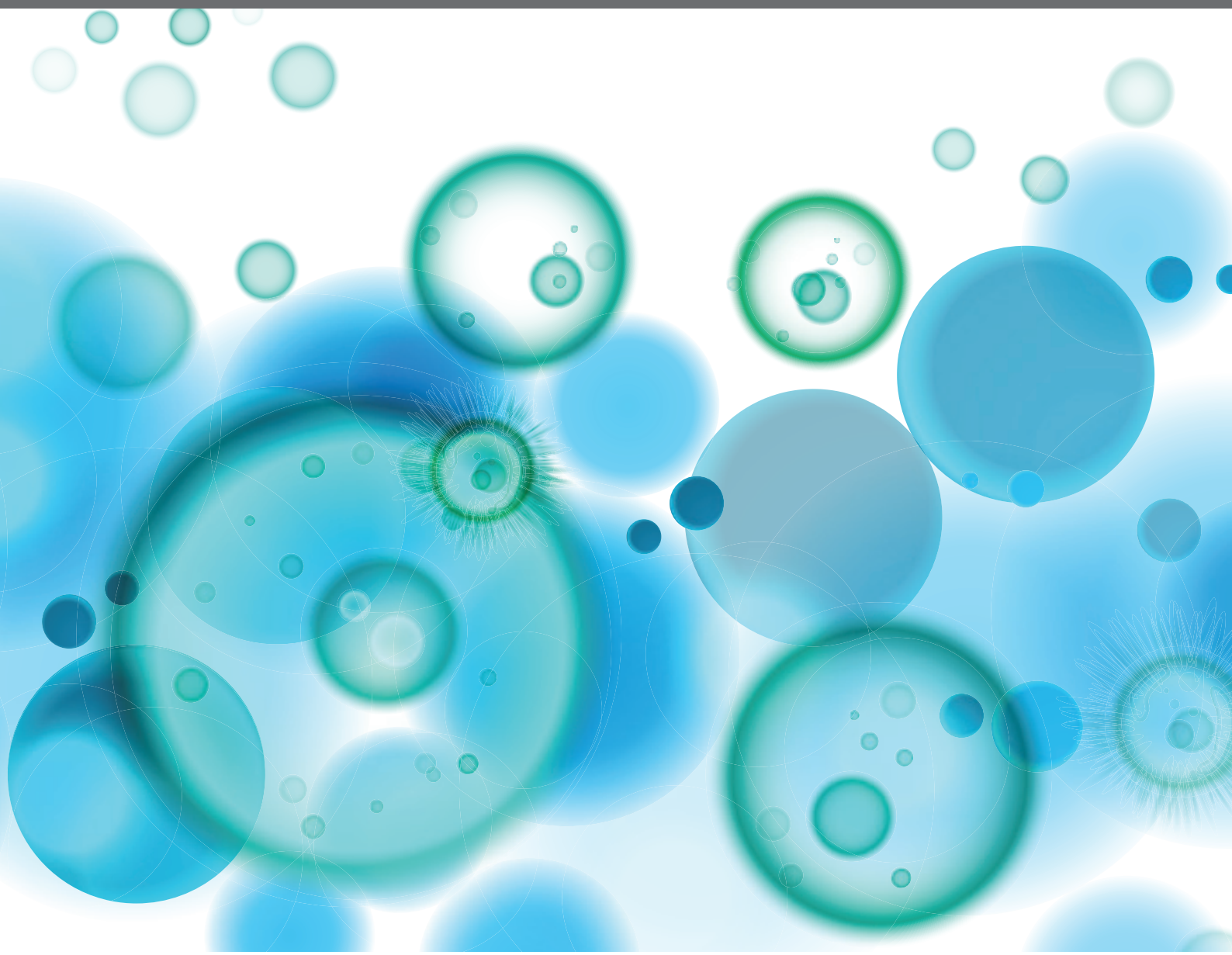

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ADAPTIVE IMMUNITY IN PREGNANCY

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EDITORIAL
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Editorial: Adaptive Immunity in Pregnancy

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Keywords: T helper cells, T reg cells, placenta, spontaneous abortion, pregnancy, maternal-fetal immune tolerance

Editorial on the Research Topic

Adaptive Immunity in Pregnancy

One of the most remarkable features of reproductive biology is the fact that a healthy woman can successfully carry her genetically disparate conceptus to full term, without immune rejection.

The juxtaposition of the placenta and decidua creates what is referred to as the 'fetal-maternal interface', where placental trophoblasts of fetal origin and maternal uterine lymphocytes come into close contact. Due to the presence of paternal class I HLA-C molecules on trophoblasts, the conceptus can be considered to resemble a semi-allograft. Conceptus-derived and placental-derived antigens act to both prime maternal T cells and render the conceptus potentially susceptible to inflammatory effector activity or T cell-mediated attack. After presentation of paternal alloantigens by maternal antigen presenting cells (APCs), the maternal alloantigen-specific T cells proliferate and secrete cytokines, responsible for the activation of allograft rejection or tolerance mechanisms, respectively promoting pregnancy failure or fetal survival.

Therefore, the quality and strength of the adaptive immune response is critical to healthy pregnancy. There is accumulating information that imbalance in the numbers, phenotypes and functional activity of T cell subsets can adversely impact fertility and pregnancy health. Predominant Th1, Th17 and Th17/Th1 immunity and decreased Th2, Th17/Th2 and Treg cells are associated with recurrent pregnancy loss (RPL) of fetuses with normal fetal chromosomal content. Various subsets of T cells are essential for pregnancy tolerance and interact in networks with innate immune cells to counteract inflammation and promote robust placental development. In fact, immune cells that populate the decidua are specialized not only to minimize events that might evoke conceptus attack, but also to foster placental development and function and to combat infections during pregnancy.

In addition, T cells are commonly perturbed in late gestation disorders including preeclampsia, fetal growth restriction and spontaneous preterm birth. There is some evidence that T cell disturbances precede the onset of symptoms and contribute to disease pathophysiology through events around the time of implantation and early placental development.

In this Research Topic we welcomed six original articles and four review articles, which discuss the role of novel immunosuppressive cells and molecules regulating fetal tolerance and development.

During pregnancy, sex steroid hormones like estrogen, progesterone, hCG but also a progesterone-induced mediator, the progesterone-induced blocking factor (PIBF), which conveys some of the immunological effects of progesterone, suppress effector immune activation resulting in successful pregnancy. Csabai et al. reports that the implantation rate is decreased in mice treated with anti-PIBF antibody. In these anti-PIBF-treated mice, NK activity, IL-12A mRNA expression in

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$CD8^+$ T cells and Th1 differentiation are increased, whereas the expression of mRNA for IL-4 (a Th2-type cytokine) is decreased in $CD4^+$ T cells (**Figure 1**). Thus, PIBF plays an important role in implantation by upregulating a Th2-type and downregulating Th1-type immune responses.

As well as hormones, other molecules including the prostaglandins (PG) can also regulate immune cells in pregnancy. In particular Andrade et al. show that PGE_2 also contributes to immune tolerance, by inhibiting platelet aggregation and IL-5 and IL-13 production by innate lymphoid cells (ILCs), and by suppressing neutrophil, NK cell and monocyte effector functions (**Figure 1**).

The role of Treg cells in pregnancy is extensively developed in the Research Topic. Huang et al. reviewed the role of Treg in normal pregnancy, in implantation failure, miscarriage, endometriosis and preeclampsia. Krop et al. described not only the role of well-known $Foxp3^+$ Treg cells in pregnancy, and also importantly the role of the lesser-known $Foxp3^-$ Treg cells, which include HLA-G Treg cells, Tr1 cells that secrete IL-10 and TGF-β, Th3 cells that secrete TGF-β, IL-10, and IL-4, $CD8^+$ Treg cells, NO-Treg cells, $TIGIT^+$ Treg cells, and $V\delta1^+\gamma\delta T$ cells (**Figure 1**). However, the relationship between these $Foxp3^-$ Treg cells and pregnancy disorders remains to be clarified.

Morita et al. examine $CD8^+$ T cells that are less well studied in pregnancy, in particular clonally expanded $CD8^+$ T cells, using single cell analysis of T cell receptor β (TCRβ) sequences. Clonally expanded $CD8^+$ T cells may be a surrogate marker for fetal/paternal antigen-specific $CD8^+$ T cells. The authors show that clonal $CD8^+$ T cells are more abundant in effector memory $CD8^+$ T cells ($CD8^+$EM) and that there are more $CD8^+$ EM cells in the decidua than in the peripheral blood. The clonal $CD8^+$ T cells increase from early to late pregnancy, and PD-1 expression, which suppresses cytotoxic activity, is low on clonal $CD8^+$ T cells in early pregnancy but high in late pregnancy. The clonal $PD1^-$ $CD8^+$ T cells increase in miscarriages with normal fetal

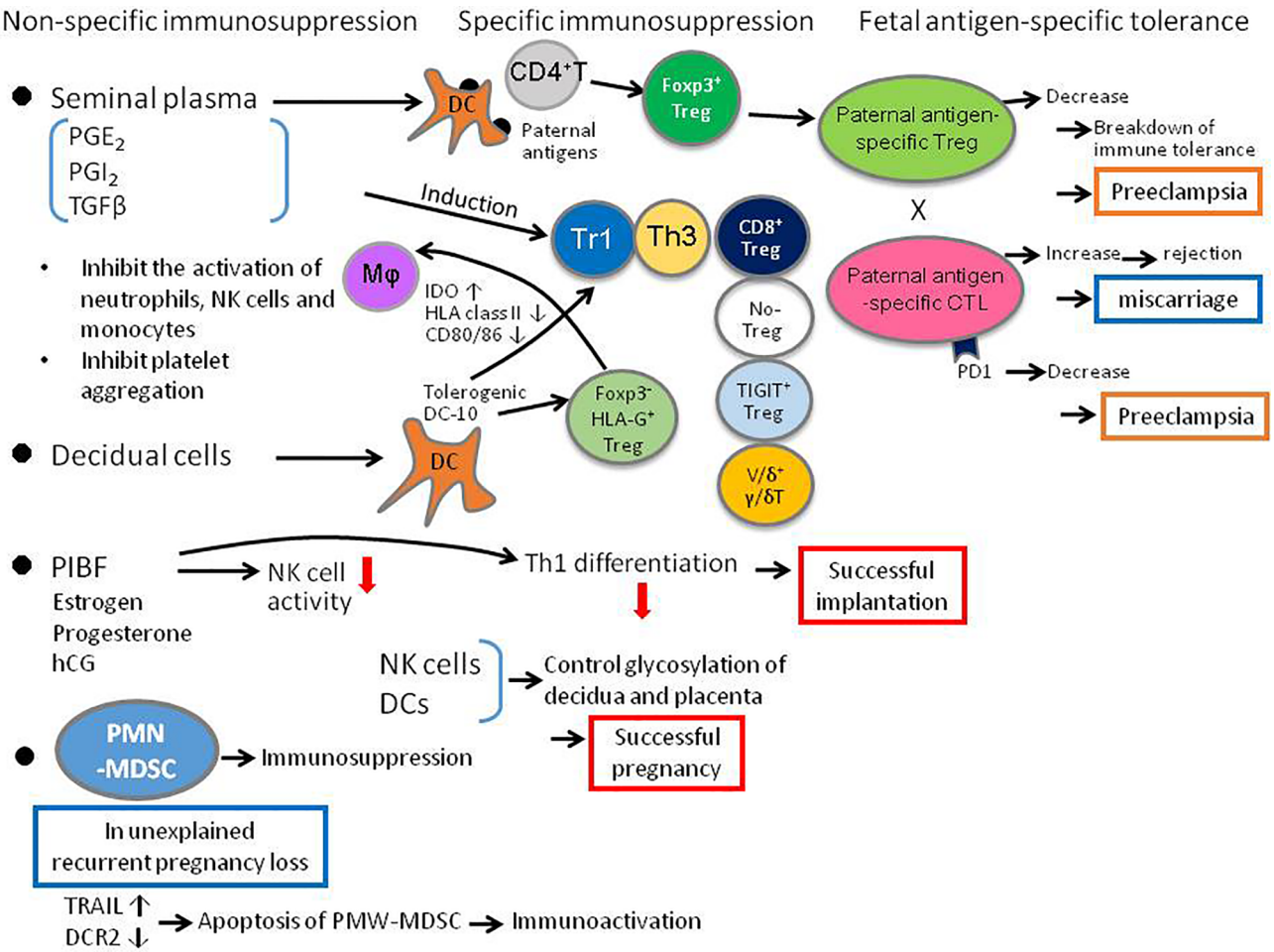

FIGURE 1 | Diagrammatic summary of critical elements of the adaptive immune response to pregnancy, that determine the balance between T cell tolerance and T effector function, and influence the outcome of pregnancy and infant health. DC, dendritic cell; IDO, indoleamine-dioxygenase; MΦ, macrophage, PD1, programmed death 1; PIBF, pregnancy-induced blocking factor; PMN-MDSC, polymorphonuclear myeloid-derived suppressor cell; PGE, prostaglandin E2; PGI, prostaglandin I: Tr1, T regulatory 1; Th, T helper; TIGIT, T cell immunoglobulin and ITIM domain; Treg, regulatory T cell; uNK, uterine natural killer cell.

karyotype (**Figure 1**). The percentage of clonal $CD8^+$ T cells is not different in preeclampsia compared to normal pregnancy, but PD-1 expression is significantly decreased, suggesting an increased cytotoxic activity against fetal antigens in preeclampsia.

Immune cells can not only act by inducing trophoblast tolerance but can also affect the structure of trophoblast by altering the glycan chains of trophoblast. Dendritic cell activity is particularly important, and may be amplified if NK cells are removed as demonstrated by Borowski et al. Thus, immune cells can influence the placental glycade and could impact placental and fetal development (**Figure 1**). The relationship between the alteration of trophoblast glycan chains and immune cells in miscarriage and preeclampsia is an interesting topic for future clarification.

Non-immune cells can also regulate immune cells responsible for immune tolerance in pregnancy. Gori et al. showed that endometrial decidualization increases tolerogenic dendritic cells named DC-10 cells, which secrete IL-10 and induce different regulatory T cells (Treg cells), including HLA-G^+ Treg cells, Tr1 cells and Th3 cells (**Figure 1**).

Recently, polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs) have been found to increase in the pregnant uterus and play an important role in maintaining pregnancy. Li et al. show that the number of decidual PMN-MDSCs decrease in patients with unexplained recurrent pregnancy loss. PMN-MDSC apoptosis, increased by elevated TRAIL and decreased by DcR2, could explain the decreased number of PMN-MDSCs in unexplained recurrent pregnancy loss (**Figure 1**). The interaction between PMN-MDSCs and immune cells such as Treg cells, need to be investigated.

Van der Zwan et al. used mass cytometry to analyze lymphocyte subpopulations in the decidua and peripheral blood. Such analysis could be helpful for the classification of immunocompetent cells and to clarify the role of each of these cells in the decidua. This promising technology, which may serve as a foundation for further identification of immune subsets in healthy and complicated pregnancy, is set to offer further advances in the future.

Finally, it is important to recognize that the maternal adaptive immune response to pregnancy has consequences not only for pregnancy outcome, but also for the health of the child after birth. Albrecht et al. review emerging studies showing that cellular immunity is transferred from mother to child not only through IgG transfer during pregnancy, but also by maternal cellular immunity transmitted to the child through lactation after birth. This article introduces the interesting possibility of transferring immunity to the fetus by vaccination during pregnancy.

In summary, this collection of papers provide a snapshot of the state of this field and provide new insight on the mechanisms and significance of the adaptive immune response to maternal and infant health. Collectively the work highlights the imperative to further delineate the underlying mechanisms by which maternal tolerance is generated and mediated, so that interventions to protect against immune-based pregnancy disorders arising from compromise maternal tolerance can be advanced.

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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ORIGINAL RESEARCH
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Altered Immune Response and Implantation Failure in Progesterone-Induced Blocking Factor-Deficient Mice

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Earlier data suggest that progesterone-induced blocking factor (PIBF) is involved in implantation. The present study therefore aims to investigate the consequences of functional PIBF deficiency during the peri-implantation period. CD1 female mice were injected intraperitoneally with 2 μg anti-PIBF monoclonal antibody on days 1.5 and 4.5 of pregnancy. The number of implantation sites and resorption rates were recorded on day 10.5. PIBF+ decidual NK cells and B cells were detected by immunohistochemistry or immunofluorescence. Decidual and peripheral NK activity was assessed by flow cytometry. A prime PCR array was used for determining the differential expression of genes involved in lymphocyte activation and Th1 or Th2 differentiation in CD4+ and CD8+ spleen cells from pregnant anti-PIBF-treated and control mice. Anti-PIBF treatment in the peri-implantation period resulted in impaired implantation and increased resorption rates in later pregnancy. The number of PIBF+ decidual NK cells decreased, while both decidual and peripheral NK activity increased in the anti-PIBF-treated mice. B cells were absent from the resorbed deciduas of anti-PIBF-treated mice. The genes implicated in T cell activation were significantly downregulated in CD4+ and increased in CD8+ of the anti-PIBF-treated animals. The gene for IL-4 was significantly downregulated in CD4+ cells while that of IL-12A was upregulated in CD8+ cells of anti-PIBF-treated animals. These data suggest that the lack of PIBF results in an impaired T cell activation, together with Th1 differentiation and increased NK activity, resulting in implantation failure.

Keywords: PIBF, decidual NK cells, T cell activation, B cells, implantation

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INTRODUCTION

The success of embryo implantation depends on embryo quality as well as on the receptivity of the maternal endometrium. The process starts with the attachment of the embryo to the endometrial epithelium (1–6), followed by invasion to the decidua. Progesterone plays a central role in this process (4, 6) via the nuclear progesterone receptor (PR) isoforms, PRA and PRB (7, 8). Studies on PR knockout mice revealed that PRA is required for endometrial receptivity and decidualization (9), and consequently, PRA-deficient mice are infertile (10, 11).

The progesterone-induced blocking factor (PIBF) is a progesterone-induced mediator which conveys some of the immunological effects of progesterone. The *Pibf1* gene contains a progesterone response element (12), which is activated following the engagement of PRA in the mouse uterus (13).

Earlier data suggest that PIBF is required for the establishment and maintenance of pregnancy, both in humans and mice. In the sera of pregnant women, PIBF concentrations increase throughout gestation and drop before labor (14). During spontaneous miscarriage or preterm delivery, serum PIBF concentrations fall below the normal levels (15). Anti-PIBF treatment or anti-progesterone treatment of pregnant mice results in increased resorption rates, together with an inversion of the Th1/Th2 cytokine balance (16). The latter is due to the fact that PIBF induces an increased synthesis of Th2 cytokines both *in vitro* (17) and *in vivo* (18). Recent data show that PIBF plays a role in implantation in mice (13).

The decidual transformation of endometrial stromal cells is a prerequisite for a successful implantation. Ablation of PRA but not PRB expression in mice results in a uterine phenotype similar to PRKO, indicating that PRA is the major isoform involved in the regulation of uterine receptivity and decidualization in the mouse (19). It is important to point out that, in humans, PRB is also involved in decidualization (20).

In our hands, during a 6-day culture, PIBF induced the decidual transformation of mouse endometrial stromal cells (13). Furthermore, in the mouse endometrium, PIBF expression significantly increased during the implantation window (13).

The immunological effects of PIBF play an important role in establishing a favorable immunological milieu for the developing fetus.

In spite of the presence of perforin and granzyme in their cytoplasmic granules, the decidual NK cells are weakly cytotoxic. High decidual NK activity might damage the fetus and result in a failed pregnancy. PIBF inhibits the degranulation of NK cells (21). Recently we demonstrated a high number of mouse decidual NK cells that contained PIBF in their cytoplasmic granules, suggesting that the local presence of PIBF might be a factor in the low decidual NK activity (22).

In the present study, we aimed to investigate the consequences of anti-PIBF treatment of pregnant mice during the peri-implantation period on reproductive performance as well as the underlying mechanisms.

MATERIALS AND METHODS

Treatment of Mice

Eight- to 12-week-old CD1 female mice (Charles River, Germany) were caged overnight with CD1 males in an environment controlled for temperature, humidity, and light. Sighting of the vaginal plug was considered as 0.5 day of pregnancy.

Females were injected intraperitoneally with 2 μg of anti-PIBF monoclonal antibody (14) on days 1.5 and 4.5 of pregnancy. The control mice were injected with 100 μl of PBS, among the same conditions. On day 10.5 of pregnancy, the mice were sacrificed, the number of implantation sites as well as resorption rates was recorded, and spleens and deciduas were removed for lymphocyte isolation.

All procedures were approved by the Animal Care Committee of the University of Pecs.

Isolation of Decidual and Spleen Lymphocytes

Isolated mouse deciduas were minced with scissors and incubated for 30 min with 10 ml (1 mg/ml) of collagenase (collagenase from *Clostridium histolyticum*, type IV, Sigma-Aldrich, USA). The fragments were then passed through a 70-μm mesh and washed with RPMI1640 (Gibco, Life technologies, Scotland).

The pellet was resuspended in 10 ml of fetal calf serum (FCS)-free RPMI and filtered on a 40 μm filter. The cell count was adjusted to 1×10^6/ml in RPMI1640 (Gibco, Life technologies, Scotland) +10% FCS (Gibco, Life Technologies, Scotland) + 1% penicillin/streptomycin (Gibco, Life Technologies, Scotland).

Spleen cells were isolated by passing the spleen through a 100-μm stainless steel mesh and centrifuging for 10 min at 1,000 rpm. The pellet was resuspended in 10 ml RPMI1640, filtered on a 70-μm mesh and further on a 40-μm mesh, washed, and resuspended in RPMI1640. The lymphocytes were isolated on Ficoll-Paque gradient, washed, and resuspended in RPMI1640 + 10% FCS + 1% penicillin/streptomycin.

Immunohistochemistry

The implantation sites were isolated on day 10.5 of pregnancy, fixed in 6% of buffered formalin, and then embedded in paraffin. Five-micrometer paraffin sections were deparaffinized, rehydrated, and revealed with DAKO Target Retrieval Solution (S1699, Dako, Denmark) at pH 6.0 in a microwave oven. Endogenous peroxidase activity was inhibited with 3% H_2O_2, and non-specific antibody binding was blocked with 3% BSA.

The slides were than reacted with 1:25 diluted biotinylated monoclonal anti-PIBF antibody produced in our laboratory (14) or biotinylated mouse IgG2a either for 1 h at room temperature or overnight at 4°C. After incubation, the slides were washed for 3–5 min and reacted with 1:100 diluted streptavidin-horseradish-peroxidase (GE Healthcare, Little Chalfont, United Kingdom) for 30 min in a humidified chamber. The reaction was developed with diamino-benzidine (DAKO, Glostrup, Denmark). The nuclei were counterstained with hematoxylin (DAKO, Glostrup, Denmark) for 3 min, and the slides were mounted.

Fluorescent Staining

For visualizing of the B cells, the sections were reacted overnight at 4°C with 1:30 diluted rat anti-mouse B220 IgG conjugated with Alexa Fluor 647. The antibody was produced at the Department of Immunology and Biotechnology, University of Pécs, and was provided by Dr. Peter Balogh. The anti-B220 IgG was purified from the supernatant of rat hybridoma RA3-6B2 (obtained from ATCC) using Protein G affinity chromatography. The purified antibody was dialyzed into 0.1 M $NaHCO_3$ buffer and conjugated with Alexa Fluor 647 NHS dye (ThermoFisher Scientific) as recommended by the vendor. The

conjugated immunoglobulin was separated using Sephadex-G25 size exclusion chromatography.

For identifying of PIBF-positive decidual B cells, the sections were reacted overnight at 4°C with 1:25 diluted FITC-conjugated anti-mouse PIBF antibody, together with the 1:30 diluted Alexa647-conjugated anti-mouse B220. The nuclei were stained with Hoechst33342 (Calbiochem, San Diego, CA, USA) for 5 min, washed, and then mounted with Vectashield mounting medium (Vector Laboratories, Peterborough, United Kingdom) and examined with an Olympus FV-1000 laser scanning confocal system.

NK Cytotoxicity Test

A flow cytometric assay was used for the determination of the cytotoxic activity of peripheral and decidual natural killer cells from the control and the anti-PIBF-treated pregnant mice. The assay is based on the quantitative and the qualitative flow cytometric analysis of cell damage on a single-cell level. The mouse lymphoma cell line YAC-1 was used as the target cell population. The target cells were pre-stained with PKH-67 (PKH67 Green Fluorescent Cell Linker Midi Kit for General Cell Membrane Labeling, Sigma- Aldrich, USA), a lipophilic dye that stably integrates into the cell membrane without disturbing its surface marker expression. It, thus, permits the distinction between target and effector cells. Freshly isolated peripheral and decidual lymphocytes from pregnant mice served as the effector cells. The target cells and the lymphocytes, at a ratio of 1:12.5, were centrifuged and incubated in RPMI1640 containing 10% FCS and 1% penicillin/streptomycin medium for 4 h at 37°C and in 5% CO_2. After incubation, the cell mixture was centrifuged and stained with propidium iodide [PI, 50 μg/ml (Sigma-Aldrich, USA)]. PI staining allows the discrimination between death and living target cells. Data analysis is performed first by gating on PKH-67-positive target cells, followed by the analysis of the PI-positive subpopulation killed (gating strategy is shown in **Figure 1**). The percentage of cytotoxicity in the PKH-67-gated cell population was calculated by subtracting unspecific PI+ positive target cells (spontaneous cell death), measured in appropriate controls without effector cells from the experimental samples. Flow cytometric analysis was performed on a FACS Calibur flow cytometer (BD Immunocytometry Systems, Erembodegen, Belgium) equipped with the CellQuest software program (BD Biosciences, San Diego, CA, USA) for data acquisition and analysis.

Lymphocyte Activation and Th1 and Th2 Cell Differentiation

A prime PCR array from Bio-Rad was used for determining the markers for T cell activation and Th1 or Th2 differentiation in separated CD4+ and CD8+ spleen cells from anti-PIBF-treated and control pregnant mice. The mice were sacrificed on day 10.5 of pregnancy. The spleens were minced with scissors and passed through a 70-μm cell strainer. The CD4+ and CD8+ T cells were separated from splenic single-cell suspension by magnetic separation with the Mini-MACS system (Miltenyi Biotec Biotechnology Company, USA). Mouse Naive CD8a+ T Cell Isolation Kit (130-096-543) and mouse CD4+ T Cell Isolation Kit (130-104-454) were applied for negative selection, according to the manufacturer's instructions. The collected cells were washed, and the cell count was adjusted to 5×10^6. One hundred thousand separated cells were fluorescently stained with the anti-mouse CD4 or anti-mouse CD8 monoclonal antibodies for checking of the separation efficiency. Cell debris and dead cells were excluded from the analysis based on scatter signals and propidium iodide fluorescence. For the stabilization of RNA, separated CD4+ or CD8+ splenocytes were stored frozen in RNAlater®-ICE solution. Total RNA was extracted from the cells with the Qiagen RNeasy Mini Kit (Cat. No. 74104) according to the supplier's protocol. The RNA content of samples was measured with Qubit 4 Fluorometer using the Qubit RNA HS Assay Kit. The expression of 41 genes was determined using a Bio-Rad prime PCR array (Immune response-Th1 and Th2 cell differentiation M384; Bio-Rad Laboratories, Inc., California, USA). Quantitative PCR reactions were carried out on an ABI 7900 real-time PCR instrument according to the manufacturer's instructions.

Statistical Analysis

Data were analyzed by the Mann–Whitney *U* test and Student's *t* test. $P \leq 0.05$ was considered as significant.

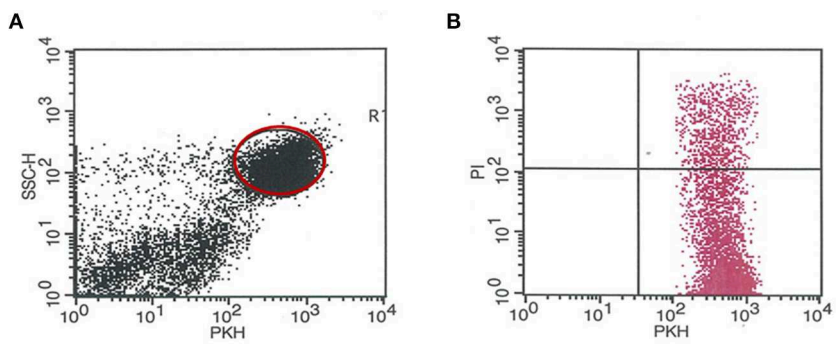

FIGURE 1 | Gating strategy for determining the number of the PKH-67-positive target cells killed (PI+). **(A)** Representative dot plot showing the PKH67 staining of YAC target cells. PKH67-positive cells were gated and were used for further analysis. **(B)** Representative dot plot shows the red fluorescence of PKH67+ YAC cells after PI staining. The percentage of PI+/PKH67+ YAC cells was defined as apoptotic cells.

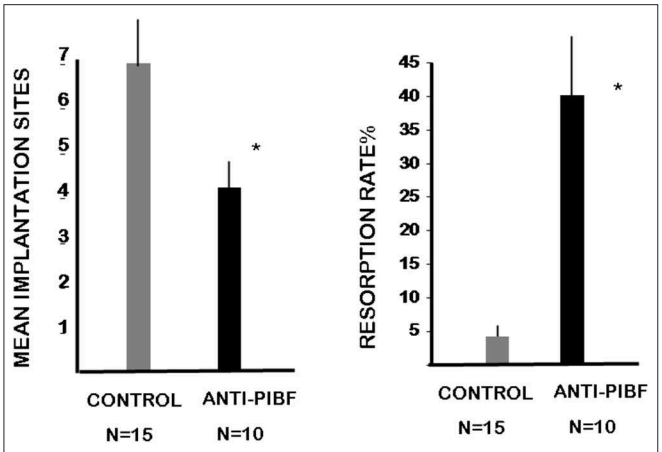

FIGURE 2 | Anti-PIBF treatment of pregnant mice in the peri-implantation period results in decreased implantation and increased resorption rates. CD1 mice were injected with anti-PIBF monoclonal antibody at days 1.5 and 4.5 of pregnancy. The controls were treated with PBS, among the same conditions. The number of implantation sites (1st panel) and resorption rates (2nd panel) were recorded on day 10.5. The implantation sites were significantly lower, while the resorption rates significantly increased in the anti-PIBF-treated mice. The columns represent the mean ± SEM of the results from 10 (anti-PIBF-treated) and 15 (control) mice. *$P < 0.05$.

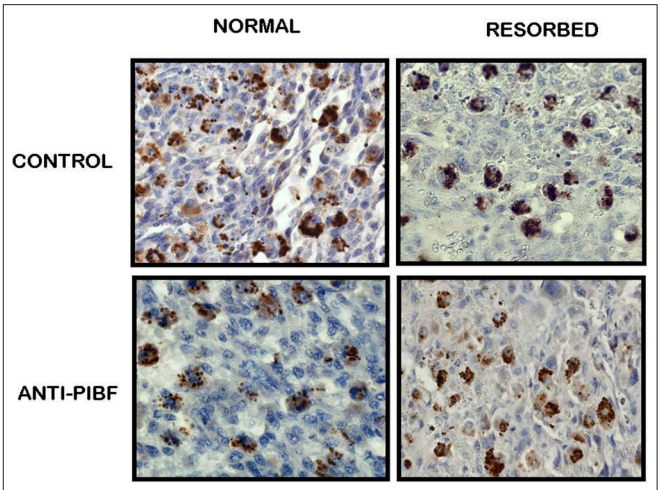

FIGURE 3 | Immunohistochemical analysis of PIBF+ NK cells in normal and resorbed deciduae from anti-PIBF-treated and control mice on day 10.5 of pregnancy (×400).

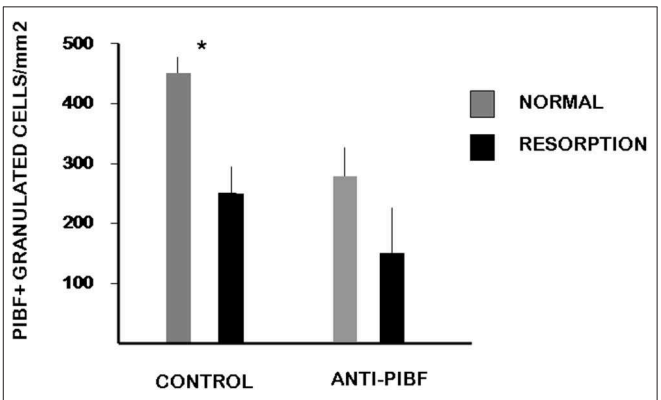

FIGURE 4 | The number of PIBF+ NK cells in deciduas from anti-PIBF-treated and control mice on day 10.5 of pregnancy. Compared to the normal deciduae from the untreated controls, the number of PIBF+ NK cells is significantly lower not only in the deciduae from both the normal and the resorbed fetuses from anti-PIBF-treated animals but also in those of spontaneously resorbed fetuses from control mice. The bars represent the mean ± SEM of 10 independent determinations. *$P < 0.05$.

RESULTS

Anti-PIBF Treatment in the Peri-Implantation Period Results in Impaired Implantation and Increased Resorption Rates in Later Pregnancy

The mice were treated with anti-PIBF antibody at days 1.5 and 4.5 of pregnancy to render them functionally PIBF deficient during the implantation window. The mice were sacrificed at day 10.5. This enabled us to record not only the implantation sites but also the resorption rate among the implanted embryos. While the average number of implanted embryos was 6.5 in the controls, in females treated with anti- PIBF antibody in the peri-implantation period, the mean implantation sites decreased to four (**Figure 2**). The unusually low 2% resorption rate in the control group increased to 40% in the functionally PIBF-deficient mice (**Figure 2**).

PIBF+ Large Granulated Cells Are Depleted From the Deciduas of Anti-PIBF-Treated Mice

In an earlier study, we demonstrated a high number of large granulated cells—with a strong PIBF reactivity in the cytoplasmic granules—in the deciduae of day 12.5 pregnant mice. These cells were identified as members of the PAS+ DBA+ uterine NK cell population. PIBF co-localized with perforin in the cytoplasmic granules of the cells (22).

In the present study, we found a significantly decreased number of PIBF+ NK cells in the day 10.5 deciduae of resorbed embryos from anti-PIBF-treated mice compared to normal deciduae from untreated mice (**Figures 3**, **4**). The number of PIBF+ granulated cells was also significantly lower in the deciduae of normal embryos from the anti-PIBF-treated mice and in those of spontaneously resorbed embryos from untreated control mice (**Figures 3**, **4**) than in the normal decidua of the untreated mice. These data suggest that the decreased number of PIBF+ decidual lymphocytes was associated with resorption rather than with the lack of functional PIBF.

Cytotoxic Activity of Decidual and Peripheral NK Cells From Anti-PIBF-Treated and Control Mice

We determined the cytotoxic activity of decidual lymphocytes and of spleen cells from anti-PIBF-treated and control mice using a flow cytometric method. Both the decidual and the

peripheral NK activity of the anti-PIBF-treated mice were significantly ($P < 0.05$) increased compared to those of the controls (**Figure 5**).

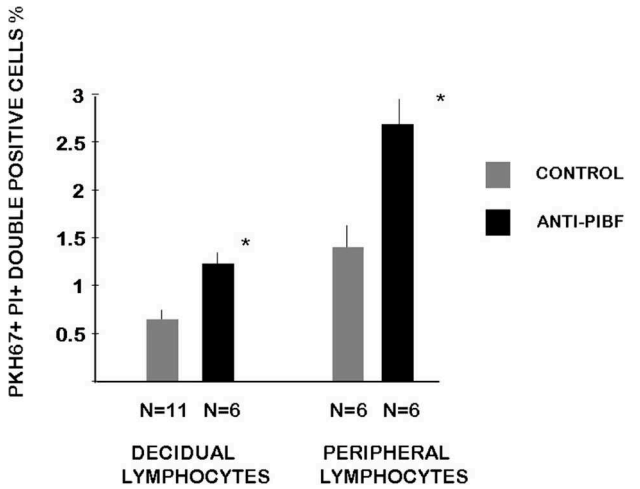

FIGURE 5 | Cytotoxic activity of decidual and peripheral lymphocytes from anti-PIBF-treated and control mice on day 10.5 of pregnancy. The cytotoxic activity of peripheral and decidual NK cells from control and anti-PIBF-treated mice was determined by flow cytometric analysis of target cell damage on a single-cell level. The target cells were labeled with PKH-67 and stained with propidium iodide after 4 h of incubation with the lymphocytes to distinguish apoptotic from non-apoptotic target cells. The bars represent the mean ± SEM of at least six experiments. *$P < 0.05$.

B Cells Are Depleted From the Deciduas of Anti-PIBF-Treated Embryos

The endometria of the control animals (**Figure 6A**) contained decidual NK cells (**Figure 6A**—a,c) and a discrete layer of B cells (**Figure 6A**—b,c) at the coriodecidual interface. While the NK cells were still present (**Figure 6B**—a,c), the B cells disappeared from the deciduas of the resorbed embryos from the anti-PIBF-treated mice (**Figure 6B**—b,c).

Functional PIBF Deficiency in the Peri-Implantation Period Results in Impaired CD4+ T Cell Activation and Th1 Type Differentiation

Peripheral Th cell subsets from anti-PIBF-treated and control mice were tested for the differential expression of 48 genes using a prime PCR array. Twelve of these showed a significantly higher or lower expression in the lymphocytes of anti-PIBF-treated mice compared to the controls. When analyzing the results, the differentially expressed genes were assigned to the following groups: (1) genes involved in T cell activation, (2) those involved in Th1 differentiation, and (3) those involved in Th2 differentiation.

The genes implicated in T cell activation, e.g., members of the CD3 complex (CD 247, CS3D, CS3E, CS3G, and IL2RG), were significantly downregulated in the CD4+ spleen cells of anti-PIBF-treated mice but significantly increased in the CD8+ cells of the same animals (**Figure 7**). In the anti-PIBF-treated mice, the beta chain of the IL2R was downregulated in the

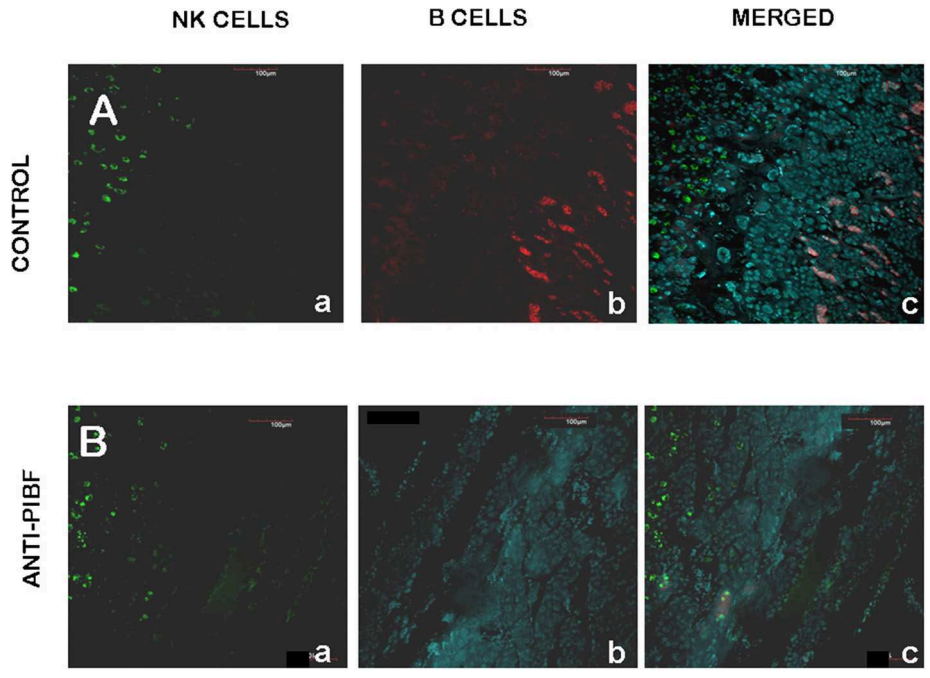

FIGURE 6 | Decidual B cells in control **(A)** and anti-PIBF-treated **(B)** mice. B cells were reacted with rat anti-mouse B220 IgG conjugated with Alexa Fluor 647 (red fluorescence), and NK cells were reacted with fluorescein-conjugated DBA lectin (green fluorescence). **(A)** Decidua of an untreated mouse. NK cells (a,c) are present in the decidua and B cells (b,c) are located at the choriodecidual interface. **(B)** Decidua of anti-PIBF-treated mouse. NK cells (a,c) are present, while B cells (b,c) are absent. (a) NK cells, (b) B cells, and (c) merged (×200).

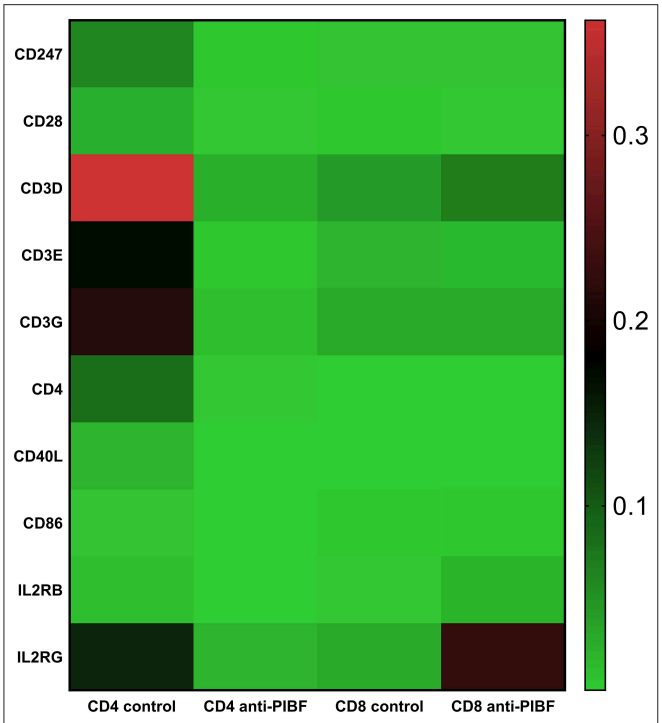

FIGURE 7 | Differential expression of genes implicated in T cell activation in splenic CD4+ and CD8+ T spleen cells of anti-PIBF-treated mice and controls. Heatmap of the T cell activation-related mRNA expression of genes in CD4+ and CD8+ splenocytes of anti-PIBF-treated and control mice. Clear separations are seen between the anti-PIBF-treated and control animals and also between the CD4+ and CD8+ cell types. Members of the CD3 complex and co-stimulatory molecules were downregulated in CD4+ cells and upregulated in CD8+ cells of anti-PIBF-treated mice. All of the results shown were significantly ($P < 0.05$) different from the values of the controls. The expression intensities were scaled on rows (genes) to Z scores to make them weigh equally in the clustering. The colors of the heatmap are mapped linearly to the Z scores (low expression in green and high expression in red).

CD4+ population, while in the CD8 population the alpha and the gamma chain of the IL2R and the IL2 increased. The genes of the co-stimulatory molecules were altered in a similar fashion. Upon anti-PIBF treatment, the genes for CD4, CD28, CD40L, and CD86 were downregulated in the CD4 and upregulated in the CD8 population (**Figure 7**). These data suggest that the absence of PIBF inhibits the activation of CD4+ cells and facilitates that of CD8+ T cells.

Among genes involved in the Th1/Th2 pathway, IL-4 was significantly downregulated in CD4+ cells of the anti-PIBF-treated mice. At the same time, IL-12 was upregulated in CD8+ cells of the anti-PIBF-treated animals (**Figure 8**).

DISCUSSION

Earlier we showed that PIBF induces the decidual transformation of mouse endometrial stromal cells. Furthermore, PIBF expression in the mouse endometrium is markedly increased during the implantation window (13). These data suggest that PIBF might play an active role in implantation. To confirm this hypothesis, we neutralized the biological activity of PIBF during the peri-implantation period in mice and investigated the consequences of functional PIBF deficiency at several levels.

The anti-PIBF treatment of pregnant mice at days 1.5 and 4.5 of pregnancy resulted in a significantly reduced number of the implantation sites, and the implantations that took place nevertheless must have been compromised as shown by the high resorption rates. In an earlier study, anti-PIBF treatment on day 8.5 of pregnancy increased the resorption rate to 40% (23). The present data show that when administered in the peri-implantation period, the anti-PIBF antibody also interferes with implantation.

Several cell types, e.g., the peripheral pregnancy lymphocytes (24), the embryo itself (25), the trophoblast (26), and the endometrial cells (13), produce PIBF. In confirmed clinical pregnancies, PIBF is detectable in the serum of IVF patients 14 days after embryo transfer (Hudic et al., manuscript in preparation). A single embryo cannot produce such a high amount of PIBF. It is more likely that the bulk of the PIBF is produced by the maternal side in response to the presence of the embryo.

We further investigated the underlying mechanisms of implantation failure and pregnancy loss in functionally PIBF-deficient mice.

Failed pregnancies are characterized by high peripheral NK activity, both in humans and in mice (27–35).

Progesterone decreases the NK activity of peripheral pregnancy lymphocytes in a concentration-dependent fashion (36), and RU 486 (a progesterone receptor antagonist) significantly augments the NK cell cytolytic activity *in vitro* (37).

Decidual NK cells constitute 60% of decidual lymphocytes (38). Is spite of the availability of perforin and granzyme in their cytotoxic granules (38), these cells have a very moderate cytotoxic potential (39, 40) but secrete angiogenic factors and cytokines (38). The dynamics of the appearance of uterine NK cells suggests that one of their functions might be the control of placentation.

The low cytotoxic activity of decidual NK cells might be due to the presence of PIBF in their cytoplasmic granules (22). PIBF blocks the upregulation of perforin expression in activated decidual lymphocytes and inhibits NK cell cytotoxicity by blocking granule exocytosis (21, 41). Bogdan et al. (22) demonstrated a high number of PIBF+ NK cells in the day 12.5 decidua of pregnant mice.

Here we show that anti-PIBF treatment during the peri-implantation period results in the reduced presence of PIBF+ NK cells in the day 10.5 decidua, together with significantly increased decidual and peripheral NK activity, compared to the controls.

Anti-PIBF treatment of mid-pregnant mice has been shown to boost both the peripheral NK activity and the resorption rates. Increased resorption rates in anti-PIBF-treated mice were corrected by simultaneous treatment of the mice with anti-NK antibodies (23), suggesting that PIBF prevents pregnancy loss in mice—at least partly—by blocking NK activity. Increased decidual NK activity owing to the loss of PIBF+ decidual NK cells

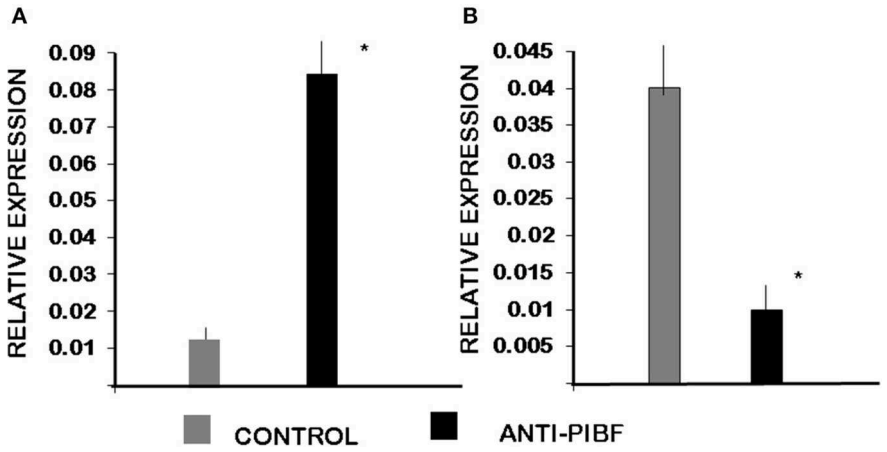

FIGURE 8 | Differential expression of genes involved in Th1/Th2 differentiation by spleen cells of control and anti-PIBF-treated mice. **(A)** IL12A mRNA is significantly upregulated in CD8+ cells of anti-PIBF-treated mice. **(B)** IL-4 mRNA is significantly downregulated in CD4+ cells of anti-PIBF-treated mice. The bars represent the mean ± SEM of four experiments. *$P < 0.05$.

could be one of the reasons for the increased resorption rates in the anti-PIBF treated mice.

B cells constitute a minor population among decidual lymphocytes, yet they might be important for the immunological balance of the decidua. A recent study showed that the IL-33-induced expression of PIBF1 by decidual B cells protects against preterm labor both in humans and in mice (42).

In the present study, we detected a distinct layer of B cells at the choriodecidual interface of control pregnant mice on day 10.5 of pregnancy. These cells were completely absent from the deciduae of mice that had been treated with anti-PIBF during the peri-implantation period. We could not detect PIBF in the B cells on day 10.5 of pregnancy; however, PIBF+ B cells were present in the late pregnancy deciduae of the control mice (data not shown).

Taken together, it is conceivable that anti-PIBF treatment—by depleting decidual B cells—will, at a later stage, put pregnancy at risk due to the lack of PIBF-positive B cells (42).

Finally, we performed a gene array on the spleen cells of anti-PIBF-treated and control mice in order to investigate whether the absence of functional PIBF had an effect on T cell activation and differentiation.

The T cell receptor is a complex of T cell receptor alpha and beta chains and the CD3 proteins. Activation of CD4+ T cells occurs through the simultaneous engagement of the T cell receptor and a co-stimulatory molecule on the T cell by the MHCII peptide and the co-stimulatory molecules on the APC. In the absence of co-stimulation, T cell receptor signaling results in anergy.

In addition to TCR alpha/beta, a whole set of cell surface receptors are also engaged by their ligands on APCs, which regulate Th differentiation. CD4 acts as a cellular adhesion molecule that binds MHC class II and stabilizes the interaction of T cells and APCs (43, 44). CD28 is a costimulatory receptor on T cells, which binds CD80 and CD86 on activated APCs (45). The TCR alpha/beta/CD3 complex provides the first signal and CD28 the second signal for T cell activation. Both signals are required for IL-2 production and T cell proliferation. CD40 ligand, expressed by activated T cells, binds to CD40 on APCs, initiating a T cell-mediated immune response (46).

In this study, we found that members of the T cell receptor CD3 complex were significantly downregulated on CD4+ T cells of anti-PIBF-treated mice, while CD3D and IL2R B and G were upregulated in CD8+ cells, suggesting that Th cell activation is severely inhibited in the anti-PIBF-treated pregnant mice.

IL-4 is the main cytokine driving Th2 cell differentiation. IL-4 is produced by various cell types, including mast cells, basophils, eosinophils, NK cells, activated CD4+ T cells, and differentiated Th2 cells (47).

Here we found that the gene for IL-4 was significantly downregulated in CD4+ cells, while that of IL-12A was upregulated in CD8+ cells of the anti-PIBF-treated mice.

There is now ample evidence that the recognition of paternal antigens by the maternal immune system is not simply harmless but absolutely necessary for the setting in of the mechanisms that adapt the immune response to tolerate the fetus (48). Following recognition of fetal antigens, the immune system becomes activated, and this will result in the establishment of regulatory mechanisms, e.g., a Th2 dominant immune response (49, 50).

Neutralizing PIBF in the peri-implantation period abolishes this mechanism right at the start. CD4+ T cell activation is disturbed, T cells differentiate in the Th1 direction, and as a result, implantation as well as ongoing pregnancies is compromised.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Care Committee of the University of Pecs.

AUTHOR CONTRIBUTIONS

TC performed most of the work. EP and AK did the prime pcr. EM was responsible for the flow cytometry. ZB helped with the animal experiments. This study was designed and supervised by JS-B.

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REVIEW
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Vertically Transferred Immunity in Neonates: Mothers, Mechanisms and Mediators

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Over the last years, an increasing number of outbreaks of vaccine-preventable infectious diseases has been reported. Besides elderly and immunocompromised individuals, newborns and small infants are most susceptible to infections, as their immune system is still immature. This vulnerability during infancy can be mitigated by the transplacental transfer of pathogen-specific antibodies and other mediators of immunity from mother to the fetus during pregnancy, followed postnatally by breast milk-derived immunity. Since this largely antibody-mediated passive immunity can prevent the newborn from infections, neonatal immunity depends strongly on the maternal concentration of respective specific antibodies during pregnancy. If titers are low or wane rapidly after birth, the protection transferred to the child may not be sufficient to prevent disease. Moreover, emerging concepts propose that mothers may transfer active immunity to the newborns via vertical transfer of pathogen-specific T cells. Overall, a promising strategy to augment and prolong neonatal immunity is to vaccinate the mother before or during pregnancy in order to boost maternal antibody concentrations or availability of specific T cells. Hence, a large number of pre-and postconceptional vaccine trials have been carried out to test and confirm this concept. We here highlight novel insights arising from recent research endeavors on the influence of prenatal maternal vaccination against pathogens that can pose a threat for newborns, such as measles, pertussis, rubella and influenza A. We delineate pathways involved in the transfer of specific maternal antibodies. We also discuss the consequences for children's health and long-term immunity resulting from an adjustment of prenatal vaccination regimes.

Keywords: maternal vaccination, measles, rubella, pertussis, influenza, FcRn, blunting, breastfeeding

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EARLY LIFE IMMUNITY AND TIME WINDOWS PERMITTING PATHOGEN THREATS FOR NEONATES

After birth and during their first months of life, human newborns are not yet equipped with a fully matured immune system (1, 2). Hence, they are highly susceptible to infectious pathogens, such as measles, pertussis, rubella, and influenza. These pathogens can cause a severe course of disease in neonates and infants, which may even be fatal (3–5). The availability of safe and immunogenic vaccines against infectious diseases, i.e., the combined measles-mumps and rubella vaccine, does

not mitigate this threat to neonatal health, as the vaccines contain living pathogen components; hence, their use is not recommended to be administered to children under the age of 12 months. Similarly, the vaccination with the combined tetanus-diphtheria-pertussis (Tdap) vaccine and the inactivated influenza vaccines (IIV) is not recommended until 2 or 6 months of age, respectively (6, 7). These restrictions to vaccination leave a pivotal gap of neonatal immunity against these pathogens until routine immunization can be administered (8).

This gap in immunity is – at least in part – covered by the active, transplacental transfer of maternal pathogen-specific antibodies. Mothers convey passive immunity to their newborns through the transplacental transfer of antibodies, hereby providing a shield for the infant from pathogen-mediated diseases (1, 9). The amount of transferred antibodies can differ between individuals and is mainly dependent on maternal antibody concentrations (10, 11). Based on this natural immunity mediated by the mother, maternal vaccination strategies during pregnancy are vividly discussed. Such strategies could increase maternal antibody concentrations, enhance the levels of transplacental antibody transfer and, in consequence, the degree of passive immunity for the neonate (12).

In the light of the recent outbreaks of vaccine-preventable diseases such as measles even in countries with high vaccine coverage, the topic of immunization has received significant attention by medical professionals and the lay community. Measles infection has caused more than 140,000 deaths globally in 2018, most of them among children under five years of age (13). Promoting the immunity of newborns via maternal vaccination holds the potential to become an effective and low-cost approach to prevent neonatal morbidity and mortality caused by communicable diseases (14–16). In the present article, we comprehensively discuss recent research studies on maternal vaccination against common childhood infections such as pertussis, influenza, measles, and rubella. We further highlight pathways involved in the transplacental transfer of antibodies as well as mechanisms through which neonatal immunity can be improved irrespective of maternal antibodies (**Figure 1**).

OBSERVATIONS FROM VACCINATION STUDIES AGAINST TETANUS, DIPHTHERIA AND PERTUSSIS DURING PREGNANCY

A number of recent studies confirm that vaccination with the combined tetanus, diphtheria, and acellular pertussis vaccine (Tdap) can be recommended during pregnancy, since vaccine trials carried out on a large scale and in various countries have generally demonstrated its safety and immunogenicity in mothers and their infants (**Table 1**). The World Health Organization (WHO) reports a 96% reduction of death by neonatal tetanus through implementation of recommended elimination practices from 1988 to 2015, including the vaccination of pregnant women (17). Similarly, the burden of diphtheria disease has been reduced (18). Unfortunately, comparable achievements have not been made with regard to pertussis elimination. Outbreaks of whooping cough have recently been occurring worldwide, exposing young infants to a particularly high risk of severe infections. Thus, we here mainly discuss studies that focus on the outcome of pertussis vaccination in pregnant women.

Amongst others, the authors of a recent study aimed to evaluate the safety and immunogenicity of Tdap administration during pregnancy in mothers and their infants and to assess the possible interference of maternal antibodies with subsequent infant immunizations (19). Apart from mild and self-limiting local reactions at the vaccination site, no adverse events caused by the immunization with Tdap were reported in mothers and their infants. Anti-pertussis toxin (PT) antibodies, which primarily mediate protection against Bordetella pertussis-induced disease (20), and anti-pertactin (PRN) antibodies, which convey protection by opsonization and subsequent phagocytosis of Bordetella pertussis (21), were significantly increased in mothers vaccinated with Tdap during pregnancy, compared to the placebo group. Accordingly, both anti-PT and anti-PRN were significantly higher at birth in infants of vaccinated mothers. Irrespective of prenatal vaccination, cord blood antibody titers exceeded maternal titers assessed at delivery, indicating an active transplacental transport of antibody. However, anti-PT and anti-PRN decreased quickly until the age of 2 months.

The investigators also pointed out differences in anti-PRN and anti-PT seroresponses following routine infant vaccinations at 2 and 4 months of age with a combined tetanus, diphtheria, pertussis, polio and Hib vaccine (19). After vaccination, infants of placebo-receiving mothers showed a greater increase of anti-PT levels compared to infants of Tdap-vaccinated mothers, indicating an interference of maternal antibodies with the child's seroresponse to vaccination. Surprisingly, opposed to the response to PT, an anti-PRN response was not mounted in these infants, irrespective of maternal Tdap vaccination. This is in contrast to a study focusing on infants' response to Tdap vaccination during early life, in which a significant seroresponse to both PT and PRN was mounted (22). An explanation for the ambiguity between the vaccination responses observed in these two studies cannot be deduced from the respective articles, but may be due to different cohort sizes, variations in the procedure of specimen preparation or the different ELISA kits used to determine antibody concentrations.

Another study focusing on the influence of maternal vaccination with Tdap during the second trimester of pregnancy (23) revealed that anti-PT IgG could be detected in 92% of infants born to vaccinated mothers, whilst anti-PT IgG was undetectable in infants of unvaccinated mothers. Although this study has some limitations, for example the lack of initial maternal anti-PT levels and the ELISA-based analysis allowing for detection of antibody presence or absence only, but no concentrations, it shows that maternal immunization with Tdap during the 2nd trimester of pregnancy significantly increases the percentage of seropositive newborns.

Not only immune responses of mother and child toward Tdap immunization during pregnancy have been investigated,

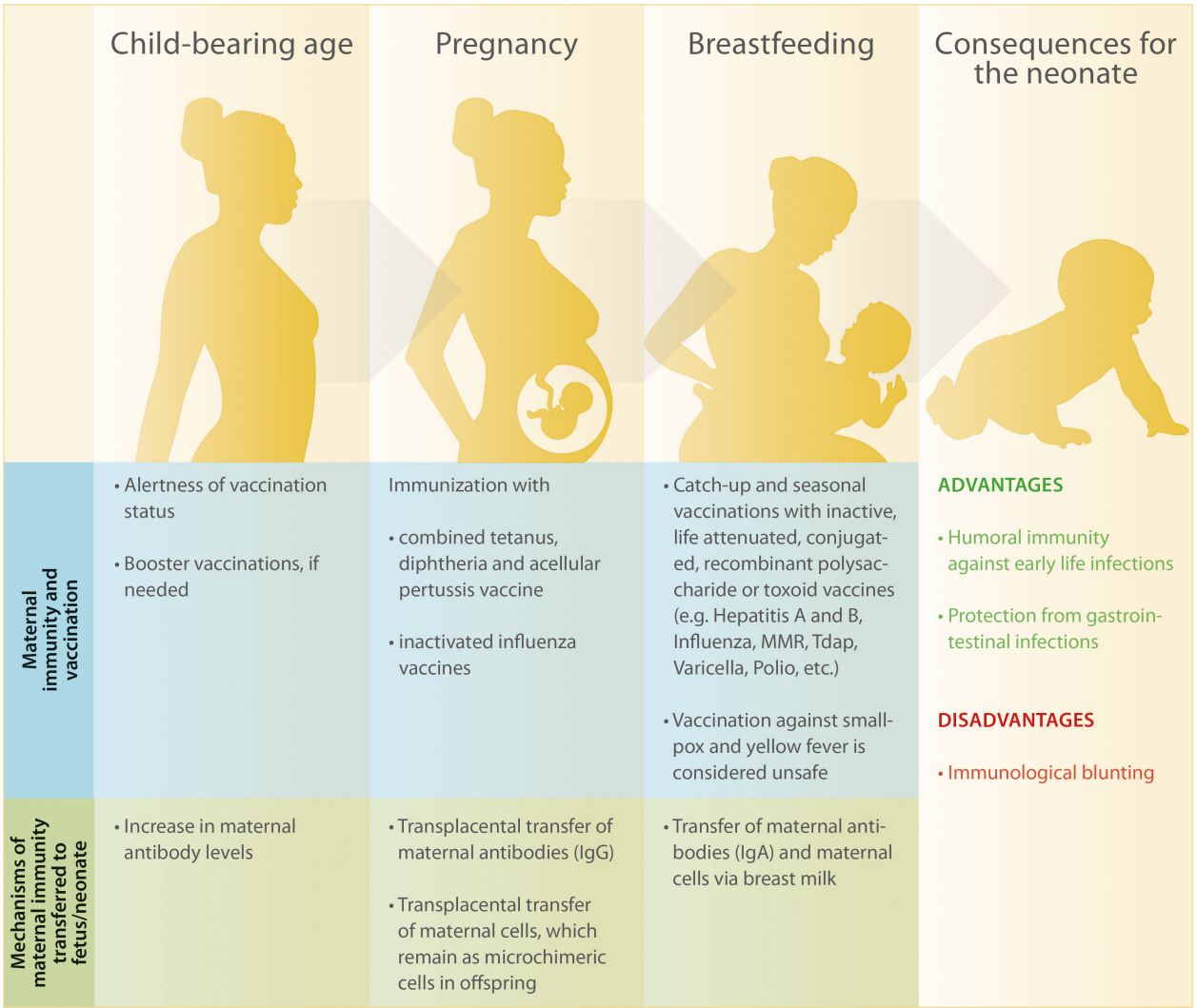

FIGURE 1 | Overview of maternal immunity and recommended vaccinations before, during and after pregnancy as well as consequences for maternal and children's health.

but also vaccine safety. By using information from different national databases, Griffin et al. (24) identified a large cohort of women who were eligible to receive governmental funded Tdap vaccination between gestational week 28 and 38. Hospitalization for severe pregnancy complications was set as the primary outcome and hospitalizations for less critical pregnancy complications as secondary outcomes. Key finding of this study was that the hazard ratio for primary or secondary outcomes did not increase when Tdap was administered during pregnancy. Intriguingly, the authors also report that Tdap vaccination during pregnancy significantly reduced the risk for hospitalization due to severe pre-eclampsia, as well as the risk for antenatal bleeding and preterm labor and delivery. Upon inspection of the studied population, these risk reductions might be biased by the demographic characteristics that distinguish vaccinated and unvaccinated women. Vaccinated women tended to be European, have a higher income level and receive care from an obstetrician. Since pregnancy complications as well as mother and infant mortality are rather associated with lower socioeconomic status and non-caucasian ethnicity (25–27), it is tempting to assume that higher rates for primary and secondary outcomes observed in this study may be due to confounders.

Noteworthy, New Zealand had been facing a large pertussis epidemic from 2011 to 2013. However, only 11.9% of the individuals eligible to receive Tdap in the study by Griffin et al. have been vaccinated. This example shows the urgent need for further education of the population regarding the effectiveness of immunization against pertussis.

Whilst the evidence for safety and immunogenicity of Tdap is steadily increasing, Saul et al. also emphasized on the effectiveness of maternal Tdap vaccination with regard to infant hospitalization due to pertussis infection (28). The authors report a 39% vaccine effectiveness (VE) to prevent pertussis infection

TABLE 1 | Overview of studies and trials assessing safety, effectiveness and outcome of vaccinations with Tdap, IIV, and MMR during child-bearing years, pregnancy or infancy in humans.

Aim of study	Study design	*N*	References
Pathogens: *C. tetani, C. diphtheriae,* and *B. pertussis*			
Assessment of immunity against vaccine preventable diseases	Prospective, observational study	194	(1)
Safety and immunogenicity of Tdap matVac, interference of matAB	Randomized double-blind controlled clinical trial	171	(19)
Effect of 2 doses of pertussis vaccine before 2 months of age	Randomized non-blinded clinical trial	76	(22)
Assessment of B. pertussis titers in third trimester and newborns	Observational, cross-sectional study	111	(23)
Maternal outcome upon Tdap matVac	Retrospective observational study	68,550	(24)
Assess effectiveness of Tdap matVac	Matched case-control study	234	(28)
VE in protecting newborns from pertussis infection	Matched case-control study	88	(29)
Comparative analysis of Tdap matVac timepoint and AB yield in newborn	Prospective study	81	(30)
Determination of optimal GW for Tdap matVac in third trimester	Prospective study	154	(31)
Comparative analysis of Tdap matVac in second or third trimester	Prospective observational study	335	(32)
Effect of Tdap booster dose between two pregnancies	Prospective study	144	(72)
Comparative analysis of maternal and cord blood AB and proteins at term	Observational study	16	(73)
Analysis of neutralizing antibodies in infants after vaccination against diphtheria	Prospective study	44	(94)
Effect of matVac with Tdap and IIV on infant AB responses	Prospective study	369	(95)
Influence of Tdap booster dose during pregnancy on infant's matAB levels and immune responses	Prospective controlled cohort study	99	(96)
Safety and immunogenicity of Tdap matVAc and effect on infant immune responses	Randomized, double-blind, placebo-controlled trial	80	(97)
Pathogen: Influenza A			
Assessment of safety and immunogenicity of seasonal trivalent IIV matVac	Prospective, randomized, double-blind clinical trial	100	(40)
Risk assessment for neonatal birth defects after first trimester IIV exposure	Observational study	425,944	(41)
Persistence of HAI titers and VE of IIV3 in subsequent influenza season in women	Double-blind, randomized, placebo-controlled trial	479	(43)
Duration of infant protection upon IIV matVac	Substudy of randomized, double-blind, placebo-controlled clinical trial	322	(44)
Clinical effectiveness of IIV matVac; safety and immunogenicity of pneumococcal vaccines	Prospective, controlled, blinded, randomized study	340	(45)
Risk assessment for infant hospitalization due to lower respiratory infection after IIV matVac	Secondary analysis of randomized controlled trial	52	(46)
Effect of IIV matVac on risk for influenza in infants < 6 months of age	Non-randomized, prospective, observational cohort study	1169	(47)
Influence of IIV matVac on subsequent B. pertussis infection rates in mothers	Retrospective testing of samples collected in randomized controlled trial	3125	(48)
Effect of vitamin A supplementation on immune response to IIV matVac	Prospective study	112	(70)
Investigation of sensitization to IIV antigens *in utero*	Prospective observational study	126	(74)
Effect of maternal influenza vaccination on influenza-specific IgA levels in breast milk	Prospective, blinded, controlled trial	340	(80)
Effect of cross-reactive cellular immunity on symptomatic influenza illness in AB- naïve individuals	Prospective study	342	(90)
Pathogens: Measles, Mumps, and Rubella Virus			
Repertoire of maternal anti-viral AB in newborns at birth	Prospective study	78	(10)
Assessment of safety of MMR vaccination in adults	Retrospective observational study	3175	(51)
Assessment of B cell impairment upon measles-associated immunosuppression	Prospective observational study	29	(54)
Identification of measles infection long- term effects on immune system	Prospective study	196	(55)
Association of maternal age and vaccination status with cord blood matAB	Observational study	206	(57)
MatAB transfer in vaccinated or naturally immune mothers to preterm/term infants	Prospective study	195	(58)
Quantification of AB against MMR and varicella zoster in mothers and infants	Prospective observational study	138	(59)
Duration of presence of matAB to measles in infants	Prospective study	207	(60)

(Continued)

TABLE 1 | Continued

Aim of study	Study design	*N*	References
Seronegativity in infants < 6 months and serologic response to measles vaccine	Cross-sectional study	203	(61)
Prenatal fetal infection among women (re-) infected with rubella during pregnancy	Prospective observational study	40	(62)
Detection of rubella-specific IgM in subclinical rubella reinfection in pregnancy	Case report	8	(63)
Criteria for defining rubella reinfection	Case report	5	(64)
Fetal infection after maternal rubella reinfection during pregnancy	Case report	1	(65)
Seroepidemiology of anti-measles, -mumps and -rubella AB in pregnant women and neonates	Prospective study	353	(71)
Assessment of transplacental transport of IgG immune complexes	Prospective study	152	(75)
Immunogenicity of measles vaccine in infants < 12 months	Cohort study	72	(92)
Studies on breast milk immunity			
Assessment of gut microbiota bound by breast milk IgA	Observational study	69	(81)
Effects of infections during pregnancy on colostrum IgA levels	Cross- sectional study	900	(82)

matVac, maternal vaccination during pregnancy; Tdap, Tetanus, diphtheria, acellular pertussis vaccine; matAB, maternal antibody; VE, vaccine effectiveness; GW, gestational week; AB, antibody; IIV(3), (trivalent) inactivated influenza vaccine; MMR, measles, mumps, rubella vaccine; HAI, hemagglutination inhibition assay.

for infants < 6 months and of 69% for infants younger than 3 months of age; the overall VE against hospitalization due to severe pertussis infection was 94%. These results clearly demonstrate that maternal Tdap vaccination is predominantly effective in preventing severe cases of pertussis disease, with maternal vaccination attenuating the intensity of the illness rather than preventing it. Furthermore, the authors identified that breastfeeding may have a protective effect on pertussis infection of the infant. These findings are in line with a very similarly set up of a study conducted in the same year (29). Here, the authors found a 90.9% VE of maternal Tdap vaccination during pregnancy in protecting infants < 3 months from laboratory confirmed pertussis; yet, VE was calculated from a small cohort. Also, apart from maternal vaccination, breastfeeding was identified as the only other significant influence on infant protection against pertussis. This effect could be observed not only in mothers vaccinated during pregnancy, where maternal IgA could be passed via the breast milk, but also in those who had not been vaccinated against or in contact with pertussis for the last 10 years. The authors suggest that this might be attributed to other breast milk components which were not further specified.

There is still ambiguity with regard to vaccination timepoint recommendation by national health services. The National Health Service (NHS) in the United Kingdom and the Advisory Committee on Immunization Practices (ACIP) in the US suggest two different vaccination schedules. While the NHS recommends Tdap administration between 16 and 32 weeks of gestation (30), the ACIP proposes that Tdap should be administered at a later timepoint between 27 and 36 weeks of gestation (6). Using cord blood concentrations of pertussis-specific IgG as a read out parameter, one study reports highest levels if mothers had been vaccinated with Tdap between 27 and <31 weeks of gestation, as compared to vaccination at 31 weeks or later (31). Another study suggested that the optimal timepoint for Tdap administration is between 28 and 32 weeks of gestation, based on higher cord blood anti-pertussis antibody concentrations resulting from vaccination at this timepoint as compared to later in gestation (32). Conversely, another study with a higher number of participants reports that maternal Tdap vaccination between gestational week 13 and 25 results in higher cord blood anti-pertussis antibody concentrations than immunization after 26 weeks of gestation (33). A longer period of time between vaccination and childbirth allows for a greater transfer window, which may explain the observed higher cord blood titers. Re-scheduling the recommended vaccination to an earlier timepoint during pregnancy might therefore be beneficial, not only for preterm neonates (33).

Taken together, Tdap immunization should be recommended to each pregnant woman in every pregnancy, regardless of the previous vaccination status. This will yield to high maternal antibody concentrations toward the end of pregnancy, so that antibodies can be transferred at greater extent to the fetus. Whilst vaccination of the mother during the 2nd or 3rd trimester of pregnancy is safe and efficacious, the best strategy to ensure high neonatal anti-pertussis antibody concentrations seems to be vaccination between gestational week 13 and 25. Besides maternal vaccination, passive protection of the neonate via reduction of pathogen exposure can result from a so-called cocooning effect, achieved by vaccination of family members and caregivers of the newborn (34, 35). By combining these protective techniques, the risk for pertussis infection during the first months of life, until the neonate has mounted humoral and cellular immunity against this pathogen, can be reduced.

INSIGHTS FROM VACCINATION STUDIES AGAINST INFLUENZA DURING PREGNANCY

Apart from Tdap, vaccination against influenza using inactivated influenza vaccines (IIV) is the only other recommended

vaccination during pregnancy. Pregnant women are at high risk for severe influenza disease outcomes due to a multi-faceted failure to mount an anti-viral response. As shown in basic science approaches, this less stringent selective environment can promote the emergence of mutated influenza variants which mediate increased viral pathogenicity (36). The Robert Koch Institute, the governmental central scientific institution safeguarding public health such as the surveillance and prevention of infectious diseases in Germany, recommends vaccination against influenza for all pregnant women during the second and third trimester. For women with increased morbidity risk or preexisting medical conditions, vaccination is even recommended during the first trimester (37). Similar recommendations have been made by the ACIP in the United States (38), where vaccination against influenza is recommend at any time during normally progressing pregnancy.

These recommendations result from a wealth of studies carried out worldwide on safety, immunogenicity and efficacy of influenza vaccination during pregnancy. These studies have clearly demonstrated the advantages of protecting mother and infant from influenza disease, as extensively reviewed elsewhere (14, 15, 39).

Moreover, independent studies (**Table 1**) have assessed the impact of influenza vaccination on pregnancy outcomes and confirmed that the risk for structural birth defects or pregnancy complications is unaffected by maternal vaccination against influenza (40, 41). On the contrary, the frequency of infants born small for gestational age was lower among vaccinated women and the overall birth weight was higher (42). Immunogenicity analyses using hemagglutination inhibition assay (HAI) revealed that the overall reactogenicity to the inactivated influenza virus vaccine was similar between non-pregnant and pregnant individuals (40). Here, it was also reported that higher maternal age negatively correlates with seroconversion and -protection, whilst data supporting this observation have not been shown. However, another study showing that HAI titers were likely to remain elevated one year after immunization especially in women younger than 25 years of age supports the link between maternal age and immunogenicity to IIV (43).

Besides the maternal response to influenza vaccination during pregnancy, the subsequent children's outcome upon maternal vaccination has also been the focus of a number of studies. Here, an overall beneficial response could be identified, such as a lower hospitalization rate and milder disease course of infants < 6 months, not only related to influenza infection (44, 45), but also to all-cause lower respiratory tract infection (ALRI), including diseases induced by pathogens such as B. pertussis, respiratory syncytial virus (RSV) and rhinovirus (42, 46, 47). This broad protection from lower respiratory tract infections has been explained by an increased susceptibility to pathogens affecting the airway system subsequent to an influenza infection, from which neonates with maternally inherited passive immunity against influenza are protected to a higher degree (46, 48). However, large-scale studies are urgently needed to confirm this suggestion. Once confirmed, such insights will likely increase the vaccination compliance of pregnant women, which is still surprisingly low (49).

VACCINES CONTRAINDICATED FOR IMMUNIZATION DURING PREGNANCY

Unlike vaccinations against tetanus, diphtheria, pertussis and influenza, which can be recommended during pregnancy, live attenuated vaccines like the combined measles-mumps-rubella (MMR) vaccine are contraindicated in pregnant women due to the hypothetical risk of transplacental viral transmission and infection of the fetus (50). However, observations from prenatal MMR immunization administered during the first trimester to women unaware of their pregnancy revealed that the risk for adverse pregnancy outcomes such as spontaneous abortion, hydrocephalus, vaginal bleeding and preterm birth is not significantly increased compared to the general population. Also, fetal infection has not been reported (51).

Resulting from the global rise of vaccine hesitancy, one of the 10 threats to global health (52), transmission of measles is rapidly spreading, which poses a significant hazard to children's health. Besides common complications related to measles infection in children, such as diarrhea, middle ear infection and pneumonia (53), it has recently been identified that measles can obliterate existing humoral immune memory against a repertoire of pathogens (54, 55). The incomplete reconstitution of the naïve B cell pool and the depletion of previously expanded B memory clones account for this obliteration of immune memory (54). Hence, the susceptibility toward subsequent infections is greatly enhanced after measles infection, which strongly underpins the urgency not only for vaccination of children, but also for women with the intention to become pregnant. This will close a vulnerable gap of neonatal susceptibility toward measles prior to the recommended vaccination at the age of one year and allow to achieve global measles elimination.

In Germany, immunization of adults with MMR is only recommended for individuals with an incomplete or unclear vaccination history (37). Since the age of women at the time of giving birth to their first child has increased by approximately a decade during the last 50 years (56), the window between routine childhood vaccination and onset of pregnancy has also increased. Hence, antibody concentrations might have waned substantially at the time of pregnancy. It has been observed that a MMR vaccination dose administered close to pregnancy induces higher matAB levels in the offspring, irrespective of the total number of vaccine doses given to the mother (57). In countries where pathogens such as measles still circulate within the population and hence, natural infections and boosting through recurrent exposures to the wild-type pathogens are frequent, antibody concentrations are higher compared to those mounted by immunization (57). *Vice versa*, in highly vaccinated populations with low pathogen circulation, antibody concentrations in mothers and their children tend to be lower due to faster decrease of vaccine-induced antibodies and a lack of natural boosting through pathogen exposure (58–60). Gonçalves et al. quantified this observation by measuring anti-measles-IgG in

cord blood and found that in infants born to MMR-unvaccinated mothers, who most likely gained their immunity through natural infection, anti-measles-IgG reached 1849 mIU/ml, while cord blood of infants born to MMR-vaccinated mothers only contained 987 mIU/ml of anti-measles-IgG (57). It has been suggested that immune responses toward measles may differ if mounted by natural infection or by vaccination, because different antibody subclasses may be induced and that infants of vaccinated mothers lose passive acquired immunity at an earlier age compared to naturally immune mothers (61).

Studies observing the impact on the neonate in case of rubella reinfection during pregnancy (62–64) have reported that rubella reinfection can occur both in naturally immune women and in women immunized against rubella during childhood. Noteworthy, immunized women are at greater risk for such reinfection, which might be due to differences in the immune response following vaccination or natural infection. The course of rubella reinfection is mostly subclinical, but may have severe consequences such as the congenital rubella syndrome (CRS), though this has been described only in one case (65). Thus, MMR booster doses can be recommended to women planning to become pregnant in order to avoid serious illness of the child if exposed to measles or rubella virus during gestation or during the first months of life.

MECHANISMS OF TRANSFERRING IMMUNITY TO THE NEWBORN: TRANSPLACENTAL TRANSPORT OF MATERNAL ANTIBODIES

The wealth of studies summarized so far highlights that maternal antibodies against specific pathogens can be vertically transferred to the fetus and subsequently protect the neonate from infections. Thus, the mechanism of such vertical transfer is a key modulator of neonatal immunity and shall be reviewed in the following.

Generally, the placenta poses a barrier which can – at least partially – control and hinder the transmittance of harmful substances from mother to fetus. Hence, a specific and active transport mechanism is needed in order to transfer maternal pathogen-specific antibodies to the fetus. In this respect, the neonatal Fc-receptor (FcRn) plays a key role. It is, amongst other tissues, expressed in placental syncytiotrophoblasts and belongs to the Fcγ receptor family, which characteristically binds the Fc fragment of IgG antibodies and promotes their transport to body sites where specific immunity is needed (66). The IgG binding characteristics of FcRn are highly pH-dependent (67); in acidic environments, FcRn shows a much higher affinity to IgG compared to the physiological pH of 7.4, which is present in maternal and fetal blood. Thus, maternal antibodies are unable to bind to FcRn at the apical side of the syncytiotrophoblasts, which is bathed on maternal blood, but need to be taken up by endocytosis (**Figure 2**). The amount of antibody that can be transferred to the fetus depends on the amount of FcRn expressed by syncytiotrophoblasts. If all FcRn are engaged in IgG transport, additional IgG molecules will be degraded in the lysosome, as they are not receptor-bound. Thus, antibody transfer is a saturable process and will stagnate once maternal antibody concentrations reach a certain level, which has been defined as a total IgG of 15 g/L (68). Transplacental IgG transport starts early in gestation (10, 69), though still at low efficacy. With the continuation of pregnancy, FcRn expression and transplacental transport increase, peaking during the last four weeks of gestation (9). It is tempting to speculate that the increased cell mass of the growing placenta accounts for the mere increase in FcRn and related higher antibody transport rate.

To date, research on factors influencing the FcRn expression is scarce. In one study, the effect of vitamin A supplementation during pregnancy on the immune response following maternal influenza vaccination during pregnancy has been assessed. Here, a possible influence of vitamin A on FcRn expression has been proposed, but this aspect is still highly speculative and data are based on a small sample size (70). Data on sex-specific differences in placental FcRn expression is also currently lacking.

Interestingly, not all IgG subclasses are equally transferred, as FcRn mainly transports IgG1, with decreasing efficacy for IgG4, IgG3, and IgG2 (69). Structurally different antigens have been shown to induce different IgG subclasses and thus, are transferred in varying amounts. While protein antigens, such as pertussis toxin and pertactin, tetanus toxin or the measles virus elicit IgG1, polysaccharide antigens, as found on the surfaces of bacteria like Haemophilus influenzae type b or Neisseria meningitidis, induce IgG2 (2). Since the latter subclass is being transported less efficiently, newborns might lack specific immunity toward pathogens which mainly present polysaccharide antigenic structures, such as most bacteria.

The most predictive factor of transplacental antibody transfer is the level of maternal antibody (10). Higher gestational age, recent maternal vaccinations, a balanced maternal nutritional status and male gender of the newborn have been shown to positively influence maternal antibody concentrations in the infant (1, 71, 72).

Observations dating back some decades indicate that apart from IgG being transported across the placenta as a single molecule, it can also be transported as an IgG-immune complex (IgG-IC) involving IgG and it's respective antigen (73). In this study, serum tetanus antigen reached nearly the same levels in mother and infant at birth, suggesting an active transfer of anti-tetanus IgG-IC. Active transfer could not be observed for different pregnancy-related proteins such as alpha-fetoprotein (AFP) and human chorionic gonadotropin (hCG), as their concentrations highly differed between mother and child, indicating a transmission by low-rate diffusion. More recently, influenza-specific fetal IgM could be detected in cord blood upon maternal influenza vaccination during pregnancy, suggesting that anti-influenza IgG-IC had been transferred to the fetus, followed by a fetal B- and T-cell immune response against influenza elicited by the IC *in utero* (74). Together with the observation that IgE, which plays a major role in allergy pathogenesis, can also be transported via the placenta as an IgG-IgE-IC (75), these findings have a great impact on understanding neonatal immunity and the development of atopy in children.

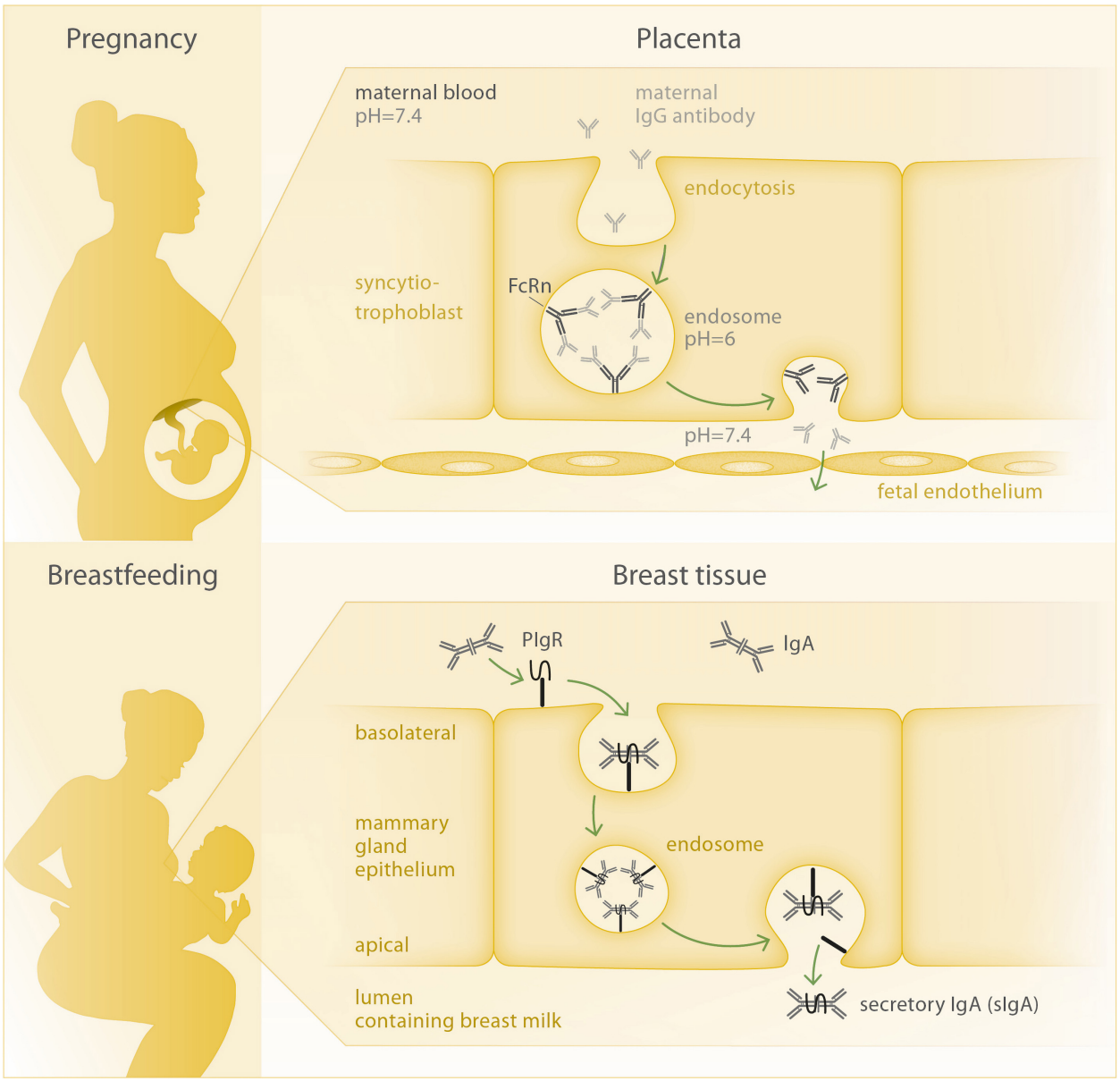

FIGURE 2 | Mechanisms of antibody transfer via placenta and breast tissue. Top: Circulating IgG antibody is taken up into the syncytiotrophoblast cell, where two IgG molecules per FcRn bind at the inner membrane of the acidic endosome. Upon opening of the endosome at the basolateral side of the cell facing the fetal circulation, FcRn releases the IgG molecules due to the increased pH and can then be recycled to perform another transport cycle. Bottom: The joining chain of the dimeric IgA molecule is bound by the polymeric Ig-receptor (pIgR) and both are internalized via endocytosis. At the apical membrane, secretory IgA (sIgA) is being released to the breast milk, as the secretory component of pIgR remains bound to the IgA antibody.

MECHANISMS OF TRANSFERRING IMMUNITY TO THE NEWBORN: TRANSFER OF MATERNAL ANTIBODIES VIA BREASTMILK

Another substantial element of neonatal immunity is the intake of breast milk, which contains a significant amount of secretory IgA. Also, maternal immune cells, such as IgG-producing memory B cells and $CD4^+$ T cells, can be detected in breast milk (9, 76).

The dimeric IgA antibodies are produced by plasma cells in the mammary gland as well as in other tissues associated with mucosal surfaces. The epithelial cells of the mammary acini transport the IgA molecules from the connective tissue to the breast milk via transcytosis, involving the polymeric Ig receptor (pIgR) (77, 78) (**Figure 2**). The two IgA subclasses present in humans, IgA1 and IgA2, are distributed differently along mucosal membranes, with IgA1 being mainly present in the respiratory tract, saliva, serum and skin and IgA2 being the main secretory

antibody of the intestine (79). In their seminal review, Hanson and Winberg already concluded that breast milk IgA is not absorbed by the infant's gut, but rather coats the mucosal surface of the intestine to protect it from pathogens (80).

Several studies have unveiled that the consumption of breast milk by the neonate is beneficial to its health (**Table 1**). One example is the enhanced transfer of influenza-specific and neutralizing IgA to the neonate upon influenza vaccination of the mother during pregnancy (81), which was associated with a decreased number of respiratory illness of the infants during the first six months of life. Whether this effect results from the increased amount of specific breast milk IgA or from the transplacental transfer of maternal influenza-specific IgG remains to be elucidated. Cross-fostering may provide an answer and considering the growing number of milk banks, such studies may become feasible.

Another study recently highlighted a breast milk IgA-mediated protection from necrotizing enterocolitis (NEC) in preterm infants (82). The pathogenesis of NEC seems to be mainly driven by an altered sIgA binding pattern of intestinal bacteria in the newborn, since the proportion of IgA-bound bacteria was much lower in infants developing NEC compared to healthy newborns. Formula-fed infants were more likely to develop NEC than breastfed infants, presumably due to the absence of maternal IgA in formula alimentum.

Moreover, in women with respiratory tract infections during pregnancy, the proportion of IgA1 in colostrum was higher, while in women with gastrointestinal infections, levels of IgA2 were increased (83). These observations suggest that the mother's immune system seeks to shield the infant from the specific pathogens of the surrounding environment. Similarly, as shown in basic science models, maternal antibodies can potentially retain microbial molecules and transmit them to the offspring via the placenta and breastfeeding. Subsequently, the offspring are able to avert an inflammatory response to microbial molecules and allow colonization of intestinal microbes (84).

Overall, the beneficial effect of breastfeeding for infant's health seems to affect various mucosal membranes, such as the respiratory and gastrointestinal tract, hereby protecting the neonate from infections. Thus, the current recommendations of the WHO to exclusively breastfeed an infant during its first 6 months of life (85) may indeed provide optimal starting conditions for the child's postnatal immunity.

MECHANISMS OF TRANSFERRING IMMUNITY TO THE NEWBORN: MATERNAL MICROCHIMERISM

Besides antibody-mediated immunity transferred during pregnancy, it is also conceivable that pathogen-specific maternal immune cells migrate to the unborn child. It is well known that maternal immune cells can be transferred to the fetus via the placenta (86), and also via breast milk (76). These cells can then remain in the offspring until adulthood, as shown among lymphoid and myeloid compartments of peripheral blood in healthy adult women (87). Due to the low frequency of these cells in the offspring, they are referred to as maternal microchimeric cells and a considerable percentage of such cells are T cells, which can be retained for a long period of time (88).

In general, upon infection, pathogen-specific $CD8^+$ T cells remain in peripheral tissues and act as sentinels. Upon antigen re-encounter, they rapidly produce inflammatory cytokines and thereby induce a state of alertness in the local environment and recruit inflammatory cells. Thereby, a small number of pathogen-specific T cells can provoke a fast and fulminant response (89, 90). Interestingly, in the context of pregnancy, there is direct evidence for such transfer of protective maternal T cells. In a human infant with severe combined immunodeficiency suffering from Epstein-Barr virus (EBV) infection, large numbers of maternal $CD8^+$ T cells could be detected. These cells were phenotypically activated and secreted IFN-γ in response to EBV antigen. Other hematopoietic cells were of offspring genotype, indicating that the $CD8^+$ T cells originated from mature maternal T cells and not form transferred hematopoietic stem cells (91). Moreover, high frequencies of pre-existing effector $CD8^+$ T cells directed against conserved core protein epitopes of influenza virus strains correlate with a milder course of influenza infection caused by other influenza virus strains, thus providing strong evidence for a cross protective function of memory $CD8^+$ T cells against heterologous influenza strains (92). Based on these empirical evidences, it is appealing to speculate that pathogen-specific maternal microchimeric T cells also convey passive cellular immunity to the offspring.

THE DOWNSIDE OF NEONATAL PASSIVE IMMUNITY: MATERNAL ANTIBODIES INTERFERE WITH THE INFANT'S RESPONSE TO VACCINATION

Despite the significant health benefits resulting from maternal vaccination during pregnancy for mother and infant, there is also a downside to it. Many studies have demonstrated that high levels of maternal antibodies in the infant hamper the immune response required to mount humoral immunity upon routine childhood vaccinations (68). This inhibitory effect of maternal antibodies on the antibody generation by the infant's immune system, which is commonly referred to as "blunting," can affect neonatal immunity for up to more than one year of age, depending on the level of maternal antibodies in the neonate at birth. Interestingly, blunting occurs irrespective of the type of vaccine applied, including measles, influenza and pertussis vaccines (68).

The most common explanation for blunting involves a cross-link between the B cell receptor (BCR) and the Fcγ receptor FcγRIIB (68, 93), both expressed on the surface of B cells. Each BCR has a unique affinity to a certain pathogen epitope, which can also be recognized by specific maternal antibodies. These again can be ligated to the FcγRIIB by their Fc fragment. When the infant is being vaccinated, pathogen fragments enter its circulation and can be bound both by BCR and maternal antibodies at the same time, which leads to contradicting signals

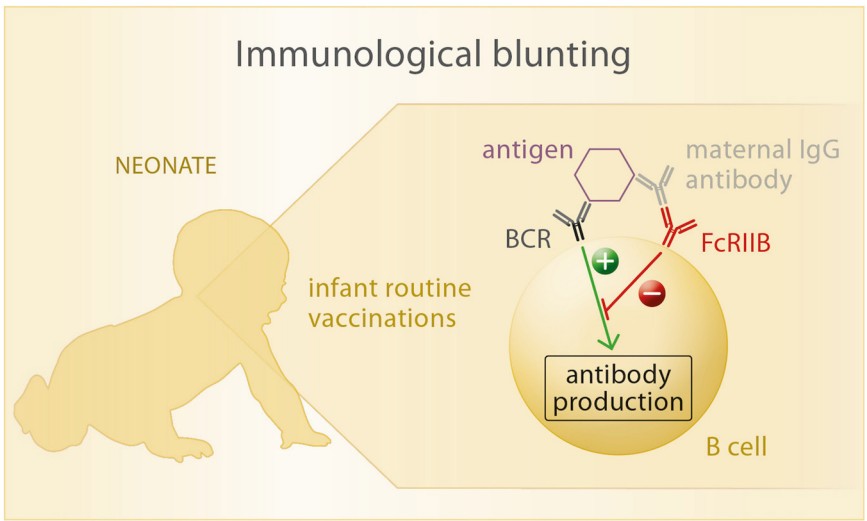

FIGURE 3 | Upon exposure of the neonate to vaccine antigens, the antigen is recognized by its specific B cell receptor (BCR). If maternal antibodies are present in the child's circulation, they bind to the vaccine antigen as well as to the Fc-receptor FcRIIB that is also expressed on B cells. Thus, a cross-link between BCR and FcRIIB is formed, which inhibits antibody production of the B cell in response to antigen recognition.

within the B cell. While the BCR recognizes the new antigen and emanates signals leading to plasma cell differentiation and antibody production, FcγRIIB signalizes the presence of specific antibody to this particular antigen and inhibits further antibody production. In consequence, the stimulatory BCR signal is being inhibited and no antibody production can be initiated by the infant's immune system (**Figure 3**).

Very recently, a large study has thoroughly addressed the topic of blunting by maternal antibodies (94). Here, children's antibody responses to routine early life vaccinations against Hepatitis B, tetanus, diphtheria, pertussis, polio, pneumococcus, rotavirus, MMR, and meningococcus have been associated with maternal vaccine responses using inactivated influenza vaccine or Tdap during pregnancy. While maternal influenza vaccination did not affect the infant's vaccine responses, maternal immunization with Tdap resulted in significantly lower vaccine responses to specific (diphtheria and pertussis) and heterologous antigens (polio and pneumococcus) in the child. This observation has sparked the notion that maternal antibodies present in the neonate bind to the diphtheria-toxin derived carrier protein of the pneumococcal vaccine before the neonatal host can mount an immune response against the heterologous antigens bound to the carrier protein. Also, reduced blunting has been described upon infant immunization with acellular as compared to the whole cell pertussis vaccine (19). In order to support maternal vaccination strategies, the consequence of Tdap-booster immunization at 13 or 15 months of age upon maternal Tdap vaccination during pregnancy has been assessed. Here, Tdap booster doses overcame an initially observed blunting effect caused by high maternal antibody levels (95, 96).

Approaches seeking to bypass the process of blunting are nowadays tested, such as alternative vaccination routes and the simultaneous injection of antigen-specific IgM or agents that stimulate the production of interferon-α along with the vaccine (68). These adjuvants have been suggested to counteract the inhibitory signal produced by FcγRIIB, thus leading to B cell activation and antibody production following immunization even in the presence of maternal antibodies. Additionally, as reported by studies dating back two decades and more, maternal antibodies do not interfere with T cell priming of the infant (97–99). These observations support that sufficient protection can still be reached at the time when maternal antibodies have completely waned in the infant at an age of approximately 6 months, even if the first vaccination did not trigger a significant humoral immune response.

There is still ambiguity regarding the occurrence of a blunting effect, as it was not confirmed in all studies assessing it (22). Hence, future studies are required to confirm the underlying mechanisms of blunting and T cell priming in order to ensure highest efficacy of neonatal immunization. Clearly, blunting of vaccine responses in infants might increase the susceptibility to certain early life infections. Considering the advantages related to maternally derived passive immunity for the neonate, blunting however, has been described as an acceptable trade-off (94).

Noteworthy, a number of articles published in the 1980s support that anti-idiotypic antibodies are also transferred from the mother to the fetus via the placenta (100) and by breast milk. Anti-idiotypic antibodies are directed against molecular patterns (idiotypes) located close to the antigen-binding site of pathogen-specific antibodies and are being elicited as part of the regular immune response. A proportion of anti-idiotypic antibodies carry an "internal image" of the antigen for which their idiotype antibodies are specific. Thus, anti-idiotypic antibodies are thought to stimulate B-cells in an antigen-independent manner and subsequently lead to the production of antigen-specific, idiotype-carrying antibodies with neutralizing ability

(101–104). Low levels of maternally derived anti-idiotypic antibodies have been shown to provide a significant priming effect on the immune system of the neonate, protecting neonatal mice from pathogen challenges (104). Conversely, a high dose of maternally derived idiotype and anti-idiotypic antibodies, acquired via transplacental transfer or breastfeeding, may yield to the observed blunting effect. Hence, based on the immune network theory by Jerne (105), complex regulatory mechanisms involving idiotype and anti-idiotypic antibodies underlie the infant's vaccine responses. Strikingly, using monoclonal anti-idiotypic antibodies as a vaccine to immunize against measles, mumps and rubella, against which to date can only be vaccinated later in life, could allow to induce protection already at birth. This would overcome a major window of vulnerability and reduce the burden of disease in young infants.

VACCINATION COMPLIANCE DURING PREGNANCY

Despite all these evidences highlighting the benefit of vaccinations during pregnancy for mother and child, poor vaccination compliance among women during their reproductive years is still an alarming clinical problem. This poor vaccination compliance is the result of a number of factors, including the neglect of healthcare providers to offer vaccination, limited availability and high costs of vaccines, doubts of the effectiveness of vaccinations, concerns about the safety of the vaccine for mother and fetus (106, 107). Continuous accumulation of evidence that vaccination strategies can yield to significant health advantages for mother and child and the communication to researchers, lay individuals and stake holders will hopefully improve the vaccination compliance in the near future.

CONCLUSION

A wealth of published evidence strongly underpins that vaccination during pregnancy is advantageous not only for maternal health, but also for children's well-being. Especially maternal vaccination against tetanus, diphtheria, pertussis and influenza has been convincingly demonstrated by a large number of studies to be safe, immunogenic and to provide significant immunity to the newborn. The latter could not only be confirmed by the mere presence of maternally derived pathogen-specific antibodies in newborns, but indeed a reduced risk for pertussis and a broad protection from lower respiratory tract infections, even beyond infection with the influenza virus. Noteworthy, the downside of high levels of maternal antibodies against pathogens, the induction of immunological blunting in the infant, seems to dampen the neonatal response to early life vaccinations and causes a threat to neonatal health. The reduced risk for neonatal infections due to maternally derived immunity however, clearly proves that blunting-related disadvantages are outweighed by the advantages. This has been confirmed by a recent study which reports that measles vaccination of infants in the presence of maternal anti-measles antibody significantly reduced overall infant mortality, compared to vaccinated infants without maternal antibodies (108). Lastly, the poor vaccination compliance is a challenge that must urgently be met, for example by implementing maternal immunization platforms through which education and communication of vaccination-related benefits are facilitated and vaccines are routinely offered in order to increase the willingness and subsequently the vaccination rate of pregnant women.

AUTHOR CONTRIBUTIONS

MA and PA jointly outlined the article, developed the figures, and wrote the review article.

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Analysis of TCR Repertoire and PD-1 Expression in Decidual and Peripheral CD8$^+$ T Cells Reveals Distinct Immune Mechanisms in Miscarriage and Preeclampsia

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CD8$^+$ T cells, the most abundant T cell subset in the decidua, play a critical role in the maintenance of pregnancy. The majority of decidual CD8$^+$ T cells have an effector memory phenotype, while those in the peripheral blood display a naive phenotype. An increased amount of highly differentiated CD8$^+$ T cells in the decidua indicates local antigen stimulation and expansion, albeit these CD8$^+$ T cells are suppressed. In decidual CD8$^+$ T cells, co-inhibitory molecules such as PD-1, TIM-3, LAG-3, and CTLA-4 are upregulated, reflecting the suppression of cytotoxicity. Previous studies established the importance of the PD-1/PD-L1 interaction for feto-maternal tolerance. CD8$^+$ T cells could directly recognize fetal-specific antigens, such as HLA-C, expressed by trophoblasts. However, although fetal-specific CD8$^+$ T cells have been reported, their TCR repertoires have not been identified. In this study, we analyzed the TCR repertoires of effector memory CD8$^+$ T cells (CD8$^+$ EM cells) and naive CD8$^+$ T cells (CD8$^+$ N cells) in the decidua and peripheral blood of women with normal or complicated pregnancy and examined PD-1 expression at a single-cell level to verify whether antigen-specific CD8$^+$ T cells accumulate in the decidua and to identify immunological differences related to the suppression of antigen-specific CD8$^+$ T cells between normal pregnancy, miscarriage, and preeclampsia. We observed that some TCRβ repertoires, which might recognize fetal or placental antigens, were clonally expanded. The population size of clonally expanded CD8$^+$ EM cells was higher in the decidua than in the peripheral blood. CD8$^+$ EM cells began to express PD-1 during the course of normal pregnancy. We found that the total proportion of decidual CD8$^+$ EM cells not expressing PD-1 was increased both in miscarriage and in preeclampsia cases, although a different mechanism was responsible for this increase. The amount of cytotoxic CD8$^+$ EM cells increased in cases of miscarriage, whereas the expression of PD-1 in clonally expanded CD8$^+$ EM cells was downregulated in preeclampsia cases. These results demonstrated that decidual CD8$^+$ EM cells were able to recognize fetal-specific antigens at the feto-maternal interface and could easily induce fetal rejection.

Keywords: effector memory CD8$^+$ T cell, human pregnancy, miscarriage, preeclampsia, T cell repertoire

INTRODUCTION

Immune tolerance to the "semi-allogeneic" fetus is particularly important for successful pregnancy. Decidual $CD4^+$ T cells, $CD8^+$ T cells, and NK cells are activated during pregnancy (1); in particular, decidual $CD4^+$ T cells are activated when an HLA-C mismatch is present between the mother and the fetus (2). Maternal regulatory T cells (Treg cells) play an important role in the maintenance of pregnancy by preventing rejection (3, 4). During pregnancy, $CD8^+$ T cells become predominant among decidual immune cells and play a major role in feto-maternal tolerance. The main population among decidual $CD8^+$ T cells ($dCD8^+$ T cells) is represented by effector memory $CD8^+$ T cells ($CD8^+$ EM cells) that are thought to potentially induce fetal rejection, while the predominant population among peripheral $CD8^+$ T cells ($pCD8^+$ T cells) are naive $CD8^+$ T cells ($CD8^+$ N cells) (5). Previous studies revealed functional differences between decidual and peripheral $CD8^+$ T cells. Decidual $CD8^+$ EM cells ($dCD8^+$ EM cells) exhibit higher production of interferon-γ (IFN-γ) and interleukin-4 (IL-4), as well as reduced perforin and granzyme B expression, compared to peripheral $CD8^+$ EM cells ($pCD8^+$ EM cells) (5, 6). $dCD8^+$ EM cells express higher levels of inhibitory checkpoint molecules such as programmed cell death-1 (PD-1), T cell immunoglobulin mucin-3 (TIM-3), lymphocyte-activation gene-3 (LAG-3), and cytotoxic T lymphocyte associated protein-4 (CTLA-4) compared to $pCD8^+$ EM cells (6–8). A high PD-1 expression was reported in decidual immune cells such as $CD8^+$ T cells, regulatory T cells (Treg cells), and NKT-like cells (7, 9, 10), and programmed cell death ligand-1 (PD-L1) was found to be highly expressed in extravillous trophoblasts (EVT), syncytiotrophoblasts (ST), and other immune cells at the feto-maternal interface (11–15). The blockade of the PD-1/PD-L1 pathway results in increased fetal resorption in mice (16), suggesting that this axis is necessary for immune tolerance in the decidua. Therefore, the cytotoxicity of $CD8^+$ T cells in the decidua is regulated so as to promote immune tolerance against fetal antigens during pregnancy, albeit these cells maintain a cytotoxic potential against virus-infected cells (17).

Immunological differences have been reported between normal pregnancy, miscarriage, and preeclampsia. Ramhorst et al. demonstrated that in non-pregnant women undergoing recurrent pregnancy loss, the proportion of effector memory T cells in the peripheral blood is higher than in fertile non-pregnant women (18). Several studies reported that miscarriage and preeclampsia are associated with a decreased number of Treg cells (4, 19–23). Interestingly, clonally expanded decidual Treg cells are decreased in preeclampsia but not in miscarriage (24). In light of this increasing evidence, successful pregnancy seems to require an appropriate functional change in cytotoxic $CD8^+$ T cells as well as a correct balance between cytotoxic $CD8^+$ T cells and Treg cells.

Paternal antigen-specific tolerance is necessary for the maintenance of allogeneic pregnancy (4). Previous studies have identified fetal antigen-specific $CD8^+$ T cells and Treg cells in mice (25, 26). However, the detection of fetal antigen-specific $CD8^+$ T cells and Treg cells is technically difficult in humans, because of the high diversity of CDR3 amino acid sequences in TCRβ, with a lower boundary of 2×10^7 in young humans (27). We have previously reported the existence of clonally expanded Treg cells by performing single-cell DNA sequencing of T cell receptor β (TCRβ) (24). The population size of clonally expanded Treg cells that are able to recognize fetal antigens at the feto-maternal interface is increased in the decidua, but not in the peripheral blood (24). In serial pregnancies, Treg cells expressing the same TCR clonotypes across different pregnancies were observed in the decidua, suggesting that these cells might recognize fetal antigens (24). The clonal population of decidual effector Treg cells is less abundant in preeclampsia than in normal late pregnancy, suggesting that paternal antigen-specific tolerance mediated by Treg cells might be disturbed in this condition (24). As $CD8^+$ T cells can recognize fetal antigens at the feto-maternal interface, we hypothesized that antigens recognizing $CD8^+$ T cells would be clonally expanded in the decidua, but that their function would be suppressed during human pregnancy. In addition, we postulated that the maldistribution or functional alteration of antigen-specific $CD8^+$ T cells could underlie pregnancy complications.

In this study, we analyzed the TCRβ repertoire of decidual and peripheral $CD8^+$ EM cells and $CD8^+$ N cells in women undergoing normal pregnancy and in cases of miscarriage or preeclampsia. We further examined the expression of PD-1 in these cells, to clarify whether antigen-specific $CD8^+$ T cells accumulated in the decidua, and to identify the mechanisms underlying their suppression during normal pregnancy, miscarriage, and preeclampsia. If decidual $CD8^+$ T cells recognize fetal antigens, $CD8^+$ T cells with the same TCRβ repertoire should be clonally expanded, and express elevated levels of PD-1 during normal pregnancy. Therefore, a difference in the proportion of antigen-recognizing $CD8^+$ cells or in the expression of PD-1 should be observable between normal pregnancy, miscarriage, and preeclampsia.

MATERIALS AND METHODS

Blood and Tissue Samples

Paired samples of peripheral blood mononuclear cells (PBMC) and decidual tissues were collected from 10 cases of artificial abortion in the 1st trimester (1st trimester normal pregnancy), 6 cases of miscarriage with normal fetal chromosomes in the 1st trimester (1st trimester miscarriage), 9 cases of uncomplicated pregnancy with delivery in the 3rd trimester (3rd trimester normal pregnancy), and 9 preeclampsia cases with delivery in the 3rd trimester (3rd trimester preeclampsia). As a control group, 6 samples of peripheral blood from age-matched healthy donors who had never been pregnant were collected. Written informed consent was obtained from all women in accordance with a protocol approved by the Ethical Review Board of the University of Toyama (Rin-28- 144). In the artificial abortion cases, the fetal heartbeat was confirmed before dilation and evacuation. For miscarriage cases, the diagnosis was formulated when the fetal heartbeat was lost or when the fetal heartbeat had not been detected inside the gestational sac for at least 2 weeks. In miscarriage cases, isolated chorionic villi were examined for

TABLE 1 | Demographic and clinical characteristics.

	Control	1st trimester		3rd trimester	
	No pregnancy history	Normal pregnancy	Miscarriage with normal fetal chromosomes	Normal pregnancy	Preeclampsia
	(*n* = 6)	(*n* = 10)	(*n* = 6)	(*n* = 9)	(*n* = 9)
Maternal age (years)[a]	30 (25–35)	26 (22–39)	34 (26–41)	32 (22–36)	36.5 (28–41)
Body Mass Index (kg/m^2)[a]	NA	18.6 (16.7–21.2)	21.4 (16.4–27.6)	18.4 (17.7–24.9)	23.7 (17.6–26.0)
Gravidity[a]	0 (0–0)	4 (1–7)	4 (3–7)	2 (1–3)	2 (1–5)
Parity[a]		2 (0–4)	0.5 (0–2)	1 (0–2)	0 (0–3)
Live birth[a]		2 (0–4)	0.5 (0–3)	1 (0–2)	0 (0–3)
Miscarriage[a]		0 (0–3)	2 (0–4)	0 (0–1)	0 (0–4)
Still Birth *n* (%)		0 (0.0)	0 (0.0)	2 (22.2)	0 (0.0)
Nullipara *n* (%)		3 (30.0)	3 (50.0)	4 (44.4)	6 (66.7)
Gestational week (weeks)[a]		8 (6–9)	8 (6–8)	38 (37–40)	35.5 (32–39)
Cesarean section (patient number) *n* (%)				5 (55.6)	5 (55.6)

[a]*Data are presented as median (range). NA: not available. Steel–Dwass test and Fisher's exact test were used for continuous and categorical variables, respectively. No statistically significant differences were observed between the groups.*

fetal chromosomal karyotype by G-band staining, and only cases with normal fetal chromosomes were enrolled. The diagnosis of preeclampsia was based on the guidelines of the International Society for the Study of Hypertension in Pregnancy (28). Both the peripheral blood (10 mL) and the decidual tissues were obtained at dilation and evacuation, or after vaginal delivery or elective cesarean section. First-trimester decidual samples were derived from uterine content. Third-trimester decidual tissues were macroscopically dissected from the maternal surface of the placenta. The clinical and demographic characteristics of the enrolled patients are summarized in **Table 1**.

Mononuclear Cell Isolation

Peripheral blood samples were layered on Ficoll Hypaque gradients (LymphoprepTM; Alere Technologies, Norway) for density gradient centrifugation (453 × g for 30 min). Mononuclear cells were isolated and washed twice with phosphate-buffered saline (PBS). Decidual tissues were rinsed thoroughly with PBS and minced into 1–2 mm pieces by a pair of scalpel blades in Dulbecco's Modified Eagle Medium. Then, the suspensions were filtered through a 32 μm nylon mesh as reported elsewhere (24). All samples were cryopreserved until single-cell analysis.

Single-Cell Sorting

The following monoclonal antibodies were used for cell staining: anti-CD3 (FITC; BD Biosciences, San Jose, CA, USA), anti-CD8 (APC; eBioscience, San Diego, CA, USA), anti-CD45RA (PE; BD Biosciences), anti-CCR7 (PerCP/Cy 5.5; BioLegend, San Diego, CA, USA), anti-PD-1 (PE/Cy7; BioLegend), and Fixable Viability Dye (APC-Cy7; eBioscience). Both PBMC and decidual cells were stained with anti-CD3, anti-CD8, anti-CD45RA, anti-CCR7, and anti-PD-1 for 20 min on ice and then incubated for 5 min with Fixable Viability Dye to exclude dead cells. After staining, the cells were washed with PBS. Flow cytometric analysis and single cell sorting were performed using a FACSAria II flow cytometer (BD Biosciences). $CD3^+CD8^+CD45RA^+CCR7^+$ cells (naive $CD8^+$ T cells; $CD8^+$ N cells) and $CD3^+CD8^+CD45RA^-CCR7^-$ cells (effector memory $CD8^+$ T cells; $CD8^+$ EM cells) were single cell sorted into 96-well PCR plates (**Supplementary Figures 1A,B**). PD-1 expression in each cell was analyzed by the index sort method (**Supplementary Figure 1C**) (29).

TCR Repertoire Analysis

TCR cDNAs were amplified from single cells using one-step RT-PCR, as previously described (30). All primers are listed in **Supplementary Table 1**. The contents of the PCR reaction mixture are listed in **Supplementary Table 2**. For the one-step RT-PCR, 5 μL of the RT-PCR mixture were added to each well containing a single $CD8^+$ T cell. The program for the one-step RT-PCR was as follows: 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, 72°C for 1 min. The amplification products were diluted 10-fold and 2 μL of each were added to 18 μL of the second PCR mixture. The PCR program for the second PCR cycle for TCRβ was as follows: 98°C for 1 min and 35 cycles of 98°C for 10 s, 52°C for 5 s, 72°C for 30 s. PCR products were electrophoresed to confirm their amplification (**Supplementary Figure 2A**) and then analyzed by direct sequencing. The TCR repertoire was analyzed with the IMGT/V-QUEST tool (http://www.imgt.org/). We defined $CD8^+$ T cells in which the same TCRβ clonotype was detected two or more times as clonally expanded populations (clonal populations), and $CD8^+$ T cells with a unique TCRβ clonotype as unique populations (**Supplementary Figure 2B**). The frequency of clonal populations and their PD-1 expression were compared in all groups.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 8 (GraphPad Software, San Diego, CA, USA). The differences

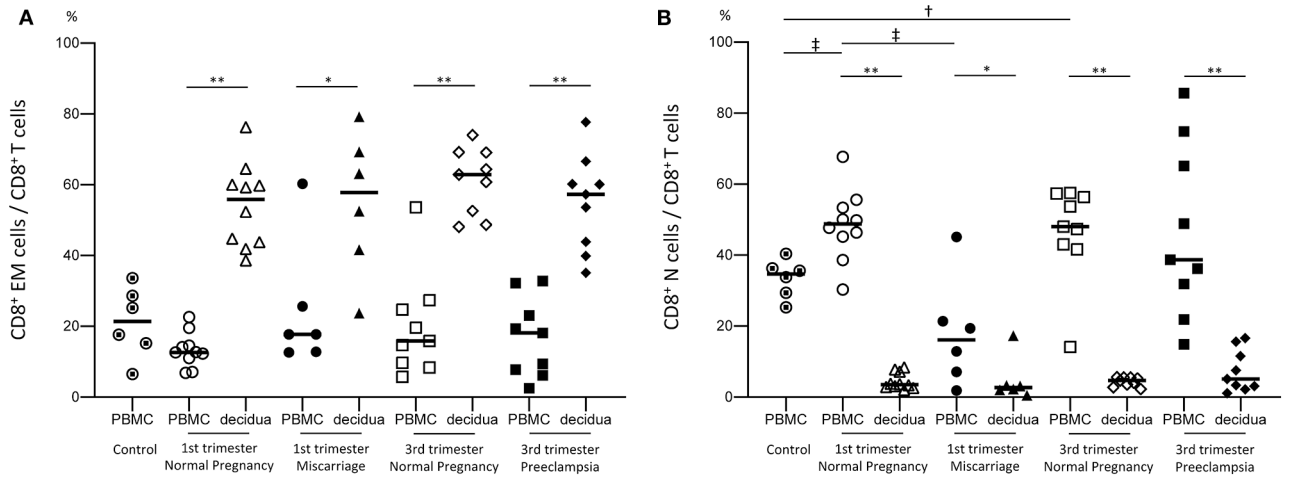

FIGURE 1 | Proportion of CD8$^+$ EM and CD8$^+$ N cells among total CD8$^+$ T cells. The proportion of CD8$^+$ EM cells among CD8$^+$ T cells **(A)** and that of CD8$^+$ N cells among CD8$^+$ T cells **(B)** are shown. Statistical analysis was performed using Wilcoxon matched-pairs single rank test (PBMC vs. decidua in each group); *$p < 0.05$; **$p < 0.01$. Mann-Whitney *U*-test (control vs. 1st or 3rd trimester normal pregnancy, 1st vs. 3rd trimester normal pregnancy, 1st trimester normal pregnancy vs. miscarriage, 3rd trimester normal pregnancy vs. preeclampsia); $^{\dagger}p<0.05$; $^{\ddagger}p<0.01$.

between PBMC and decidua in each group were assessed using the Wilcoxon matched-pairs single rank test. A Mann-Whitney U test was performed to determine the differences between PBMC or decidual samples of different groups via two-group comparisons (control vs. 1st trimester or 3rd trimester normal pregnancy, 1st trimester vs. 3rd trimester normal pregnancy, 1st trimester normal pregnancy vs. miscarriage, and 3rd trimester normal pregnancy vs. preeclampsia). $p < 0.05$ were considered indicative of statistical significance (*$p < 0.05$; **$p < 0.01$ in Wilcoxon matched-pairs single rank test; $^{\dagger}p < 0.05$; $^{\dagger}p < 0.01$ in Mann-Whitney U test; NS, not significant).

RESULTS

CD8$^+$ T Cell Phenotype in PBMC and Decidua

To examine functional differences between peripheral CD8$^+$ T cells (pCD8$^+$ T cells) and decidual CD8$^+$ T cells (dCD8$^+$ T cells), we compared the proportion of effector memory CD8$^+$ T cells (CD8$^+$ EM cells) and naive CD8$^+$ T cells (CD8$^+$ N cells) in the PBMC and decidua. A significantly higher number of CD8$^+$ EM cells was observed in the decidua compared to the PBMC throughout the pregnancy period in normal pregnancy subjects, miscarriage cases, and preeclampsia cases (**Figure 1A**). In contrast, CD8$^+$ N cells were significantly more abundant in the PBMC than in the decidua (**Figure 1B**). Therefore, dCD8$^+$ T cells showed a distinct phenotype compared to pCD8$^+$ T cells.

Clonal Populations of CD8$^+$ T Cells

To verify our hypothesis that clonally expanded CD8$^+$ T cells accumulate in the decidua, we analyzed the TCRβ clonotype of CD8$^+$ T cells and their clonality ratio. As shown in **Figure 2A** and **Supplementary Figure 3A**, clonally expanded CD8$^+$ T cell populations were observed both in the peripheral and decidual CD8$^+$ EM cells. However, they were rarely detected in CD8$^+$ N cells (**Figure 2B**, **Supplementary Figure 3B**). The clonality ratios of pCD8$^+$ EM and dCD8$^+$ EM cells among CD8$^+$ EM cells were similar in early pregnant subjects, miscarriage cases, late pregnancy subjects, and preeclampsia cases (**Supplementary Figure 3A**). However, as shown in **Figure 2A**, the total amount of clonally expanded CD8$^+$ EM cells among CD8$^+$ T cells in the decidua was significantly higher than in the peripheral blood.

The total proportion of clonally expanded dCD8$^+$ EM cells was significantly higher in miscarriage cases than in subjects with normal early pregnancy ($p < 0.05$). On the other hand, this population did not significantly differ between preeclampsia and normal late pregnancy (**Figure 2A**). These results demonstrated that dCD8$^+$ EM cells are likely to recognize fetal or placental antigens in the decidua and are clonally expanded. An increased proportion of clonally expanded CD8$^+$ EM cells was found to be associated with miscarriage.

Common TCRβ Clonotype Between PBMC and Decidua

In each subject the TCRβ clonotype of CD8$^+$ T cells was compared in paired PBMC and decidua to identify differential immunological functions (**Figure 3**). One representative sample of normal late pregnancy is shown in **Figures 3A–C** (case number #2). The proportion of clonally expanded CD8$^+$ EM cells was comparable in PBMC and the decidua (**Figure 3A**). TCRβ clonotypes of clonally expanded CD8$^+$ T cells and/or TCRβ clonotypes that are common to both pCD8$^+$ T cells and dCD8$^+$ T cells are shown in a row (A to Q) in **Figure 3B** (the different color density indicates the number of clones). Eight TCRβ clonotypes were shared between pCD8$^+$ EM cells and dCD8$^+$ EM cells (clonotypes A, B, C, D, I, N, O, and P in **Figures 3B,C**). Common TCRβ clonotypes between pCD8$^+$ EM cells and dCD8$^+$ EM cells

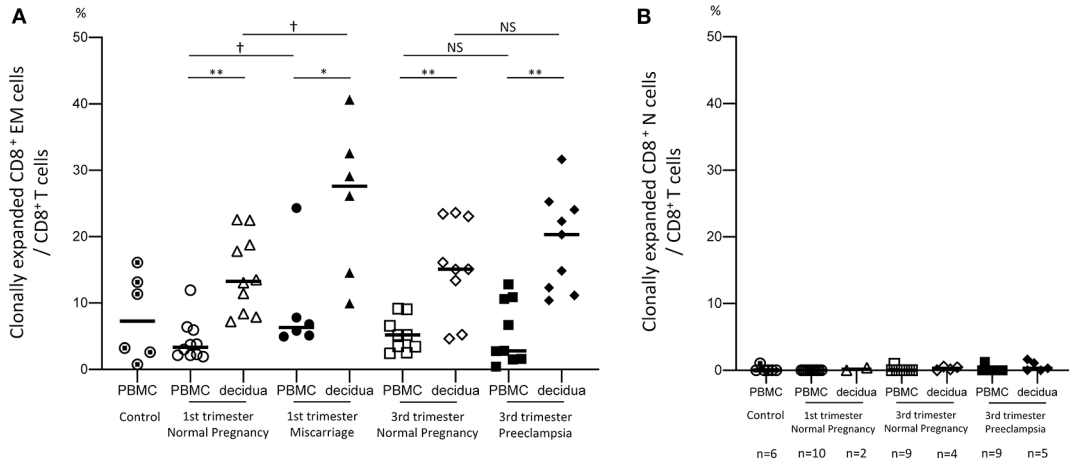

FIGURE 2 | Clonal populations of $CD8^+$ EM and $CD8^+$ N cells among total $CD8^+$ T cells. Clonally expanded $CD8^+$ EM cells **(A)** and $CD8^+$ N cells **(B)** among $CD8^+$ T cells. Due to their small amount, $CD8^+$ N cells could not be sorted in all samples. The number of cases analyzed in each group is shown under the horizontal axis. Statistical analysis was performed in $CD8^+$ EM cells **(A)** by using Wilcoxon matched-pairs single rank test (PBMC vs. decidua in each group); $^*p < 0.05$; $^{**}p < 0.01$. Mann-Whitney U test (control vs. 1st or 3rd trimester normal pregnancy. 1st vs. 3rd trimester normal pregnancy, 1st trimester normal pregnancy vs. miscarriage, 3rd trimester normal pregnancy vs. preeclampsia); $^\dagger p < 0.05$; NS not significant.

were detected in all groups (**Figure 3D**). We calculated the ratios of $pCD8^+$ EM cells and $dCD8^+$ EM cells expressing common TCRβ clonotypes among the total $CD8^+$ EM cells analyzed in each group. The ratios were comparable in all groups: 17.3% in 1st trimester normal pregnancy, 14.2% in miscarriage, 14.5% in 3rd trimester normal pregnancy, and 16.3% in preeclampsia (see the numbers above the bars in **Figure 3D**). These findings indicated that the immunological differences between normal pregnancy, miscarriage, and preeclampsia did not depend on the proportion of $CD8^+$ EM cells expressing common TCRβ clonotypes in PBMC and the decidua.

TCRβ Clonotype Comparison of the Serial Pregnancies

If decidual $CD8^+$ T cells recognize fetal antigens of paternal origin, $CD8^+$ T cells with the same TCRβ clonotypes should be detected in different pregnancies of the same couple. To verify this hypothesis, we examined the TCRβ clonotypes of peripheral and decidual $CD8^+$ EM cells in two pregnancies of the same subject with normal early pregnancy (**Figure 4A**). As shown in **Figure 4B**, the same TCRβ clonotypes were detected. Three TCRβ clonotypes (clone E, I, and J in **Figure 4B**) were shared by $pCD8^+$ EM cell populations during the two pregnancies. Twelve TCRβ clonotypes (clone F, L, M, N, O, R, S, T, U, V, W, and X in **Figure 4B**) were found in $dCD8^+$ EM cells from both pregnancies. Three TCRβ clonotypes (clone B, F, and K in **Figure 4B**) were shared between $pCD8^+$ EM cells from the former pregnancy and $dCD8^+$ EM cells from the subsequent pregnancy. Two TCRβ clonotypes (clone E and L in **Figure 4B**) were shared by $dCD8^+$ EM cells from the former pregnancy and $pCD8^+$ EM cells from the subsequent pregnancy; some clones were expanded in both pregnancies. $CD8^+$ EM cells exhibiting the same TCRβ clonotypes in different pregnancies might be able to recognize fetal antigens.

PD-1 Expression in $CD8^+$ T Cells

Finally, the expression of PD-1 was analyzed in $CD8^+$ T cells to identify immunological differences between normal pregnancy, miscarriage, and preeclampsia. Most $CD8^+$ N cells were PD-$1^{low/-}$ cells, both in the PBMC and the decidua (**Supplementary Figure 4C**). Among $CD8^+$ EM cells, the size of the PD-$1^{low/-}$ population was significantly lower in the decidua than in PBMC (**Supplementary Figure 4A**). In normal pregnancies, decidual PD-$1^{low/-}$ $CD8^+$ EM cells were significantly less abundant in late than in early pregnancy ($p < 0.05$) (**Supplementary Figure 4A**). When we focused on clonally expanded $CD8^+$ EM cell populations, significant differences in decidual PD-1 expression were observed between normal pregnancy and preeclampsia cases during late pregnancy (**Figures 5A,B**). The population size of PD-1^{high} $dCD8^+$ EM cells was significantly lower in preeclampsia than in normal late pregnancy ($p < 0.05$) (**Figure 5A**), indicating that the proportion of PD-$1^{low/-}$ $dCD8^+$ EM cells with high cytotoxic potential was increased in the clonal population of preeclampsia cases (**Figure 5B**). These findings indicated that $dCD8^+$ EM cells had a higher level of PD-1 expression compared to $pCD8^+$ EM cells, and that they begin to express PD-1 during pregnancy. An increased number of PD-$1^{low/-}$ $CD8^+$ EM cells would result in miscarriage, whereas, on the contrary, the downregulation of PD-1 in clonally expanded $CD8^+$ EM cells would be associated with preeclampsia.

DISCUSSION

This study is the first to analyze the TCRβ repertoire and the expression of PD-1 in $CD8^+$ T cells during

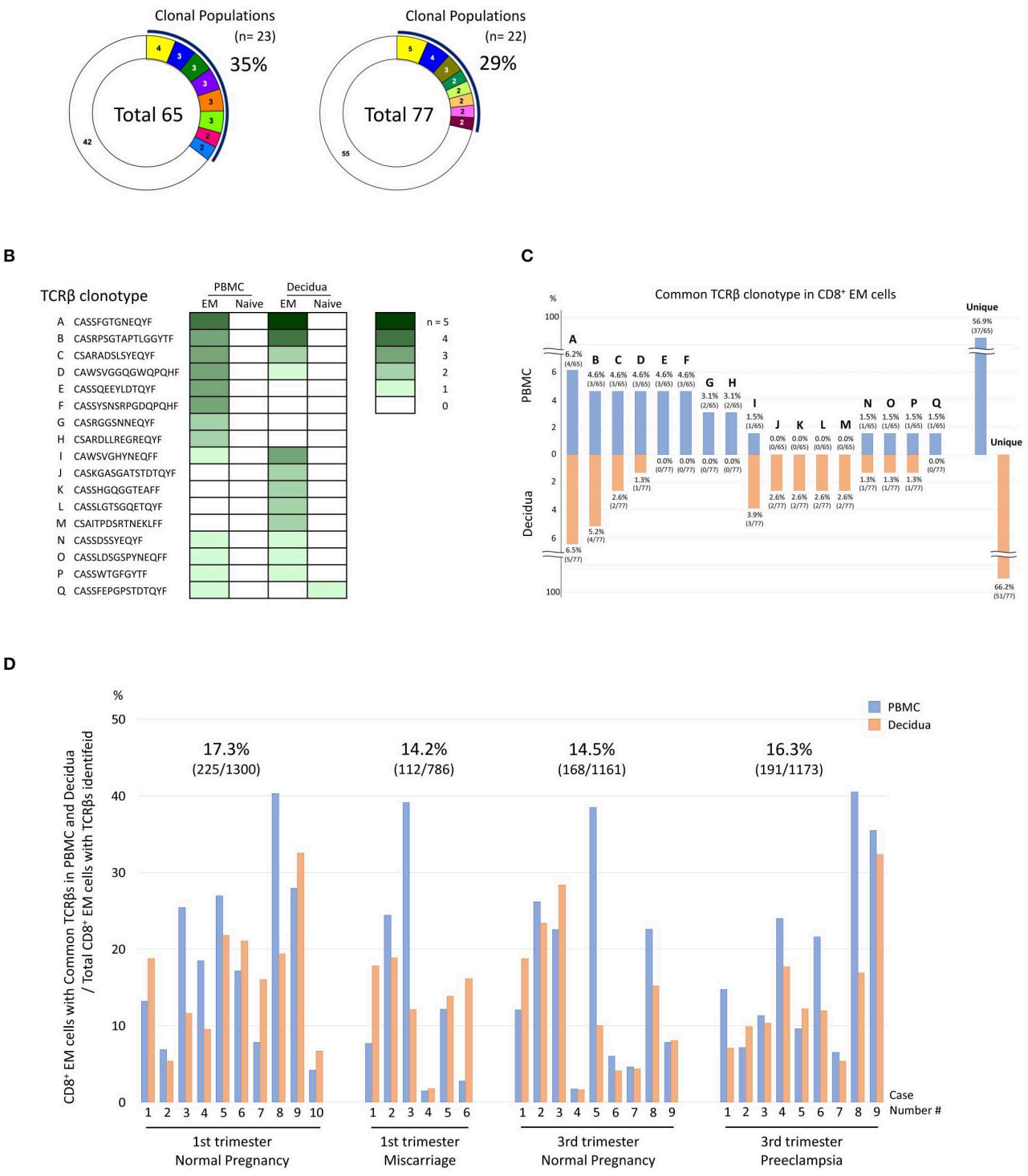

FIGURE 3 | Comparison of TCRβ clonotype between PBMC and decidua. **(A–C)** A representative example of normal late pregnancy (case number #2). **(A)** Analysis of the TCRβ repertoire in peripheral CD8$^+$ EM cells (pCD8$^+$ EM cells) and decidual CD8$^+$ N cells (dCD8$^+$ N cells). Each pie slice in color indicates the T-cell population expressing the same clonotypic TCRβ. The numbers of CD8$^+$ T cells expressing the same TCRβ are shown in the pie charts. The white slice in each pie chart indicates the T cell population with unique TCR. The number in the center of the pie charts is the total number of analyzed T cells. The proportion of clonal cells was calculated as follows: clonal population (%) = clonal CD8$^+$ T cells/total CD8$^+$ T cells analyzed. **(B)** The TCRβ clonotypes were compared between pCD8$^+$ EM,

(Continued)

FIGURE 3 | pCD8$^+$ N dCD8$^+$ EM, and dCD8$^+$ N cells. TCRβ clonotypes of clonally expanded CD8$^+$ T cells in PBMC or decidua and/or TCRβ clonotypes in common between pCD8$^+$ T and dCD8$^+$ T cells are shown in a row (A to Q) (the color density reflects the number of clones). **(C)** Clones of clonally expanded CD8$^+$ EM cells and/or clones that were shared between pCD8$^+$ EM and dCD8$^+$ EM cells are shown. The number above or under each bar is the percentage of the CD8$^+$ EM cells with a particular TCR clone among total CD8$^+$ EM cells with an identified TCRβ repertoire. **(D)** Comparison of the TCRβ clonotype between pCD8$^+$ EM and dCD8$^+$ EM cells in all cases. The blue bar indicates the CD8$^+$ EM cell population with common TCRβ clonotype among total CD8$^+$ EM cells with an identified TCRβ repertoire in PBMC; the orange bar indicates the corresponding population in the decidua. The total number of pCD8$^+$ EM and dCD8$^+$ EM cells expressing common TCRβ repertoires in all CD8$^+$ EM cells of each group was calculated (the numbers above the bars in each group).

pregnancy. The most abundant population of clonally expanded CD8$^+$ EM cells was observed in the decidua. The proportion of clonally expanded CD8$^+$ EM cells increased in cases of miscarriage, whereas PD-1 expression was downregulated in clonally expanded CD8$^+$ EM cells in preeclampsia cases.

CD8$^+$ T cells have a crucial role in immune tolerance at the feto-maternal interface. Previous studies have examined the distribution, phenotypes, gene, and cell surface protein expression, as well as functional properties of CD8$^+$ T cells in both normal and complicated pregnancies (5, 7, 16, 17, 31, 32). Local expansion of highly differentiated decidual CD8$^+$ T cells implies direct response to fetal-specific antigens. However, due to CD8$^+$ T cell heterogeneity, it is unclear whether these decidual CD8$^+$ T cells recognize fetal antigens. In recent studies, Zeng et al. performed a transcriptional analysis of paired pCD8$^+$ T and dCD8$^+$ T cell populations in the 1st trimester and revealed differences in gene regulation (33). Powell et al. identified fetal antigen-specific CD8$^+$ T cells, both in the peripheral blood and the decidua, using HY-specific dextramers in humans (6, 34). The proportion of HY-specific CD8$^+$ T cells was significantly increased in the decidua and most of them were CD8$^+$ EM cells expressing the co-inhibitory molecule, PD-1 (6). These findings support the notion that CD8$^+$ T cells recognizing fetal antigens exist but are functionally suppressed due to PD-1 expression at the feto-maternal interface. However, although minor populations of CD8$^+$ T cells can be detected by the MHC-multimer approach, the extent of diversity of antigen-specific CD8$^+$ T cells is still unclear. To our knowledge, this study is the first to examine the TCRβ repertoire of CD8$^+$ EM cells and CD8$^+$ N cells during pregnancy at the single-cell level. We also analyzed the clonotypes and PD-1 expression in these cells to identify tolerogenic differences between normal pregnancy, miscarriage, and preeclampsia.

Previous studies demonstrated that effector memory cells are the major subset of decidual CD8$^+$ T cells, whereas naive cells predominate in the peripheral blood (5). Our results were consistent with previous studies (**Figure 1**) (5); we found that the proportion of CD8$^+$ EM cells among decidual CD8$^+$ T cells neither differed between normal early pregnancy and miscarriage, nor between normal late pregnancy and preeclampsia (**Figure 1A**). These findings indicated that pregnancy failure was not due to alterations in the proportion of CD8$^+$ EM cells in early or late pregnancy.

Clonally expanded CD8$^+$ T cells were observed in the effector memory subset but not in the naive subset (**Figure 2**), reflecting previous TCR stimulation and CD8$^+$ T cell differentiation. A larger volume of clonally expanded CD8$^+$ EM cells was observed in the decidua than in PBMC, and in late pregnancy compared to early pregnancy (**Figure 2A**). This suggested that CD8$^+$ EM cells were expanded by antigen stimulation at the feto-maternal interface.

We observed common clonotypes between peripheral and decidual CD8$^+$ EM cells (**Figures 3B–D**), reflecting the presence of effector memory T cell signatures in the systemic circulation. The ratio of CD8$^+$ EM cells with common TCRβ clonotype was comparable in each group (**Figure 3D**), suggesting that the immunological differences between normal pregnancy, miscarriage, and preeclampsia did not depend on the proportion of CD8$^+$ EM cells expressing the same TCRβ clonotype in PBMC and the decidua. CD8$^+$ EM cells with common clonotypes in PBMC and the decidua might react with microchimeric fetal cells in the periphery (35–38).

If clonally expanded CD8$^+$ EM cells recognize fetal antigens, the same TCRβ clonotype should be detected in different pregnancies of the same couple. Indeed, we found that some clonotypes were maintained across different pregnancies of the same patient (**Figures 4B–D**). Because of the high diversity of the CDR3 amino acid sequence in TCRβ, with a lower boundary of 2×10^7 in young humans (27), these clonotype matches are unlikely to occur by chance. CD8$^+$ EM cells with these TCR clonotypes might recognize the same fetal or placental antigens. In case of HLA-C mismatch between mother and fetus, and in the presence, in consecutive pregnancies, of the same paternal HLA-C, this could be recognized by CD8$^+$ EM cells with the same TCRβ clonotype. Similarly, if the fetal sex of both pregnancies is male, the CD8$^+$ EM cells might be HY-specific. Further studies are necessary to determine the antigens recognized by clonally expanded CD8$^+$ EM cells. Another question to be addressed is whether clonally expanded CD8$^+$ EM cells increase in parous women. As shown in **Supplementary Figure 5**, no differences in the size of clonally expanded CD8$^+$ EM cell populations seemed to occur between nullipara and parous women. These findings suggested that the proportion of clonally expanded CD8$^+$ EM cells did not increase in subsequent pregnancies.

The proportion of decidual PD-1$^{low/-}$ CD8$^+$ EM cells was larger in normal early pregnancy than in normal late pregnancy (**Figure 5B**); therefore, dCD8$^+$ EM cells acquired PD-1 expression during late pregnancy. Wang et al. reported increased PD-1 expression in CD8$^+$ T cells after co-culture with trophoblasts (7), suggesting that cell-cell interactions may induce PD-1 expression in CD8$^+$ T cells at the feto-maternal interface. Other studies proposed the existence of CD8$^+$ regulatory T cells with high PD-1 expression (39, 40). Therefore, the clonally expanded PD-1^{high} CD8$^+$ EM cells detected in our study might be regulatory cells.

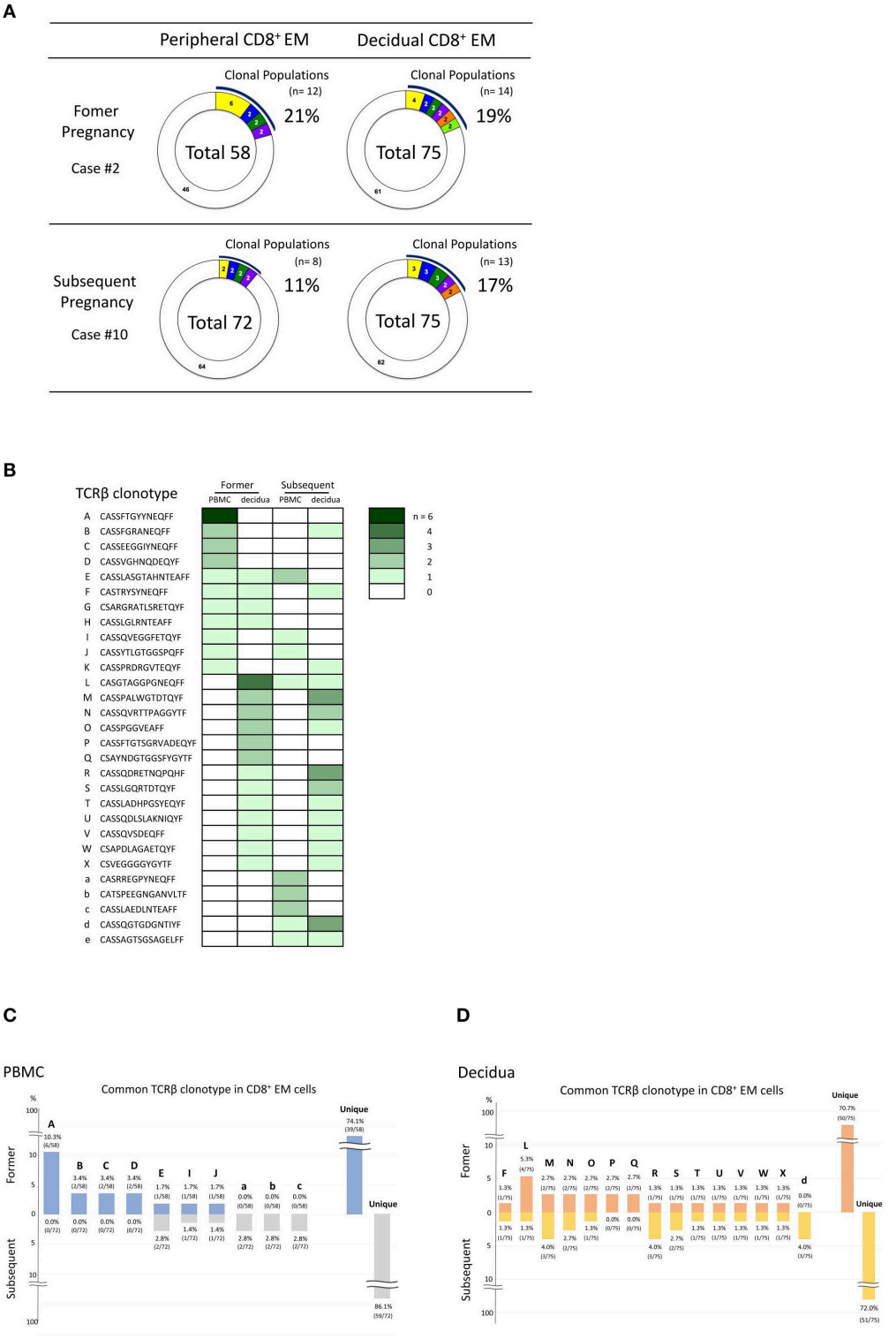

FIGURE 4 | Comparison of the TCRβ clonotype during normal early pregnancy in consecutive pregnancies of the same subject (case numbers #2 and #10, obtained from the same patient). **(A)** Analysis of the TCRβ repertoire in pCD8$^+$ EM and dCD8$^+$ EM cells in different pregnancies of the same series. **(B)** TCRβ clonotype comparison in different pregnancies of the same series. TCRβ clonotypes of clonally expanded CD8$^+$ EM cells in PBMC or decidua and/or TCRβ clonotypes in common between pCD8$^+$ EM and dCD8$^+$ EM cells are shown in a row (A to W, a to e) (the color density indicates the number of clones). Comparison of CD8$^+$ EM TCRβ clonotype between two different pregnancies in PBMC **(C)** and the decidua **(D)**. The number above or under each bar is the percentage of CD8$^+$ EM cells with a particular TCR clone among total CD8$^+$ EM cells with an identified TCRβ repertoire.

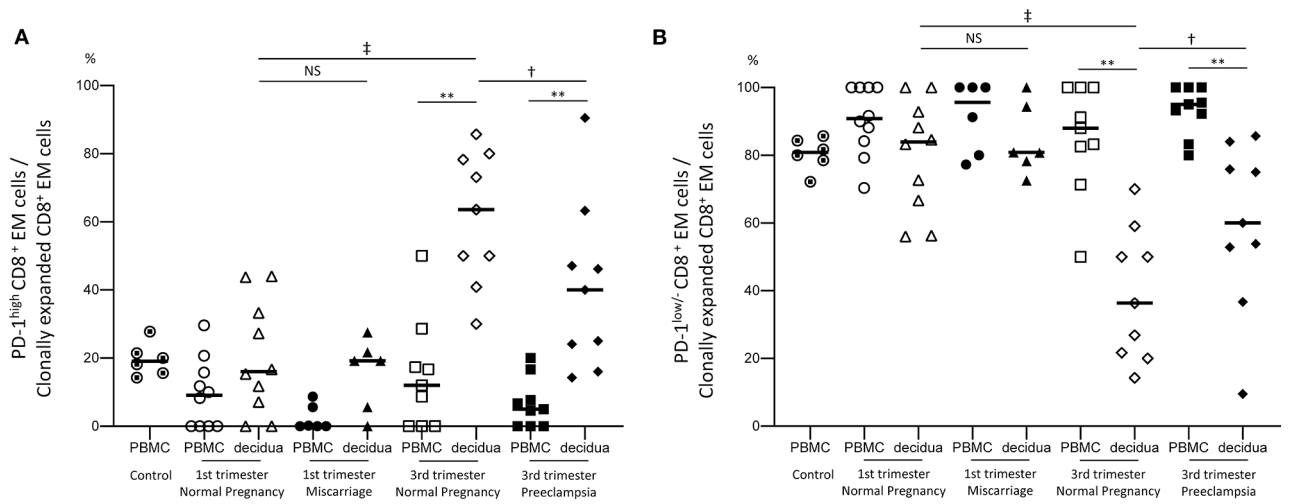

FIGURE 5 | PD-1^{high} and PD-1$^{low/-}$ CD8$^+$ EM cell populations. **(A)** PD-1^{high} CD8$^+$ EM cells among clonally expanded CD8$^+$ EM cells. **(B)** PD-1$^{low/-}$ CD8$^+$ EM cells among clonally expanded CD8$^+$ EM cells. Statistical analysis was performed using Wilcoxon matched-pairs single rank test (PBMC vs. decidua in each group); $^{**}p < 0.01$. Mann-Whitney U-test (1st vs. 3rd trimester normal pregnancy, 1st trimester normal pregnancy vs. miscarriage, 3rd trimester normal pregnancy vs. preeclampsia); $^{\dagger}p<0.05$; $^{\ddagger}p<0.01$; NS, not significant.

Clonally expanded CD8$^+$ EM cells exhibited informative differences between normal pregnancy, miscarriage, and preeclampsia in terms of clonality and PD-1 expression. In the 1st trimester, a significant increase in the proportion of clonally expanded dCD8$^+$ EM cells was observed in cases of miscarriage compared to normal pregnancy. In early pregnancy, most clonally expanded CD8$^+$ EM cells were PD-1$^{low/-}$. This suggested that an increase in antigen specific PD-1$^{low/-}$ cytotoxic CD8$^+$ EM cells at the feto-maternal interface might lead to miscarriage.

In contrast, in the 3rd trimester, the proportion of PD-1$^{low/-}$ CD8$^+$ EM cells among clonally expanded CD8$^+$ EM cells was significantly increased in preeclampsia cases compared to normal pregnancy, despite a similar proportion of clonally expanded CD8$^+$ EM cells (**Figures 2A**, **5B**). Remarkably, the amount of PD-1$^{low/-}$ CD8$^+$ EM cells with unique TCRs did not differ between preeclampsia and normal pregnancy (**Supplementary Figure 4B**). These data suggest that antigen-specific CD8$^+$ EM cells are less exhausted in preeclampsia.

We have previously reported that the total amount of decidual effector Treg cells is decreased in cases of miscarriage, whereas the size of clonal populations of decidual effector Treg cells is comparable in normal early pregnancy and miscarriage cases (24). On the other hand, the proportion of clonally expanded effector Treg cells in the decidua is lower in preeclampsia cases than in normal late pregnancy (24). Altogether, these observations indicate that the reduced proportion of non-specific decidual effector Treg cells and the increased proportion of clonally expanded PD-1$^{low/-}$ cytotoxic dCD8$^+$ T cells might lead to miscarriage in early pregnancy. In contrast, the decreased proportion of antigen-specific decidual effector Treg cells and the decreased expression of PD-1 in clonally expanded dCD8$^+$ T cells might induce fetal rejection in preeclampsia. These results suggested that, in preeclampsia, antigen-specific tolerance was disrupted both in Treg cells and CD8$^+$ T cells. This is in good accord with the epidemiology of human preeclampsia. The risk of preeclampsia increases in women at the first pregnancy following a partner change and after pregnancy intervals of more than 10 years (41–43). Increased risk of preeclampsia has also been reported in association with long-term condom usage and artificial insemination by donor, indicating that insufficient paternal antigen-specific tolerance mediated by seminal plasma priming may underlie preeclampsia (44–46). Pregnancy following oocyte donation, in which the fetus is completely allogeneic, associates with a significantly high risk of preeclampsia (45, 47). These epidemiological data demonstrate that the failure or lack of paternal antigen-specific tolerance may be responsible for preeclampsia. In addition, Barton et al. demonstrated that after the reencounter of fetal antigens by fetal antigen-specific CD8$^+$ T cells, PD-1 expression was more effectively promoted in parous mice than virgin mice (48). The exposure to fetal antigens could promote PD-1 expression in CD8$^+$ T cells, explaining the relatively high expression of PD-1 in late normal pregnancy. These epidemiological and experimental data suggest that the disruption of paternal antigen-specific tolerance in preeclampsia possibly affects PD-1 expression in CD8$^+$ T cells, consistent with the PD-1 downregulation that we observed in clonally expanded CD8$^+$ EM cells of preeclampsia cases.

Nevertheless, there are several limitations in this study. First, we assumed that clonally expanded CD8$^+$ T cells might recognize fetal-specific antigens, but the target specificity of TCRs from CD8$^+$ T cells has not been assessed. Second, although we assumed that clonally expanded dCD8$^+$ EM cells were cytotoxic, this assumption was not verified. Third, we focused on PD-1 expression, while alterations of other co-inhibitory molecules and cytokine expression have been reported in miscarriage and preeclampsia. Extensive analysis of

these factors as well as the TCR repertoire may help understand the immunological differences between normal pregnancy, miscarriage, and preeclampsia. An additional limitation is that the detrimental immune reactivity of $CD8^+$ T cells observed in miscarriage and preeclampsia may be partly due to different timing of sample collection (at 6–8 gestational weeks in miscarriage, and at 32–39 gestational weeks in preeclampsia), albeit this problem cannot be overcome.

In conclusion, $CD8^+$ EM cells might recognize some antigens at the feto-maternal interface, which are clonally expanded in the decidua. Clonally expanded $dCD8^+$ EM cells expressed PD-1 on their surface during late pregnancy, although most of them did not express PD-1 during early pregnancy. The total proportion of PD-$1^{low/-}$ clonally expanded $CD8^+$ EM cells increased in both miscarriage and preeclampsia cases, but the mechanisms behind this phenomenon were distinct. In miscarriage cases, the proportion of clonally expanded $CD8^+$ EM cells increased. On the other hand, in preeclampsia, clonally expanded $dCD8^+$ EM cells exhibited low PD-1 expression. Based on the results of this and our former study, we can conclude that, in miscarriage, the total proportion of decidual effector Treg cells decreased, while that of clonally expanded $dCD8^+$ EM cells increased. Moreover, in preeclampsia, the proportion of clonally expanded decidual effector Treg cells decreased and PD-1 expression was downregulated in the clonally expanded $dCD8^+$ EM cells. Thus, the recognition of fetal antigens by clonally expanded Treg cells and $CD8^+$ EM cells would easily induce fetal rejection. In future studies, we will attempt to clarify which antigens are recognized by clonally expanded TCRβ.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because the ethics review committee of the University of Toyama, which approved our protocol, did not give permission for data sharing. Requests to access the datasets should be directed to s30saito@med.u-toyama.ac.jp.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the 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 the University of Toyama (Rin 28- 144). All subjects gave written informed consent in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

SS and HK: conception and design. KM, ST, and AU: acquiring and processing samples. KM, EK, HH, and KT: execution of experiment. KM and ST: analysis of data. KM, ST, EK, HH, HK, and SS: interpretation of data. KM, ST, and SS: drafting manuscript. ST, EK, HH, TS, AN, HK, and SS: 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.2020.01082/full#supplementary-material

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REVIEW
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Regulatory T Cells in Pregnancy: It Is Not All About FoxP3

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In pregnancy, the semi-allogeneic fetus needs to be tolerated by the mother's immune system. Regulatory T cells (Tregs) play a prominent role in this process. Novel technologies allow for in-depth phenotyping of previously unidentified immune cell subsets, which has resulted in the appreciation of a vast heterogeneity of Treg subsets. Similar to other immunological events, there appears to be great diversity within the Treg population during pregnancy, both at the maternal-fetal interface as in the peripheral blood. Different Treg subsets have distinct phenotypes and various ways of functioning. Furthermore, the frequency of individual Treg subsets varies throughout gestation and is altered in aberrant pregnancies. This suggests that distinct Treg subsets play a role at different time points of gestation and that their role in maintaining healthy pregnancy is crucial, as reflected for instance by their reduced frequency in women with recurrent pregnancy loss. Since pregnancy is essential for the existence of mankind, multiple immune regulatory mechanisms and cell types are likely at play to assure successful pregnancy. Therefore, it is important to understand the complete microenvironment of the decidua, preferably in the context of the whole immune cell repertoire of the pregnant woman. So far, most studies have focused on a single mechanism or cell type, which often is the FoxP3 positive regulatory T cell when studying immune regulation. In this review, we instead focus on the contribution of FoxP3 negative Treg subsets to the decidual microenvironment and their possible role in pregnancy complications. Their phenotype, function, and effect in pregnancy are discussed.

Keywords: regulatory T (Treg) cells, pregnancy, preeclampsia, Tr1 regulatory cells, Th3 regulatory cells, HLA-G Treg, immune tolerance, recurrent pregnancy loss (RPL)

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PLACENTAL DEVELOPMENT AND IMMUNE EVASION BY TROPHOBLASTS

The most striking feature of pregnancy is that a semi-allogeneic fetus is tolerated by the maternal immune system. This is in sharp contrast with solid organ transplantation, where an allograft will be rejected by the patient's immune system unless the patient takes immunosuppressive drugs. Since direct contact between maternal and fetal cells occurs at the maternal-fetal interface in the placenta, it is thought that maternal immune cells in the placenta do not attack the fetal cells (trophoblasts) because of the tolerogenic microenvironment created by regulatory T cells (Tregs) and other immune cells.

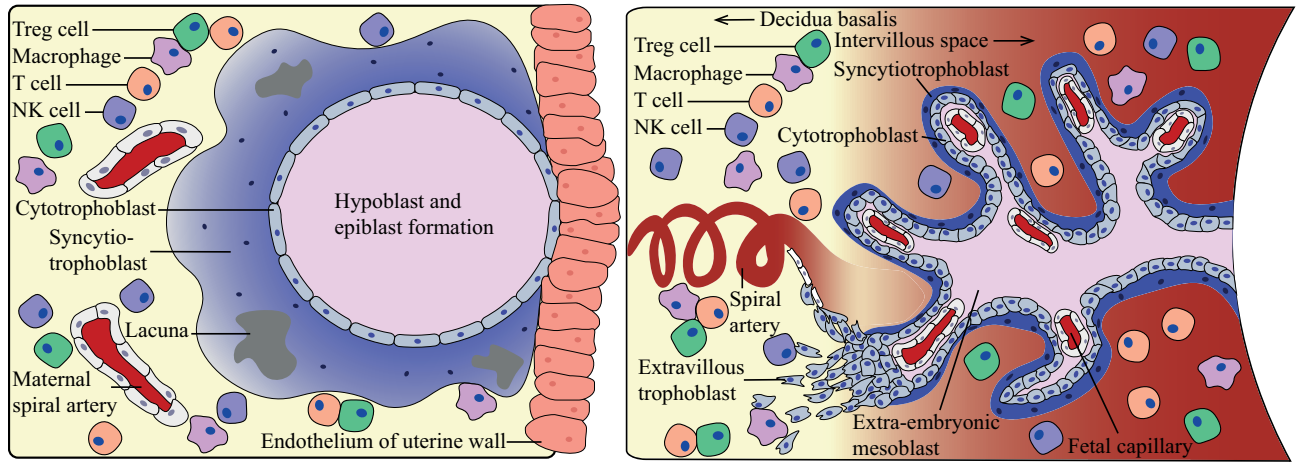

FIGURE 1 | Schematic overview of the maternal-fetal interface at different trimesters. **(A)** During first-trimester, the maternal immune cells in the decidua can come into contact with fetal syncytiotrophoblasts, when around weeks 11–12 the maternal blood flow commences **(B)** a second maternal-fetal interface occurs. The maternal immune cells in the periphery can come into contact with fetal syncytiotrophoblasts, while the maternal decidual immune cells are in contact with the fetal extravillous trophoblasts. Indicating immunotolerance needs to adapt during the shift in gestation.

Trophoblast Development

The main function of the placenta is to provide oxygen and nutrients to the developing fetus. In the first-trimester, nutrients are mainly provided by uterine glands in a hypoxic environment as no active maternal blood flow has been established yet. Once active maternal blood flow in the placenta has commenced around weeks 11–12 of gestation, oxygen and nutrients are exchanged over a thin lining of fetal cells. Since the fetus is semi-allogeneic, as it inherits both maternal and paternal antigens, the fetal trophoblast cells may potentially be recognized as foreign by maternal immune cells. Three main types of trophoblasts can be distinguished: cytotrophoblasts (CTBs), syncytiotrophoblasts (SCTs), and extravillous trophoblasts (EVTs). At the beginning of the first trimester, the maternal-fetal interface consists of the maternal parenchymal cells in the decidua and the fetal SCTs (**Figure 1A**). Later in pregnancy, this interface is mainly represented by maternal decidual cells and the EVTs (**Figure 1B**), where a distinction is made between decidua basalis and decidua parietalis. Importantly, a second maternal-fetal interface is established when active maternal blood flow in the placenta has commenced. The maternal peripheral blood then comes into contact with the SCTs lining the fetal villi. From the moment these maternal-fetal interfaces have been established, it is of utmost importance for maternal immune cells to keep the balance between tolerizing the semi-allogeneic fetus, and at the same time maintaining the ability to form a robust immune response against pathogens upon infection.

Abbreviations: Tregs, regulatory T cells; tTreg, thymic derived regulatory T cell; pTreg, periphery induced regulatory T cell; EVTs, cytotrophoblasts (CTBs), syncytiotrophoblasts (SCTs), and extravillous trophoblasts; HLA, human leukocyte antigen; APCs, antigen presenting cells; KIR, killer-cell immunoglobulin-like receptor; TCR, T cell receptor; IDO, indoleamine 2,3-dioxygenase; dNK, decidual NK; RPL, recurrent pregnancy loss; PE, pre-eclampsia; SNPs, single nucleotide polymorphisms; NK, natural killer; ILCs, innate lymphoid cell; DCs, dendritic cells; DC-10, tolerogenic DCs; mTOR, mammalian target of rapamycin; NO, nitric oxide; TGF-β, transforming growth factor-beta; IFN-γ, interferon gamma.

Mechanisms by Trophoblasts for Avoiding and Modulating Immune Responses

The classical human leukocyte antigen (HLA) class I molecules HLA-A, -B, and -C are normally present on virtually all nucleated cells in the body and present intracellular antigens to surveilling T cells. Non-classical HLA molecules are selectively present, and have initially been described on trophoblasts in the placenta (1) and later also in other tissues (2–4). HLA class II is mainly expressed by antigen-presenting cells (APCs), including dendritic cells (DCs), macrophages, and B cells. Since the fetus inherits half of its genes from the father, it also inherits half of the paternal HLA alleles, which can potentially be recognized as foreign by the maternal immune system.

One way for the trophoblasts to evade recognition by the maternal immune system is lack of the polymorphic HLA-A, -B, and HLA class II molecules on their cell surface. Interestingly, EVTs do express polymorphic classical HLA-C molecules. The regular function of these molecules is to present a wide variety of pathogen-associated peptides to surveilling $CD8^+$ T cells (5). Since HLA-C is polymorphic, its presence on trophoblasts can possibly also lead to allorecognition of the inherited paternal HLA-C by maternal T cells (6). EVTs may help to tip the local maternal immune balance toward tolerance by their expression of non-classical HLA-E and HLA-G (7), and possibly also HLA-F (8). The mechanisms responsible for the presence or absence of the specific HLA class I types on trophoblasts have not fully been elucidated yet (5). Expression of HLA molecules on trophoblasts allows them to escape natural killer (NK) cell recognition (9). HLA-G was first described on CTBs and has been shown to induce immune tolerance (10, 11) (described below). HLA-E also has tolerogenic properties as it can bind to the NK cell receptor

CD94/NKG2A upon which NK cell activity is inhibited (12). SCTs, which are in direct contact with the maternal blood, do not express any HLA molecules (13), which would potentially render them sensitive to NK cell-mediated killing (13). However, for NK cells killing an activating ligand needs to be present on the target cell, which is likely missing on trophoblasts (14).

Trophoblasts express several molecules that are thought to dampen alloimmune reactivity, including PD-L1, PD-L2, CD200, and FasL (15–19), some of which are differentially expressed throughout gestation (17). Trophoblasts are also known to produce soluble factors with an immune-modulatory action, such as soluble HLA-G (sHLA-G), transforming growth factor-beta (TGF-β), and indoleamine 2,3-dioxygenase (IDO). TGF-β is known to have various functions and will be extensively discussed below. Since IDO causes local tryptophan deprivation (20), which is an essential amino acid required for T cell activation, elevated local IDO levels lead to inhibition of T cell activation. Recently, the role of galectins in pregnancy has become more apparent, as they were found to play an important role in suppressing the maternal immune system (21). Galectins on human trophoblasts modulate a number of regulatory mechanisms (22), such as induction of T cell apoptosis (23) and induction of Treg cell development (24).

Maternal Immune Cells in the Decidua

Not only the composition of fetal cells in the placenta but also the composition of maternal immune cells changes throughout gestation. Already before conception, as early as seminal plasma exposure, activation and proliferation of fetus-specific maternal T cells in uterine draining lymph nodes have been observed in murine models (25). In humans, maternal APCs and $CD8^+$ T cells seem to get recruited to the ectocervix upon coitus, but their specificity remains unknown (26). In the first trimester of human pregnancy, maternal leukocytes account for 30–40% of all cells in the decidua (27). During this period, the most prominent immune cells are decidual NK (dNK) cells (∼60%), macrophages (∼20%), and T cells (∼10%) (27–29). During gestation, dNK cell frequencies decrease, macrophage frequencies remain relatively stable, and T cell frequencies increase (29). Next to these main immune cell populations, innate lymphoid cell (ILCs) other than NK cells, DCs, B cells, NKT cells, granulocytes, and mast cells are found in the decidua (30–32).

Despite the many mechanisms that trophoblasts have to evade an alloimmune response, fetus specific immune recognition has been observed in mice (33). Furthermore, fetus-specific $CD8^+$ T cells (34, 35) and inherited paternal antigen (IPA)-specific antibodies are found in maternal peripheral blood during pregnancy (36–38). Both HLA-C and HLA-E restricted $CD8^+$ T cells, specific for viral and bacterial peptides, are present in humans (39). However, maternal $CD8^+$ T cells could recognize the paternally inherited HLA-C from the fetus or fetal minor histocompatibility antigens, and if not suppressed are likely to attack the fetal trophoblasts (34, 35). Besides this, ∼30% of pregnancies result in the formation of paternal HLA-specific IgG antibodies (38, 40). Allo-antibodies directed against HLA-C of the fetus do not necessarily appear to be detrimental to pregnancy outcome (41), but some studies do show that they are associated with spontaneous preterm deliveries and recurrent pregnancy loss (RPL) (42, 43). Therefore, to inhibit the effect of maternal immune components, it is thought that local immune regulation is required to prevent anti-fetal immunity.

MATERNAL TREG CELLS DURING GESTATION

To prevent a detrimental immune reaction against the fetus, maternal immune cells need to be regulated. The level of both $FoxP3^+$ and $Foxp3^-$ Tregs is increased in the peripheral blood of pregnant women compared to non-pregnant control women (44, 45). While the proportion of total T cells in the decidua is low during the first trimester (∼10%), of which 10-30% of the $CD4^+$ T cells are Tregs (28, 29, 46, 47), later in pregnancy the proportion of Tregs significantly increases in the decidua [(46); van der Zwan et al. submitted]. In mice the importance of Tregs during implantation and for maintenance of a healthy pregnancy is evident. This was shown in murine studies by injecting abortion prone mice with $CD25^+$ Tregs from wild-type pregnant mice, which led to a significantly increased litter size (48). Alternatively, depleting $CD25^+$ Tregs during the implantation period of non-synergistically mated mice caused high fetal resorption (49). Depleting Tregs in the mid-gestation phase in non-sterile mice also resulted in high fetal resorption (50). In a systematic review of 17 studies on human pregnancy, it has been shown that the number and functionality of Tregs are diminished in women experiencing RPL, both in the peripheral blood and in the decidua, compared to control women (51). Similarly, in women with pre-eclampsia decreased Treg frequencies in both the periphery and the decidua and impairment in the signaling of peripheral blood Tregs have been found (45, 52, 53).

Using extensive mass cytometry panels containing more than 38 immune cell markers, we have previously shown that there is great heterogeneity in immune cell subsets among the different trimesters (van der Zwan et al. submitted). Interestingly, five Treg-like clusters were found to be differently distributed over the three trimesters. This could be attributed to the developmental changes in the placenta, causing a constant change in the possible cell-cell interactions between immune cells and different EVT subsets that seem to exist over different trimesters (54). Apart from that, a deficit in Treg presence and functionality has been observed in pregnancy complications such as PE, infertility, and RPL (55). Such complications arise at different periods of pregnancy, i.e., during implantation, <22–24 weeks of gestation or throughout gestation (56, 57). Taken together, as both Treg subsets and the initiation of complications can be prominent in a particular time frame of gestation, it might be that disbalances in different Treg subsets could play a role in the onset of different complications. Therefore, it is important to investigate the presence and functioning of the wide range of Treg subsets present during pregnancy.

ADVANCES IN TREG IDENTIFICATION

Regulatory T cells were originally named suppressor cells (58). Ideas and insights changed over time, and suppressor cells have endured much debate. In 1983 it was shown in mice that both

CD8 (Lyt-2$^+$) and CD4 (Lyt-2$^-$) suppressor cells were present that expressed the I-J molecule (59). When the I-J molecule turned out not to exist and suppressor cells could not be identified in any other way, interest in these cells waned. The arrival of novel molecular technologies propelled new knowledge, which made immunological tolerance become more evident and revived interest in T suppressor cells, now referred to as Tregs (60). In 2001, the *FoxP3* gene was identified in scurfy mice and later as a key transcription factor for Treg cell development and function in both humans and mice (61, 62). Subsequently, several FoxP3$^-$ Treg subsets were identified, as will be discussed below. Initially, it was hypothesized that Tregs could only be generated in the thymus (tTregs), but in the 2000s this concept was challenged by studies showing that Tregs could be induced from conventional T cells in the periphery (pTregs) (63, 64). It is thought that tTregs and pTregs function in distinct ways, recognize different types of antigens (autoantigens vs. foreign antigens), and are needed in different immunological events such as preventing T cell trafficking to an organ and preventing T cell priming by APC, respectively (65).

Because tTregs and pTregs can have different roles, there is a need for phenotypic markers to distinguish the two. While Helios and Nrp-1 have been proposed as markers for tTregs in mice (66, 67), it has been shown that Helios deficiency or Nrp-1 deficiency does not impede tTreg development (65, 68). Consequently, there is no consensus on which markers can distinguish tTregs from pTregs (65, 69, 70). Helios is associated with the promoter regions of apoptosis/cell survival genes, and Helios deficient FoxP3$^+$ Tregs show increased inflammatory cytokine expression, which suggests the importance of Helios in suppressing the production of effector cytokines (71). Even though Nrp-1 is not essential for tTreg development, it seems to increase Treg immunoregulatory properties, such as an increased capacity for tumor infiltration (69, 72). When comparing Nrp-1 and Helios there is no consistent overlap in expression of these markers (65). In humans, Helios is found on Tregs, but Nrp-1 is not found on peripheral blood Tregs and can, therefore, be excluded as tTreg marker (66, 73). More recently CNS1 has been suggested to distinguish between tTregs and pTregs. However, since CNS1 is a *FoxP3* enhancer, it is debatable whether this marker distinguishes FoxP3$^-$ tTreg and pTreg populations (74, 75).

Treg subsets are often identified by their co-signaling molecules. Many Treg subsets express co-signaling molecules, such as ICOS, PD-1, TIGIT, and TIM-3, which upon interaction with their ligand can alter their function to either activation or senescence (76–78). These co-signaling molecules, which can be present on both FoxP3$^+$ and FoxP3$^-$ Tregs, have widely been discussed in several reviews (79–81). Similarly, the heterogeneity within FoxP3$^+$ Tregs, generally described as CD4$^+$CD25$^+$CD127$^-$ in functional assays, has been extensively reviewed elsewhere (82–87). However, the heterogeneity within the FoxP3$^-$ compartment has not been elaborated on and will be discussed here in the context of pregnancy. Besides co-signaling molecules, several soluble factors affect the action of Tregs and are produced by these cells to mediate their immune regulatory effects. These will first be briefly reviewed.

SOLUBLE FACTORS

IL-10

IL-10 is an immunomodulatory cytokine that is produced by many immune cells in the decidua, including most known Treg subsets. It has an effect on trophoblasts and innate- and adaptive immune cells within the decidua (88). Single nucleotide polymorphisms (SNPs) in the promoter region of IL-10 correlate with adverse pregnancy outcomes in humans (89). Next to that, the administration of recombinant IL-10 or IL-10 producing B cells to mice leads to reduced incidence of fetal resorption (90). Concomitantly, IL-10 null mice in sterile cages showed normal litter size, whereas administration of a danger signal in the form of a low dose of LPS to these mice resulted in increased fetal resorption (91, 92). These data suggest that IL-10 is an important mediator of immune regulation during pregnancy. In human pregnancy, decreased serum IL-10 levels or IL-10 production by PBMCs are associated with the occurrence of PE and RPL (93–98). This suggests that IL-10 producing immune cells are important for maintaining an uncomplicated pregnancy.

IL-10 induces expression of HLA-G on trophoblasts, which has direct and indirect immune suppressive effects (described below) (99). IL-10, together with HLA-G, can induce monocyte-derived DCs *in vitro* to differentiate into tolerogenic DCs (DC-10) that have immunosuppressive properties (100, 101). They exert their immunosuppressive properties by the production of IL-10, expression of HLA-G, and upregulation of inhibitory receptors for HLA-G (namely ILT2, ILT3, and ILT4). Furthermore, these tolerogenic DCs downregulate co-stimulatory molecules CD80 and CD86, as well as HLA-DR (102–104). DC-10s induce Tregs by their expression of ILT4 and by IL-10 production (105). Macrophages are also regulated by IL-10 (106). It has been shown that IL-10 acts on macrophages by controlling their metabolic pathways, causing activation, proliferation, and inflammatory responses to be inhibited (106, 107). Next to that, CD4$^+$ T cell proliferation is suppressed by IL-10, antigen-experienced specific CD4$^+$ T cells can be induced into an anergic state, and conventional T cells can be induced to convert to Tregs (103, 108–110).

TGF-β

TGF-β is produced by and has an immunomodulatory effect on multiple cell types present in the decidua (111–120). In the early implantation phase, TGF-β is important for trophoblast invasion in the endometrium (121, 122). In humans, TGF-β serum levels are elevated in pregnant women compared to non-pregnant women, and serum levels are higher in early pregnancy compared to late pregnancy (123). However, women experiencing RPL display a decrease in TGF-β serum levels compared to women undergoing elective termination for non-medical reasons (124). Interestingly, there are indications from mouse studies that TGF-β induced Tregs could prevent spontaneous abortion, but this effect needs to be elucidated further (111, 125).

TGF-β can inhibit NK cell and T cell activation and proliferation by repressing the mammalian target of rapamycin (mTOR) signaling pathway (126, 127), and similarly, suppress activation of dNK cells (120). Since dNK cells are important

contributors to angiogenesis at the maternal-fetal interface, their cytotoxicity needs to be suppressed but they should still be able to execute their role in angiogenesis. A balanced TGF-β level may, therefore, be important to maintain correct functioning of dNK cells (120). Furthermore, TGF-β can affect T cells directly by inhibiting their proliferation and differentiation (128, 129), and indirectly by its inhibitory effect on APCs. HLA-class II on APCs is downregulated, activation of macrophages is downregulated, and maturation of DCs is prevented by TGF-β (116, 130–134). Next to that, the presence of TGF-β is needed for the induction of several FoxP3$^+$ and FoxP3$^-$ Treg subsets by APCs (135–138).

HLA-G

As discussed above, HLA-G was first described on trophoblasts (1). Interestingly, also myeloid and lymphoid cells, such as the below described FoxP3$^-$HLA-G$^+$ Treg, can express HLA-G and secrete sHLA-G (139–141). HLA-G is oligomorphic and has seven isoforms, of which some are membrane-bound (HLA-G1 to -G4), and others are secreted as a soluble form (sHLA-G5 to -G7) (142). Several polymorphisms in the untranslated region (UTR) of the *HLA-G* gene have been associated with lower sHLA-G levels in both blood and seminal plasma (143, 144). In both PE and RPL, a reduction in serum sHLA-G levels has been observed compared to healthy control women (145–148). Together these observations highlight the possible importance of (s)HLA-G during pregnancy.

(s)HLA-G exerts its immunoregulatory effects on a wide variety of immune cells because of its interactions with several inhibitory receptors, of which ILT2 seems to be most prominent (149). Other receptors for (s)HLA-G are ILT4, KIR2DL4, and CD8. The ITL2 receptor is expressed on monocytes/macrophages, DCs, B cells, and some NK and T cells (150), while the ILT4 receptor is mainly present on macrophages, NK cells, and neutrophils (150, 151). Upon ILT2 or ILT4 binding to HLA-G, NK cells and T cells receive a signal that leads to inhibited killing capacity (152–154). In CD8$^+$ T cells, this inhibited killing capacity is reflected by the down-regulation of granzyme B expression (155). KIR2DL4 has been identified on dNK cells and some T cell subsets. Engagement of this receptor with sHLA-G results in activation and secretion of different types of cytokines and chemokines, but does not result in direct cytotoxicity (156). Binding of sHLA-G with KIR2DL4 on NK cells results in the upregulation of a restricted set of chemokines and cytokines that can promote vascular remodeling (156). CD8 is not only expressed by cytotoxic T cells but also by some NK cell subsets (79, 157). When sHLA-G binds to CD8, this interaction inhibits cytotoxic activity and triggers FasL-mediated apoptosis in both the CD8$^+$ T cells and CD8$^+$ NK cells (158). Besides effector cells, APCs can also be affected by HLA-G. For example, in concert with IL-10, HLA-G induces DCs to differentiate into tolerogenic DC-10 cells (100, 101). Additionally, macrophages obtain a tolerogenic phenotype upon binding to HLA-G with their ILT2 or ILT4, and subsequently show reduced expression of HLA class II, CD80, and CD86. Such macrophages have been described to be similar to decidual macrophages as they also express IDO (159). Together this suggests that decidual macrophages are under the constant influence of HLA-G, produced by either trophoblasts or HLA-G$^+$ Tregs.

FOXP3$^-$ REGULATORY T CELLS

FoxP3$^-$ HLA-G$^+$ Tregs

In the lymphoid compartment, HLA-G expressing CD4$^+$ and CD8$^+$ cells show reduced proliferation in response to allogeneic and polyclonal stimuli (139). CD4$^+$HLA-G$^+$CD25$^-$FoxP3$^-$ Tregs (**Figures 2**, **5**, **Table 1**) suppress T cell proliferation through the expression of membrane-bound HLA-G1 and secretion of IL-10 and sHLA-G5 in a reversible, cell-contact independent and cell-contact dependent manner (139, 169). They have functionally been compared to other Treg populations such as FoxP3$^+$ Tregs and Tr1 Tregs (discussed below), and represent a population that is distinct from tTregs (169–171). Interestingly, CD4$^+$ and CD8$^+$ T cells can also acquire a similar HLA-G1$^+$ phenotype *in vitro* through trogocytosis (160), meaning the uptake of membrane fragments from another cell. Resting and activated CD25$^+$ T cells that acquire HLA-G1 expression by trogocytosis differ functionally from the HLA-G$^+$ tTregs, and they do not secrete sHLA-G5 and IL-10. They have been shown to exert their immune-suppressive capacity in a cell-contact dependent manner only (160), and will not be discussed further.

HLA-G$^+$ tTregs accumulate at sites of inflammation to regulate immune responses (172) and importantly, have also been found in the decidua (141, 173). CD4$^+$HLA-G$^+$ Treg frequencies are increased in peripheral blood throughout pregnancy compared to non-pregnant controls (45, 141). Interestingly, sHLA-G serum levels are also increased during pregnancy, while these levels are decreased in complicated pregnancies compared to healthy pregnancies (145–148). However, it is unlikely that a direct correlation between CD4$^+$HLA-G$^+$ Treg frequencies and serum sHLA-G levels exists, since other cells (in the placenta) produce sHLA-G as well. CD4$^+$HLA-G$^+$ Treg frequencies within the CD4$^+$ T cell compartment are even higher in the decidua compared to those in peripheral blood (141, 173), suggesting a role in local immune regulation. In women with PE, decidual CD4$^+$HLA-G$^+$ Tregs are decreased, whereas in the peripheral blood their numbers remain unchanged compared to healthy control pregnancies (45, 173), indicating that in a healthy pregnancy these cells are induced locally, but to a lesser extent during PE.

Tr1 Treg

Tr1 Tregs (**Figures 3**, **5**, **Table 1**) suppresses T cell proliferation mainly through IL-10 and TGF-β production. They also produce low amounts of IFN-γ, IL-5, and IL-2, and express granzyme B (109, 112, 174). Next to cytokine production, they can suppress other immune cells in a cell-contact dependent manner by using their KIR receptors or ectoenzymes (161). Tr1 Tregs are peripherally induced upon chronic antigen stimulation in the presence of IL-10 (175). Both HLA-G and IL-10 provided by APCs, like DC-10 cells, play a role in Tr1 Treg induction (103),

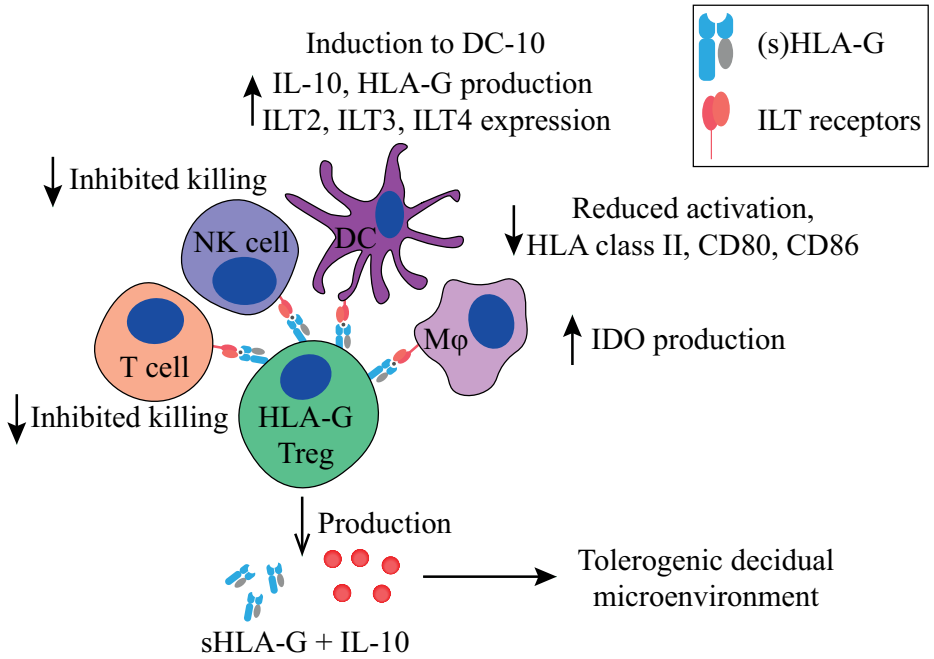

FIGURE 2 | Main tolerogenic mechanisms of the FoxP3^{-}HLA-G^{+} regulatory T cell. HLA-G^{+} regulatory T cells can suppress in a cell-contact dependent manner via HLA-G1. It inhibits the killing capacity of T cells and NK cells, downregulates HLA class II, CD80, and CD86 in DCs and macrophages, and makes them tolerogenic by inducing IDO production in macrophages and the induction of DCs to DC-10 cells. HLA-G^{+} regulatory T cells produce IL-10 and soluble HLA-G5 that helps to create a tolerogenic decidual microenvironment. HLA, human leukocyte antigen; NK, natural killer cell; DC, dendritic cell; IDO, indoleamine 2,3-dioxygenase; IL-10, interleukin-10; DC-10, tolerogenic DCs.

indicated by their reduced induction by DC-10s when anti-HLA-G is added *in vitro*. Additionally, their induction is reverted when agonistic anti-ILT4 antibodies are added, but not when agonistic anti-ILT2 antibodies are added (103). Interestingly, EVTs are also able to induce Tr1-like cells via HLA-G directly (119).

Recently, co-expression of CD49b and LAG-3 has been described as phenotypic markers for Tr1 Tregs in mice and humans (176). This observation is under debate since a subsequent study only detected a small proportion of IL-10^{+} Tregs co-expressing CD49b and LAG3 (177). Due to their lack of a clear phenotype, Tr1 Tregs are often described as Tr1-like cells, as they have similar properties, such as IL-10 production. Tr1 Tregs can express the co-signaling molecules PD-1, CTLA-4, TIM-3, and ICOS (136, 177–179), and several other molecules related to their function, including GARP, LAP, ectoenzyme CD39, and CD73 (180), as well as KIRs and ILT receptors. FoxP3 is only transiently expressed by Tr1 Tregs. Since functional Tr1 Tregs are found in patients who have a mutation in the *FoxP3* gene, FoxP3 appears not to be required for their development (110, 174).

Tr1-like Tregs have been identified in peripheral blood and various tissues (181), including the human decidua (119). These Tregs express high levels of PD-1, express granzymes, and lack FoxP3. They produce IL-10 and IFN-γ, and thereby may have a similar suppressive mechanism as bona fide Tr1 Tregs (119). Similar to Tr1 Treg, decidual Tr1-like Treg induction by EVTs can be partially reverted when agonistic anti-HLA-G antibodies are added, but not by anti-ILT2 (119). Tr1 Tregs are able to selectively lyse APCs in a cell-contact dependent manner, but not B and T cells (161). Lysis of APCs can cause amplification of the tolerogenic process since decreased numbers of activated APCs will generally lead to less activation of T cells. For this, the Tr1 Treg needs HLA-class I recognition of the APC through its KIR receptors, CD54/LFA-1 mediated adhesion, CD58/CD2 interaction, as well as CD155/CD226 ligation (161). Furthermore, the Tr1 has been described to directly affect T cells by their expression of ectoenzyme CD39 and CD73, which disrupts the metabolic state of effector T cells (180).

Th3 Tregs

The main suppressive effects of Th3 Tregs (**Figures 4**, **5**, **Table 1**) are mediated by TGF-β production, in a cell-contact independent manner (135). Phenotypically these cells are CD25^{-} and FoxP3^{-}, they are thought to express Helios, and express LAP and GARP, which can be used as surrogate markers for TGF-β production (182, 183). Th3 cells also produce IL-10, but unlike Tr1 Tregs, they produce this in conjunction with IL-4 (113, 184). Similar to Tr1 Tregs, Th3 Tregs are peripherally induced upon antigen stimulation (135). The mechanism underlying the induction into either Th3- or Tr1 Treg remains poorly understood and is thought to depend on their microenvironment during priming (114, 185). Another question that remains to be answered is whether Tr1 and Th3 Tregs truly represent different subsets or differentiation states and whether they differ depending on the microenvironment in which they reside.

With the limited markers identified so far, it is difficult to phenotypically identify Th3 Tregs, which may explain the limited number of articles describing the presence of the Th3

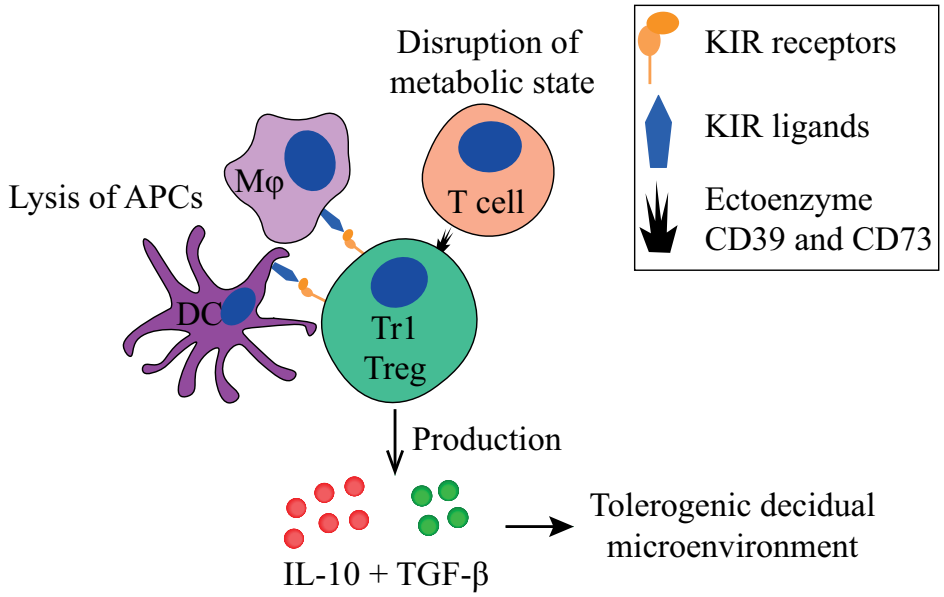

FIGURE 3 | Main tolerogenic mechanisms of the Tr1 regulatory T cell. Tr1 regulatory T cells can in a cell-contact dependent manner lyse APCs via their KIR receptors and disturb the metabolic state of T cells. They produce IL-10 and TGF-β that helps to create a tolerogenic decidual microenvironment. APC, antigen-presenting cell; KIR, killer-cell immunoglobulin-like receptor; IL-10, interleukin-10; TGF-β, transforming growth factor-beta.

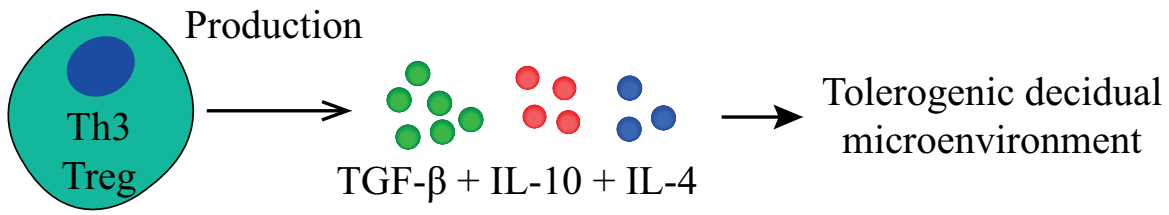

FIGURE 4 | Main tolerogenic mechanisms of the Th3 regulatory T cell. Th3 regulatory T cells suppress in a cell-contact independent mechanism only by the production of TGF-β, IL-10 and differ here from the Tr1 regulatory T cell by the production of IL-4. TGF-β, transforming growth factor-beta; IL, interleukin.

cell during pregnancy. Dimova et al. observed in paired decidua and peripheral blood samples mRNA cytokine profiles similar to Th3, the first description of a possible presence of Th3 cells in the decidua (163). Importantly, no functional testing has been performed for Th3-like cells from the decidua, and their presence and role in pregnancy remains to be confirmed. Regardless, Th3 Treg was first described to have an important role in oral tolerance (182). Interestingly, exposure to semen through oral sex has been proposed to be beneficial for subsequent pregnancy outcomes in couples experiencing PE or RPL (186–188), providing a possible mechanistic explanation for this effect.

Other Treg Populations

Besides FoxP3$^-$ HLA-G$^+$, Tr1, and Th3 Tregs, other immune regulatory T cell populations that have been described, albeit to a lesser extent, include CD8$^+$ Tregs, nitric oxide (NO) induced FoxP3$^-$ Tregs, TIGIT$^+$ Tregs, FoxP3^{dim} Tregs, and γδ T cells (**Figure 5**, **Table 1**).

CD8$^+$ Tregs are increasingly being recognized, even though they remain difficult to identify as there is no consensus on their phenotype. Both FoxP3$^+$ and FoxP3$^-$ CD8$^+$ Tregs have been described to have suppressive activities, indicating there also is heterogeneity in the CD8$^+$ Treg population (189). Shao et al. showed that a CD8$^+$ Treg subset can be activated by trophoblast cells. This activation appears not to be HLA restricted since their expansion is unaffected when cultured in the presence of pan-HLA class I blocking antibodies (164). When cultured with PBMCs, these CD8$^+$ Tregs suppress the secretion of immunoglobulins in a cell-contact dependent manner, as shown using a trans-well system. While humoral immunity seemed to be dampened, these CD8$^+$ Tregs did not have any suppressive effect on effector T cells. Phenotypically these cells can be identified as being CD101$^+$ and CD103$^+$ (164). Even though in a mixed lymphocyte reaction these CD8+ Tregs do not appear to suppress CD4$^+$ and CD8$^+$ T cells, they could potentially be important for preventing formation and suppressing production of IPA-specific antibodies.

TABLE 1 | Overview of FoxP3$^-$ immune regulating T cells discussed in this review, how they are induced or activated, their main suppressive mechanism and how they function, their localization, animal models depletion assays, master genes for differentiation, and cell volume changes in complicated pregnancies.

Subset	Induction/ activation	Suppressive mechanism	Function	Localization	Depletion in animal models	Master genes of differentiation	Cell volume changes in complications
CD4$^+$HLA-G$^+$ Treg (139, 160)	Natural occurring (139)	Secretion of sHLA-G and IL-10 (139), and cell interaction with HLA-G (160)	Induction of HLA-G expression by trophoblasts, DC-10s and Tregs by IL-10 Inhibition of macrophages, NK cells and T cell killing	Found in peripheral blood (45) and decidua (141)	Has not been performed	Not known	Found to be increased in peripheral blood of pre-eclampsia patients (45)
Tr1-(like) Treg (109, 119)	Via trogocytosis (160)	Secretion of IL-10 and TGF-β, and cell interaction (136, 161)	Induction of HLA-G expression by trophoblasts, DC-10s and Tregs by IL-10 Lysis of APCs, disruption of metabolic state of T cells	Found in peripheral blood and decidua (119)	Has not been performed	Not known	Has not been described
Th3 Treg (162)	By APC in an IL-10 dominant microenvironment (110)	Secretion of TGF-β and IL-10 (162)	Induction of HLA-G expression by trophoblasts, DC-10s and Tregs by IL-10 Inhibition of NK cell and T cells and APC by TGF-β	Found in the decidua (163)	Has not been performed	Not known	Has not been described
CD8$^+$ Treg (59)	By APC in presence of TGF-β and IL-4 (113, 135)	Suppress the secretion of immunoglobulins (164)	Prevent formation and suppressing production of IPA-specific antibodies.	Found in peripheral blood (CD8$^+$HLA-G$^+$ Treg) (45) and decidua (164)	Has not been performed	Not known	CD8$^+$HLA-G$^+$ Treg are increased in peripheral blood of pre-eclampsia patients (45)
NO-Treg (165)	CD101$^+$CD103$^+$ are induced by trophoblasts (164)	Secretion of IL-10 (165, 166)	Induction of HLA-G expression by trophoblasts, DC-10s and Tregs by IL-10.	Found in peripheral blood (165)	Has not been performed	Not known	Has not been described
TIGIT$^+$ Treg (119)	Depends on nitric oxide, p53, IL-2, and OX-40 (165)	Secretion of IFNγ and IL-2 (119)	Induction of IL-10 production by APCs. Suppression of CD4$^+$ effector T cells	Found in decidua (119)	Has not been performed	Not known	Has not been described
Vδ1$^+$ γδ T cell (167)	Unknown	Secretion of IL-10 and TGF-β (115)	Induction of HLA-G expression by trophoblasts, DC-10s and Tregs by IL-10 Inhibition of NK cell and T cells and APC by TGF-β	Found in peripheral blood and decidua (168)	Has not been performed	Not known	Decreased amount in an abortion prone mice model (111)

Niedbala et al. described NO-induced Tregs (NO-Tregs) in mice (165). These cells are characterized as CD4$^+$CD25$^+$GITR$^+$CD27$^+$T-bet^{low}, GATA3$^+$, and FoxP3$^-$, and they are induced from CD4$^+$CD25$^-$ T cells via p53, IL-2, and OX-40 (165). Experimentally, the development of NO-Tregs was induced when using adoptive transfer of CD4$^+$CD25$^-$ T cells into SCID mice, together with application of an NO synthase inhibitor. NO-Tregs produce IL-4 and IL-10, but no IL-2, TGF-β, or IFN-γ. Addition of antagonistic anti-IL4 antibodies led to reduced proliferation of NO-Tregs, whereas blocking IL-10 blocked their suppressive effect on CD4$^+$CD25$^-$ cell differentiation (165, 166). These data suggest that NO-Tregs suppress through IL-10, in a cell-contact independent manner. While NO-Tregs has not yet been studied in the context of pregnancy, NO appears to be involved in pregnancy with NO levels fluctuating throughout the different gestational ages and being lower during PE (190–193). It would, therefore, be interesting to retrospectively study first-trimester blood samples of women who develop PE, to test if NO levels are already lower at this early time point of pregnancy, and to study NO-Treg formation in these patients in comparison to healthy controls.

Salvany-Celades et al. identified three types of functional Tregs in the decidua, of which two subsets were negative or low for FoxP3 (119). One of these is the PD-1^{high}, Tr1-like cell, which has been described above. The second is the

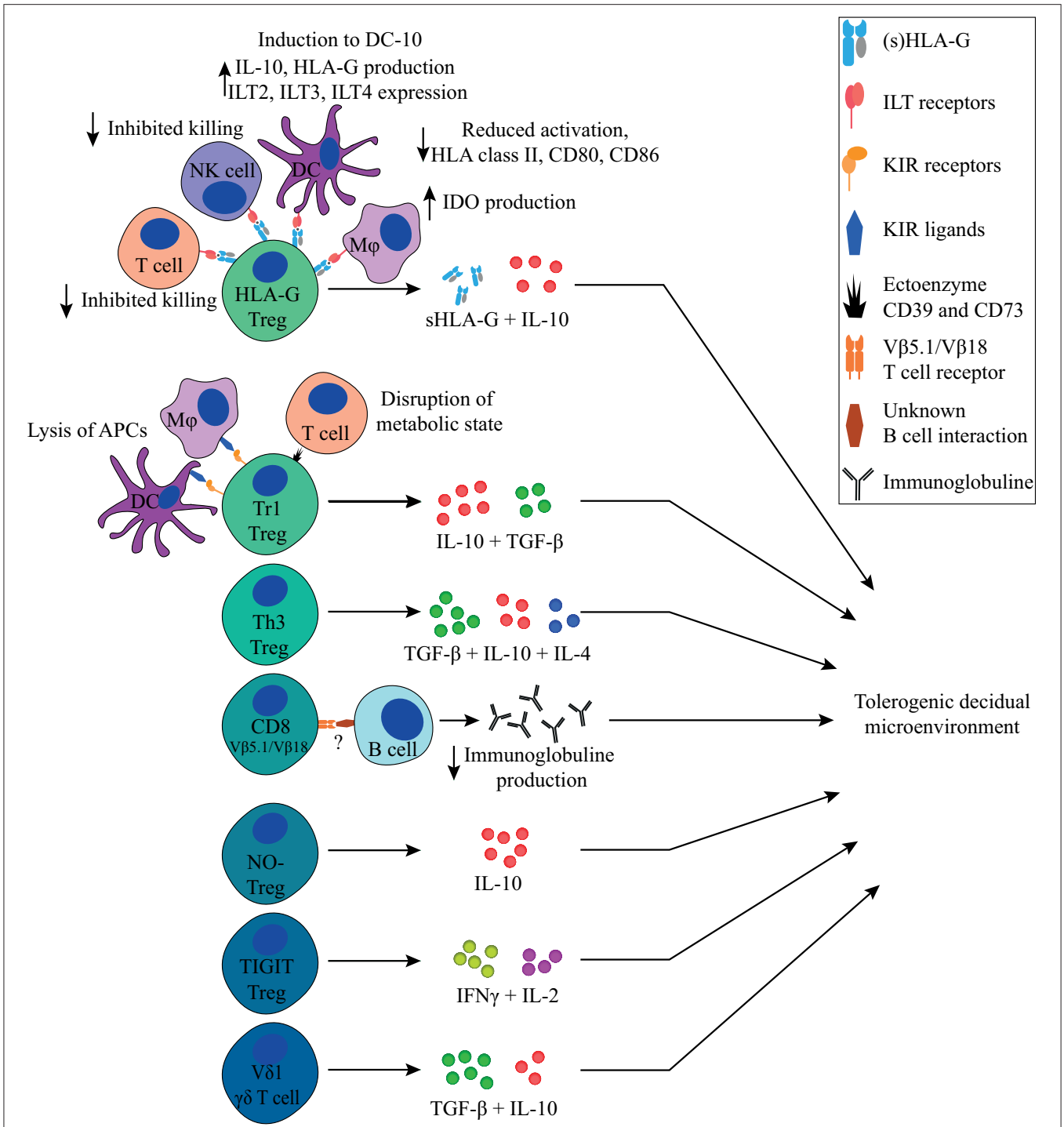

FIGURE 5 | Overview of FoxP3^{-} immune regulating T cells discussed in this review and their main tolerogenic mechanisms in pregnancy. All Tregs described in this review can exert immunosuppressive properties in a cell-contact independent mechanism that together contributes to a tolerogenic decidual microenvironment. Next to that, the HLA-G^{+} Treg, Tr1 Treg, and CD8^{+} Treg can exert their immunosuppressive properties in a cell-contact dependent mechanism.

TIGIT^{+} Treg that is characterized by TIGIT positivity, low expression of CD25 and FoxP3, and intermediate expression of PD-1. TIGIT^{+} Tregs express high levels of IFN-γ and IL-2, and low levels of IL-10. TIGIT^{+} Tregs mainly suppress CD4^{+} effector T cells in proliferation assays, but not consistently CD8^{+} effector T cells. Interestingly, TIGIT^{+} Tregs seem to vary in their characteristics, depending on the trimester in which they are encountered (119): first-trimester TIGIT^{+}

Tregs show an increased expression of IL-10 compared to term TIGIT$^+$ Tregs. This difference in trimesters could be due to the microenvironment influencing their phenotype, or because they truly represent different subsets. TIGIT has been described to be expressed on multiple Treg subsets, and it can bind CD155 on APCs, which thereby increases their IL-10 production (194, 195). Binding of TIGIT induces Tregs to produce IL-10 and fibrinogen-like protein 2 (Fgl2). By usage of Fgl2 the Tregs obtain the capacity to suppress Th1 and Th17 cells *in vitro*, but not Th2 cells (77, 195). It would be interesting to determine the presence of TIGIT$^+$ Tregs during pregnancy complications and to investigate their possible role in providing a tolerogenic microenvironment in successful pregnancies.

In the first-trimester decidua, γδ T cells produce high amounts of IL-10 and TGF-β (115, 196). As described above, these cytokines are important for establishing an immune suppressive microenvironment in the decidua. Transfer of uterine γδ T cell culture supernatant, containing a high concentration of TGF-β, into the uterus of mice before pregnancy prevents fetal resorption (111). Terzieva et al. identified the TCR repertoire from decidual γδ T cells and compared this to the repertoire of γδ T cells in peripheral blood. In 1st and 3rd trimester decidua they mostly found Vδ1$^+$ TCR, whereas this particular δ chain was hardly present in the peripheral blood (168). Vδ1$^+$ T cells are described to have a tolerogenic effect (167, 197). The possible role of γδ T cells in pregnancy is further suggested by another study showing higher numbers of γδ T cells in peripheral blood from women experiencing RPL compared to controls. The specific presence of the Vδ1 chain was not investigated (198). It would be interesting to determine the frequency and immune-suppressive effect of Vδ1$^+$ T cells in the decidua of women experiencing RPL compared to women with elective termination of pregnancy.

CONCLUDING REMARKS

In this review we have discussed several types of Tregs that may contribute to a tolerogenic environment in the decidua (**Figure 5**, **Table 1**) besides FoxP3$^+$ Tregs. Decidual Tregs seem to assist other cells in creating and maintaining a microenvironment where inflammatory signals are generally overruled by tolerogenic signals. Next to Tregs, this tolerogenic microenvironment is established and maintained by factors from paternal, maternal and fetal origin. Paternal contribution to this tolerogenic microenvironment comes early on from seminal fluid that contains tolerogenic factors such as TGF-β and paternal antigens for priming. Fetal trophoblasts contribute by their expression of tolerogenic HLA-G and HLA-E molecules, galectins, and PD-L1, and by their production of sHLA-G, IDO, and TGF-β. Next to this, the maternal contribution in maintaining a tolerogenic microenvironment in the decidua is provided by the decidual immune cells, which do not have an activated phenotype and produce IDO, TGF-β, IL-10, and sHLA-G.

It remains to be elucidated which mechanisms exactly attract Tregs to the decidua, if they are activated locally by APCs in the decidua or in the lymph nodes, where they proliferate, and if they are specific for fetal antigens. In mice, it has been shown that fetus-specific Tregs are already detectable in the uterine draining lymph nodes shortly after semen exposure and that their numbers increase upon pregnancy (199). While this could be similar in the human situation, *in vitro* fertilization with donor semen, where there is no paternal semen exposure, often results in a healthy uncomplicated pregnancy, albeit at a lower rate than in naturally conceived pregnancies (200). More information on the basic mechanisms of FoxP3$^-$ Tregs, as well as how they are initiated, is needed to provide insight in the deviations in frequencies or functionality of FoxP3$^-$ Treg subsets in pregnancy complications. Likewise, from a therapeutic point of view such basic mechanisms need to be clarified before possible novel therapeutic strategies can be developed. These therapies could be based on therapy designs similar to those proposed for FoxP3$^+$ Tregs, such as infusion of Tregs or application of the cytokines needed for induction of specific Treg subsets (201).

While it is clear that FoxP3$^+$ Tregs play a role in maintaining pregnancy, the relevance of the different types of FoxP3$^-$ Tregs herein needs to be established. FoxP3$^-$ Tregs with proven suppressive capacities are found in the decidua and are, therefore, likely to contribute to the tolerogenic microenvironment. However, studies such as depletion assays in mice need to be performed to confirm whether they play a non-redundant role in maintaining a healthy pregnancy. Since pregnancy is crucial for the existence of mankind, it is not surprising that there would be multiple mechanisms in play to establish a regulatory microenvironment to maintain a healthy pregnancy. Pregnancy complications for which no clear cause can be identified do occur, and it is plausible that many of these are related to a disbalance in maternal immune regulation. It would be helpful to get a better understanding of the function of all regulatory T cells present in the decidua, to be able to recognize their relevance in healthy and complicated pregnancies. As such, the use of multiple omics techniques to identify the decidual microenvironment by a holistic approach could give insights in the presence, frequency, and distribution of the different types of Tregs in pregnancy [(32, 202, 203); van der Zwan et al., submitted]. It is important to note that the time point of sampling is a crucial factor in such experiments, given the dynamic nature of the placental microenvironment.

AUTHOR CONTRIBUTIONS

JK contributed to the content design, writing the manuscript, and preparing the figures. FC, SH, and ME supervised the project and participated in critical discussions and evaluations of the text of the manuscript.

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REVIEW
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Role of Regulatory T Cells in Regulating Fetal-Maternal Immune Tolerance in Healthy Pregnancies and Reproductive Diseases

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Regulatory T cells (Tregs) are a specialized subset of T lymphocytes that function as suppressive immune cells and inhibit various elements of immune response *in vitro* and *in vivo*. While there are constraints on the number or function of Tregs which can be exploited to evoke an effective anti-tumor response, sufficient expansion of Tregs is essential for successful organ transplantation and for promoting tolerance of self and foreign antigens. The immune-suppressive property of Tregs equips this T lymphocyte subpopulation with a pivotal role in the establishment and maintenance of maternal tolerance to fetal alloantigens, which is necessary for successful pregnancy. Elevation in the level of pregnancy-related hormones including estrogen, progesterone and human chorionic gonadotropin promotes the recruitment and expansion of Tregs, directly implicating these cells in the regulation of fetal-maternal immune tolerance. Current studies have provided evidence that a defect in the number or function of Tregs contributes to the etiology of several reproductive diseases, such as recurrent spontaneous abortion, endometriosis, and pre-eclampsia. In this review, we provide insight into the underlying mechanism through which Tregs contribute to pregnancy-related immune tolerance and demonstrate the association between deficiencies in Tregs and the development of reproductive diseases.

Keywords: regulatory T cells, pregnancy, steroidogenesis, endometriosis, primary unexplained infertility, recurrent spontaneous abortion, preeclampsia

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INTRODUCTION

Regulatory T cells (Tregs), a key subset of T lymphocytes, play a critical role in regulating the immune response and maintaining immune tolerance both in physiological and pathological processes. Many studies have shown that Tregs are compromised in patients with autoimmune diseases as well as in patients with graft-versus-host disease after receiving transplanted organs (1), however, these cells are activated to promote tumor growth and progression, leading to the failure of immunotherapies in cancer (2). Defects in the number of Tregs and their suppressive activity are involved in the development of various systemic or organ-specific autoimmune diseases, including thyroiditis (3), gastritis (4), type I diabetes (T1D) (5), systemic lupus erythematosus (SLE) (6), multiple sclerosis (MS) (7), rheumatoid arthritis (RA) (8), and inflammatory bowel disease (IBD) (9).

During the course of pregnancy, the mother's systemic immune system is altered to tolerate the fetus, who expresses paternal major histocompatibility complex antigens. Many studies have supplied multiple lines of evidence that Tregs possess specific characteristics for preventing the development of a maternal immune response against the fetus and maintaining fetal-maternal tolerance. First, the proportion of Tregs in peripheral blood is significantly increased during pregnancy in both women and mice, and there is a specific recruitment of Tregs from maternal peripheral blood to the fetal-maternal interface, leading to a higher proportion of Tregs in the placental decidua than in the peripheral blood (10). Furthermore, a decreased proportion of Tregs has been proposed to be associated with pregnancy-related complication such as recurrent spontaneous abortion and pre-eclampsia (11–13). Second, antibody-mediated depletion of $CD25^+$ Tregs has been shown to cause implantation failure in allogeneic mated mice (14). Conversely, the adoptive transfer of Tregs attenuates the high abortion rates in the well-studied CBA/J×DBA/2J abortion-prone murine model (15).

Pregnancy is a physiological process greatly dependent on immune tolerance, which is regulated by the number of Tregs and their suppressive activity. This review of the current literature describes the role played by Tregs in regulating fetal-maternal immune tolerance. Furthermore, we demonstrate the relationship between a deficiency of Tregs and pregnancy-related complications, with the aim of identifying the mechanisms through which Tregs maintain fetal-maternal immune homeostasis, thus providing a potential target for treating pregnancy-related complications.

Differentiation and Immunosuppressive Function of Tregs

Tregs are divided into two populations, namely natural regulatory T cells (nTregs) and inducible regulatory T cells (iTregs). NTregs originate from the thymus in response to self-antigens, whereas iTregs are peripherally induced from T cells responsible for restraining immune responses to foreign antigens, such as commensal bacteria, food antigens and allergens (16, 17). The mechanism underlying how Tregs are generated remains controversial. Although some studies have suggested that Tregs are anergic to TCR (T cell receptor) stimulation *in vitro*, the process involving the formation and selection of Tregs in the thymus is highly dependent on the TCR rearrangement, as evidenced by the observation that the development of Tregs is abrogated in TCR transgenic mice with RAG-2 deficiency (18). An increasing number of studies have suggested that Tregs are positively selected from autoreactive T cells that express specific TCR with the appropriate affinity for self-peptides (19–21).

Unlike other T helper cells, Tregs lack the capacity to secrete specific cytokines, and it is therefore difficult to distinguish them from other T helper cells. Foxp3 is the most specific Tregs marker and is constitutively expressed in Tregs generated in both the thymus and the periphery irrespective of the mode or state of activation (22, 23). The Foxp3 gene contains 11 exons and maintains a high degree of conservation between human and mouse genes (24). Mice genetically deficient in Foxp3 lose the ability to properly regulate Tregs activity and succumb to a fatal and severe lymphoproliferative autoimmune syndrome at 3–4 weeks of age (25). Similar to mice, humans carrying a FOXP3 mutant gene develop an autoimmune syndrome named IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) (26, 27). Beyond its role as an indispensable factor required for the development of Tregs, continuous Foxp3 expression is required for the latter's suppressive function. Research has shown that Tregs isolated from Foxp3 deficient mice lack suppressive function. However, transduction of Foxp3 endows $CD4^+CD25^-$ T cells with the capacity to suppress the proliferation of $CD4^+$ T cells (28, 29).

The suppressive function of Tregs is achieved via two mechanisms, namely a cell-contact dependent mechanism involving the recognition of co-stimulated molecules that directly suppress the expansion of effector T cells and a cell-contact independent mechanism involving the secretion of soluble cytokines that negatively regulate the immune response (30) (**Figure 1**).

CELL-CONTACT DEPENDENT MECHANISM

Cell-contact dependent suppressive activity is mediated via the recognition of co-stimulated molecules. In this process, Tregs function is highly dependent on the normal expression of molecules located on Tregs, and a deficiency of key molecules triggers the defective expansion and suppressive activity of Tregs, leading to a disturbance of immune homeostasis. IL-2 receptor α (IL-2Rα) and CTLA4 are the most important molecules involved in cell-contact dependent mechanism.

Most Tregs abundantly express high-affinity IL-2 receptor α (CD25) and IL-2/IL-2R signaling provides indispensable signaling during the development and maturation of Tregs both in the thymus and in the periphery. Furthermore, the lack of the IL-2R cannot be compensated by other cytokine receptors (31). IL-2, IL-2Rα, and IL-2Rβ deficient mice all die from severe lymphoproliferation and autoimmune disease in early life. In addition, neutralization of circulating IL-2 by anti–IL-2 monoclonal antibodies inhibits Tregs proliferation and triggers a wide range of organ specific autoimmune diseases (32–35). IL-2-IL-2R signaling is essential for the development and maturation of both Tregs and Teff cells, however, low dose IL-2 is remarkably efficacious in promoting the expansion of Tregs rather than Teff cells, which possibly results from the higher affinity of IL-2R in Tregs (36). Based on the comparative activity and different sensitivity for IL-2, the consumption of IL-2 by Tregs has been shown to be a predominant mechanism involved in suppressing the expansion and activity of Teff cells and triggering Teff cell apoptosis due to IL2 deprivation (37, 38).

CTLA4, a key molecule constitutively expressed in Tregs, is crucial for maintaining T cells homeostasis and tolerance induction, and its expression is in part controlled by Foxp3 (39, 40). Mice deficient in CTLA4 become sick by 2 weeks of age and moribund at 3–4 weeks of age, with diffuse and

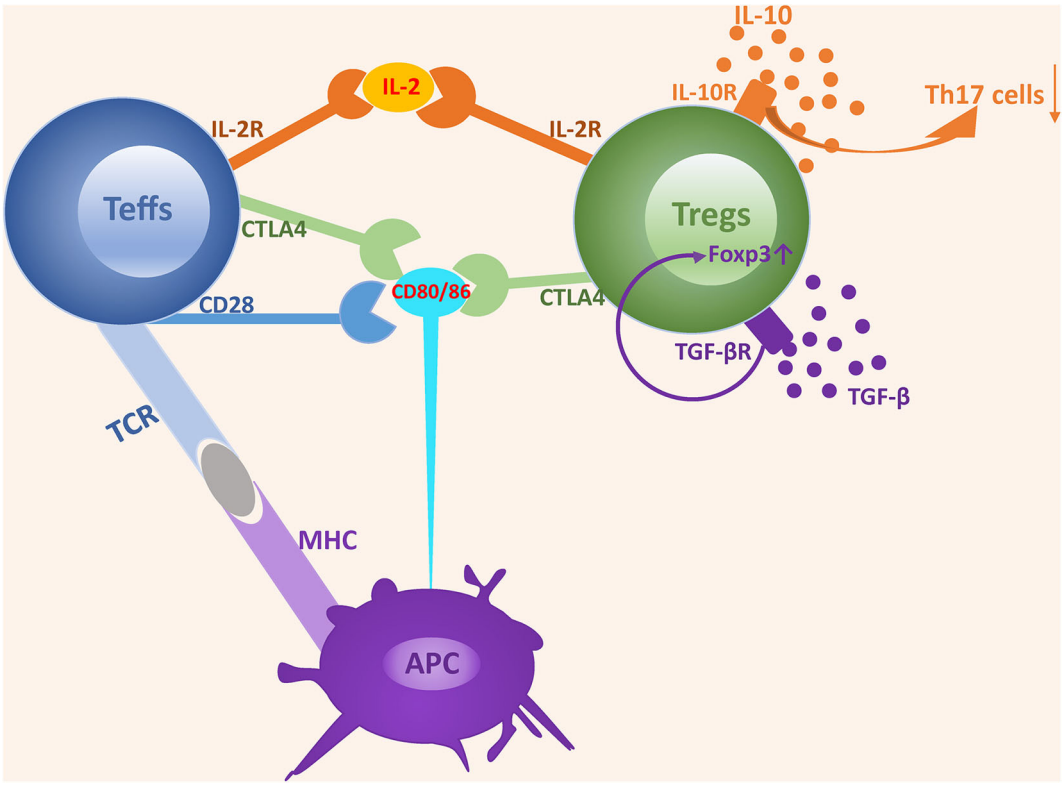

FIGURE 1 | The mechanisms underlying the suppressive function of Tregs. The suppressive function of Tregs is achieved via two mechanisms: cell-contact dependent mechanism and cell-contact independent mechanism. Tregs express a high-affinity IL-2 receptor and can competitively bind to IL-2 with Teffs, which induces IL-2 consumption and suppresses the development and expansion of Teffs. Both CD28 and CTLA4 interact with CD80/CD86 expressed on APCs. However, the affinity of CD28 is lower than that of CTLA4. CD28 plays an important role in enhancing Teffs activation, while CTLA4 acts as an inhibitor by depriving ligands and suppressing CD28 signaling. TGF-β and IL-10 are two classes of nonspecific cytokines secreted by Tregs and can promote Tregs expansion and suppressive activity by binding to their receptors.

focal lymphocytic infiltration into various organs (41, 42). Furthermore, specific deficiency of CTLA4 in Tregs results in the spontaneous development of systemic lymphoproliferation, multi-organ lymphocyte infiltrations, fatal T cell-mediated autoimmune diseases, and hyperproduction of immunoglobulin E in mice (43). CTLA-4-mediated suppressive regulation of T cell response and upregulation of Tregs activation are predominantly achieved by competition with CD28, a positive costimulatory molecule that shares common ligands (CD80/CD86) with CTLA4 (44, 45). CTLA4 possesses significantly higher affinity in binding CD80/CD86 and CTLA4 rather than CD28 removes costimulatory ligands CD80/CD86 from APCs by a process of trans-endocytosis (46, 47). These properties equip CTLA4 with the capacity to outcompete the ability of CD28 to serve as a negative immune regulator (48, 49).

CELL-CONTACT INDEPENDENT MECHANISM

In addition to the cell-contact dependent mechanism, Tregs also exert suppressive activity in a cell-contact independent manner, mainly through the secretion of inhibitory cytokines. Unlike other T cells, Tregs fail to produce exclusive cytokines. However, certain cytokines, such as TGF-β and IL-10, secreted by Tregs are essential for the expansion and suppressive activity of Tregs.

Several lines of evidence suggest that the addition of TGF-β enhances the conversion rate of native T cells into Tregs, and that TGF-β secreted by Tregs plays a partial role in maintaining suppressive properties by binding to the TGF-β receptor (50–53). Administration of neutralizing antibodies specific for TGF-β or specific deficiency of TGF-β expression in Tregs leads to a limitation or even abrogation of Tregs' suppressive activity (54, 55). Strong evidence that the role of TGF-β to maintain Foxp3 expression is supported by the observation that the expression of Foxp3 is dramatically diminished in peripheral Tregs from TGF-$\beta^{-/-}$ mice and addition of TGF-β results in increased Foxp3 expression (52).

Unlike TGF-β, the function of IL-10 in Tregs seems to be organ-specific. Recent studies have found that IL-10 and IL-35 produced by intratumoral Tregs cooperatively share a common BLIMP1 axis to promote the exhausted intratumoral T cell state and anti-tumor immunity, implying IL-10 and IL-35 contribute to maintaining immune tolerance (56, 57).

IL-10 is recognized as a potent suppressor of macrophage and T cell functions. Furthermore, IL-10 deficient mice are growth retarded and suffer from chronic enterocolitis (58). An increasing number of current studies have found that IL-10 is expressed in Tregs and plays an auxiliary role in promoting their expansion and function. IL-10^+ Tregs are mostly located in intestinal tissues and are essential for limiting immune response-induced inflammation to the diverse intestinal microbiota, which may provide a reasonable explanation as to why IL-10 deficient mice or mice treated with anti-IL-10 receptor blockers succumb to intestinal inflammation (59, 60). Although the Tregs-specific deficiency in IL-10 does not result in severe systemic autoimmunity, it does lead to immunological hyperreactivity at environmental interfaces, resulting in conditions such as spontaneous colitis, lung hyperreactivity, and skin hypersensitivity (61). Thus, while IL-10 production by Treg cells is not necessary for the regulation of systemic autoimmunity, it is essential for hindering excessive immune responses at local environmental interfaces. The suppressive activity of IL-10 is partly mediated via binding to IL-10R to restrain the Th17-induced inflammatory response, which plays a critical role in regulating intestinal homeostasis. This is illustrated by the observation that mice with IL-10R deficient Tregs produce high levels of IL-17 and are prone to developing severe colitis (62, 63).

Regulation of Fetal-Maternal Tolerance During Healthy Pregnancy

For decades, many studies have shown that successful pregnancy depends on the homeostasis of fetal-maternal tolerance. Furthermore, failure of the maternal immune system to establish fetal-maternal tolerance is the predominant trigger in the development of pregnancy-related complications. Consequently, numerous therapeutic treatments aimed at suppressing the maternal immune system are employed in clinics. However, the effect of these therapies is not always apparent and is often accompanied by various side effects. It is therefore important to identify the cellular and molecular mechanisms responsible for establishing fetal-maternal immune tolerance in healthy and abnormal pregnancies to promote the development of targeted therapeutic interventions. The immune suppressive property of Tregs confers this cell population with a fundamental role in establishing the fetal-maternal immune tolerance necessary for successful pregnancy.

Some studies consider pregnancy to be a process of mutual conversion between pro-inflammatory and anti-inflammatory conditions (64), therefore dividing pregnancy status into three distinct immunological states that correspond to different stages of fetal development: first, a pro-inflammatory stage associated with embryo implantation and placentation (65–67); second, an anti-inflammatory-oriented stage associated with fetal growth (68, 69); and third, a switch from an anti-inflammatory to a pro-inflammatory stage necessary for the initiation of labor (70, 71). Concurrent with the above stages is a dramatic change in the number of Tregs during the course of pregnancy. Following exposure of paternal alloantigens, circulating Tregs increase rapidly during the early pregnancy stage and peak during the second stage at which time trophoblast invasion of the maternal decidua is maximal; then, Tregs gradually decrease when labor begins (64). The change in the number of Tregs and crosstalk with other immune cells play a critical role throughout the entire course of pregnancy.

Tregs PRIMING AND IMPLANTATION

Embryo implantation is the initial stage of pregnancy and involves apposition of the blastocyst and the uterine endometrium followed by attachment and invasion of the blastocyst into the endometrium, and reconstruction of the decidua by the invasive trophectoderm (72). The wide application of assisted reproductive technology, such as *in vitro* fertilization-embryo transplantation (IVF-ET) and intrauterine insemination (IUI), has enabled an analysis of earlier gestational stages from oocyte fertilization to implantation in humans. Adequate endometrial receptivity is considered a pivotal precondition for successful embryo implantation. Endometrial scratching before embryo transfer has been proposed as a clinical treatment to increase uterine receptivity, and some studies have demonstrated that endometrial scratching improves the pregnancy outcome by triggering an inflammatory response and enhancing angiogenesis at the implantation site, providing indirect evidence for the role played by inflammation during implantation (73–77).

Studies based on human and animal experiments have demonstrated that the peri-implantation period is accompanied with the activation and infiltration of various immune cells (78). Uterine-specific natural killer (uNK) cells, macrophages (Mos), and dendritic cells (DCs) are recruited at the implantation site and exert prominent immune-regulatory effects during early pregnancy. uNK cells are the most abundant immune cells located in human decidua during early pregnancy, while Mos and DCs serve as antigen-presenting cells that infiltrate into the decidua. Crosstalk among these cells plays an essential role in regulating trophoblast invasion and in promoting spiral artery remodeling (79–81).

The role played by Tregs during implantation is unclear. However, some studies have reported that a reduced number of Tregs is associated with implantation failure. Mice with a depletion of Tregs exhibit a significant defect in implantation, which is reversed following an adoptive transfer of Tregs (82). A study showed that compared with fertile women, endometrial tissue from women with unexplained infertility displayed a significant reduction Foxp3 mRNA expression, the fate-determining transcription factor especially expressed in Treg cells (83). Other evidence has also revealed a correlation between the level of Tregs in peripheral blood and the implantation rate. Women with implantation failure after IVF or artificial insemination by donor sperm (AID) had a significantly decreased percentage of Tregs compared with women with a successful pregnancy (84, 85). Therefore, the presence of peripheral or local

Tregs may create a limited but necessary immunomodulatory function during the course of implantation.

ENLARGEMENT OF Tregs FUNCTION AND PREGNANCY MAINTENANCE

Successful implantation is followed by a phase of fetal growth and development. The establishment of fetal-maternal immune tolerance lays the foundation for this stage, with a shift from a pro-inflammatory immune response to a Th2/Treg-predominant anti-inflammatory immune tolerance (64). The proportion of Tregs begins to rise and peaks at this stage, and a paucity of Tregs could lead to pregnancy-related complications such as spontaneous abortion. Tregs exert a strong immunosuppressive function to maintain an anti-inflammatory environment and protect the fetus from maternal immunological rejection. Tregs can effectively suppress the expansion and activation of effector T cells via a classic cell-contact mechanism or by secreting suppressive cytokines as described previously. One study described a class of functionally distinct Tregs with expression of a co-inhibitory molecule TIGIT, which induces selective suppression of Th1 and Th17 cells but not Th2 cells. However, whether this Tregs subset is expanded and activated during pregnancy is still unknown (86).

The pivotal role played by Tregs in fetal-maternal tolerance raises several questions about the mechanisms responsible for their expansion during pregnancy and underscores the need for studies investigating these mechanisms. Previous studies suggest that the activation and regulation of Tregs is primarily impacted by antigen exposure and the dynamic changes of steroid hormones that occur during pregnancy.

ANTIGEN-MEDIATED Tregs EXPANSION: PATERNAL SPERM ANTIGEN AND FETAL ANTIGEN

Investigators have proposed that exposure to male seminal fluid delivered during mating elicits the expansion of maternal Tregs, as evidenced by the increase in the number of Tregs within the period of time subsequent to mating and before embryo implantation (87, 88). Immune tolerance to the fetus is necessary for successful pregnancy, and transmission of seminal fluid seems to play a priming role prior to implantation by promoting expansion of Tregs, thereby activating specific tolerance to paternal alloantigens. Seminal fluid contains various components, including a cellular fraction that contains sperm, leukocytes and epithelial cells and a non-cellular fraction of compounds such as TGF-β and prostaglandins. The cellular and acellular fractions in semen both contain several antigens, including classical class Ia, non-classical class Ib and minor antigens such as H-Y antigen, which drive an antigen-dependent expansion of Treg cells (89, 90). The non-cellular components are also required to confer tolerance. As mentioned above, TGF-β is a critical cytokine for Tregs proliferation. One study found that intravaginal pre-treatment with TGF-β at mating enhances successful pregnancy *in vivo* in a well-established murine model (91). An *in vitro* experiment also indicated a role for prostaglandins in upregulating Foxp3 expression and enhancing Tregs function (92). Collectively, both sperm and seminal plasma may contribute to driving an expansion of Tregs and providing an immune-privileged environment that is beneficial for subsequent embryo implantation.

Embryo implantation and fetal growth are the most important stages during pregnancy. Some studies have proposed that the implanted blastocyst should be considered a semi-allograft and constant immunosuppression is required for a pregnancy to be successful. Although a seemingly opposite pro-inflammatory process is involved in both implantation and initiation of labor, immunosuppression is an indispensable response to maintain immune homeostasis during the fetal growth stage, and this is highly dependent on the expansion and activation of Tregs triggered by the fetal alloantigens (93). When Tregs are depleted, fetal outcome is normal in syngeneic pregnancies rather than allogeneic pregnancies, suggesting that Tregs suppress maternal immune responses directed against fetal alloantigens rather than male-specific minor histocompatibility antigens (94, 95). When encountered with parental alloantigens presented by a fetus, peripheral Tregs, generated extrathymically and induced by non-self-antigens, serve as the predominant subset suppressing immune response. The development of peripheral Tregs is dependent on the expression of a Foxp3 enhancer CNS1, a deficiency of which leads to an increased resorption of embryos in mice (96).

STEROID HORMONE-MEDIATED Tregs EXPANSION: ESTROGEN, PROGESTERONE AND HUMAN CHORIONIC GONADOTROPIN

Serum levels of the pregnancy-associated hormones such as estrogen, progesterone, and human chorionic gonadotropin (HCG) increase dramatically during pregnancy. These hormones play an essential role in maintaining immune tolerance and in supporting successful pregnancy. Currently, there is increasing evidence that the mechanisms through which hormones contribute to immune homeostasis during pregnancy are in part due to the expansion of Tregs and their suppressive activity (**Figure 2**).

Estrogen-based therapy has been reported to alleviate symptoms associated with several autoimmune diseases, such as collagen-induced arthritis (97), type1diabetes (98), and autoimmune encephalomyelitis (99). Furthermore, the mechanisms underlying these protective effects seem to be associated with changes in immune cells and cytokines (100–102). The number of Tregs in human peripheral blood change continuously during the menstrual cycle and peak before ovulation, which is concurrent with the change of the concentration of estrogen, suggesting that estrogen may be a powerful factor in promoting Tregs expansion (103). Some studies have demonstrated that the proliferation and

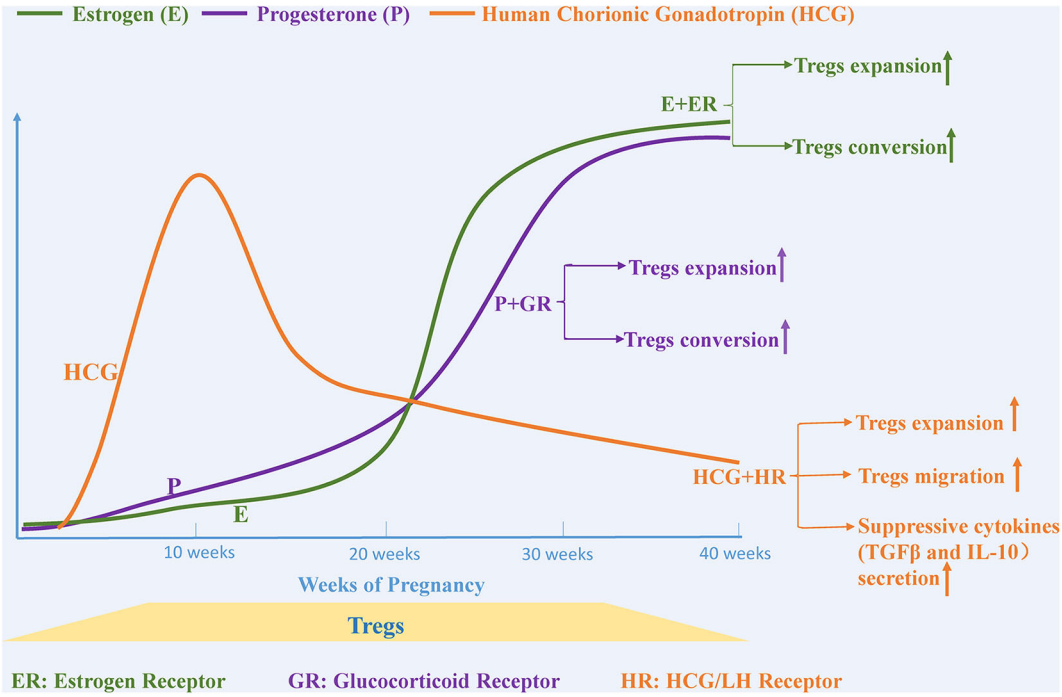

FIGURE 2 | Pregnancy-related hormones affect the expansion and migration of Tregs. The levels of various steroid hormones, such as estrogen, progesterone and human chorionic gonadotropin, change dramatically during pregnancy. Estrogen and progesterone promote Tregs expansion and trigger the conversion of CD4+CD25– T cells to Tregs separately by binding to estrogen and glucocorticoid receptors. The level of human chorionic gonadotropin (HCG), another essential hormone for maintaining a healthy pregnancy, begins to increase after fertilization, peaks at the 11th week, and then gradually decreases until birth. HCG functions as a regulator that not only upregulates the expansion of Tregs but also provokes migration of Tregs from the circulation to the decidua.

suppressive activity of human Tregs observed with estrogen treatment is mediated through estrogen receptor α (104, 105). In both *in vivo* and *in vitro* experiments, estrogen treatment triggers the expansion of Tregs. Furthermore, the addition of estrogen in combination with TCR stimulation enhances Foxp3 mRNA expression in $CD4^+CD25^-$T cells *in vitro*, suggesting that estrogen may potentially induce the conversion of $CD4^+CD25^-$T cells to Tregs (106, 107).

Progesterone, which is mainly produced by the placenta and is markedly elevated during pregnancy, functions as a regulator that maintains homeostasis at the maternal-fetal interface. Similar to estrogen, progesterone is considered to be another important hormone that promotes the expansion of Tregs and their suppressive capacity (107). The proportion of Tregs and the conversion rate of $CD4^+CD25^-$ T cells into Tregs has been shown to increase significantly in the peripheral blood, spleen, and inguinal lymph nodes of ovariectomized mice after progesterone injection (108). Progesterone-mediated immune tolerance is achieved by progesterone binding to the glucocorticoid receptor rather than to the progesterone receptor (109, 110). Progesterone promiscuously binds the glucocorticoid receptor and promotes immune suppression by inducing enrichment of Treg cells and triggering apoptosis of effector T cells, which is based on the preferred sensibility in effector T cells for glucocorticoid receptor-mediated T cells death compared with that in Tregs (110, 111). Progesterone is also present at high levels in human cord blood where it has been reported to have an immune-suppressive function. Progesterone drives a shift of native cord blood T cells into suppressive Tregs, while impeding the conversion from native T cells into Th17 cells, another potential pathway through which progesterone regulates immune tolerance (112).

HCG is another hormone that is increased during pregnancy, and is produced in the blastocyst after fertilization, reaching its maximum level at the 11th week and then gradually decreasing until birth (113). Khil et al. reported that HCG prevents the development of autoimmune-mediated diabetes in NOD mice by downregulating immune effector cells and cytokines and simultaneously upregulating the proportion of Tregs and the levels of TGF-β and IL-10, suggesting that HCG is an effective regulator for immune tolerance (114). Increased HCG during pregnancy provokes many Tregs-related responses including (1) augmenting the number of Tregs, (2) increasing their local and systemic suppressive function, (3) enhancing attraction of circulating Tregs into decidua, and (4) increasing the secretion of suppressive cytokines (115–117). HCG-mediated expansion of Tregs is achieved in part by retaining DCs in an immature state, leading to the generation of Tregs and a loss of the capacity to activate a T cell-mediated immune response (117, 118). *In vitro* migration assays further confirmed the chemoattractant properties of HCG that promote migration of Tregs from the periphery into the uterus, which is potentially

mediated via binding to HCG/LH receptors located on Tregs (116, 119).

DECLINE OF Tregs ACTIVITY AND LABOR

There is a decline in the number of Tregs as pregnancy progresses into the third gestational period. The reduction in the number of Tregs in late gestation may be a contributing factor for the initiation of spontaneous labor. This is supported by the finding that the proportion of Tregs in the decidua following a spontaneous vaginal delivery is significantly lower than that following an elective cesarean section (120). Shah et al. conducted a longitudinal analysis from 20-weeks gestational age to labor and observed a reduction in the number of activated Tregs (defined as Tregs with HLA-DR$^+$) and a significant shift toward a Th1/Th17 response with the onset of labor (121). Compared with women undergoing spontaneous term labor, the proportion of activated Tregs is significantly decreased in women in preterm labor (122, 123). The change is similar to the reduction in activated Tregs observed in patients who experience an acute rejection after kidney transplantation, supporting that the reduction in the proportion and activity of Tregs promotes the conversion from an anti-inflammatory to a pro-inflammatory stage and plays a critical role in initiating spontaneous labor. The mechanism underlying the reduction in Tregs during labor remains an enigma. The alteration in hormone levels and in the microbial environment may be stimuli for activating an inflammatory response, however, the specific molecular mechanisms needs to be further investigated.

The level of Tregs progressively decreases after delivery. However, there is a retention of "memory" Tregs with fetal specificity, which retain the ability to generate a more effective and accelerated suppressive response when re-exposed to the same fetal antigens in subsequent pregnancies (124). The primary pregnancy confers Tregs with a protective regulatory memory, which may provide an immunological basis for protection against complications such as pre-eclampsia in a subsequent pregnancy (125, 126).

Dysfunction of Tregs in Reproductive Diseases

Since it has been determined that Tregs maintain fetal-maternal tolerance during the normal course of embryo implantation and pregnancy, it is of interest to investigate whether systemic and local maldistribution and dysfunction of Tregs play a role in the etiology of infertility and pregnancy-related complications. Increasing evidence suggests that a deficiency in the expansion and function of Tregs as well as an abnormal expression of key molecules are linked to pregnancy-related complications.

RECURRENT SPONTANEOUS ABORTION

Recurrent spontaneous abortion (RSA), defined as the loss of three or more consecutive pregnancies, affects ∼1% of women attempting to conceive (127, 128). RSA is a complex pregnancy-related complication that is due to multiple factors including chromosomal abnormalities, congenital or acquired anatomical defects in the uterine fundus and cervix, and other endocrine diseases such as PCOS, diabetes, thyroid disorders, and others related to aberrant immune responses (128, 129). Increasing evidences suggests that the proportion of various immune cells and cytokines is altered in patients with RSA, supporting that immune dysfunction may be a contributing factor to its etiology (130, 131). Although there have been detailed guidelines describing clinical interventions for managing women with RSA, treatment based on immune rejection as a potential etiology is controversial, because no definite cellular and molecular mechanism has been discovered to date (17, 129).

The mechanisms through which Tregs contribute to RSA primarily involve an imbalance of the Th1/Th2/Th17/Treg cells paradigm and the abnormal proportion and activity of Tregs. Dysregulation of T lymphocyte homeostasis is also involved in the etiology of RSA. In peripheral blood from patients with RSA, the balance between Th1 and Th2 cells is disrupted in favor of Th1 cells, and the ratio of Th17/Treg cells is skewed toward Th17 cells (132, 133). It is widely accepted that there is a close interaction between the expansion of Tregs and the secretion of IL-17. When IL-17 combines with the IL-17 receptor, Tregs are upregulated. Conversely, Tregs suppress the proliferation of Th17 cells and the secretion of IL-17 via cell-cell contact and via Il-10/TGF-β-mediated effects (134, 135). However, this suppressive function of Tregs is abrogated in patients with RSA (134). Transfusion of Tregs into mice pretreated with IL-17 has been shown to significantly increase the expression of IL-10 and TGF-β, two key cytokines that mediate the suppressive activity of Tregs in decidua and lower the fetal resorption rates in mice (136). Furthermore, insufficient generation of pregnancy-induced Tregs triggers the accumulation of paternal alloantigen specific Th1 cells and directly results in the failure to establish appropriate maternal-fetal immune tolerance (137).

Numerous studies have also confirmed that the reduction in the number of Tregs are involved in the pathogenesis of RSA (**Table 1**). Sasaki et al. first reported the presence of Tregs in the decidua and demonstrated the proportion of Tregs in decidua from spontaneous abortions was significantly lower than that in decidua from induced abortion (11). Other studies have also demonstrated that the proportion of Tregs and the expression of Foxp3 in both the decidua and peripheral blood from patients with unexplained RSA patients are significantly lower than those from women with normal pregnancies (13, 139). In addition to the reduction in number, Lourdes et al. reported that the suppressive function of Tregs is significantly impaired in RSA as assessed by a co-culture technique with CD4$^+$CD25$^-$T cells (103). Inadequate number of Tregs and downregulation of Treg cell activity impair the anti-inflammatory environment, weaken the immune tolerance against fetal rejection and thereby increase the risk of RSA.

ENDOMETRIOSIS

Endometriosis is a benign gynecological disease affecting ∼6–10% women of childbearing age, and is characterized by

TABLE 1 | The change of the proportion of Tregs in patients with recurrent spontaneous abortion compared with normal pregnant women.

Proportion of Tregs		References
Peripheral blood	**Decidua**	
↓	↓	(11)
↓	↓	(13)
↓	↓	(138)
↓	↓	(139)
↓	Not mentioned	(132)
↓	Not mentioned	(140)
Not mentioned	↓	(141)
↓	Not mentioned	(142)
↓	↓	(143)

↓: Decreased.

TABLE 2 | The change of the proportion of Tregs in patients with endometriosis compared with patients without endometriosis.

Proportion of Tregs in patients with endometriosis			References
Peripheral blood*	**Peritoneal fluid***	**Ectopic peritoneal lesions#**	
Not mentioned	Not mentioned	↑	(156)
Not mentioned	↑	Not mentioned	(157)
↓	↑	Not mentioned	(158)
→	→	Not mentioned	(159)
→	Not mentioned	Not mentioned	(155)
→	→	↓	(160)
→	↑	Not mentioned	(161)
→	→(Early) ↑(Advanced)	Not mentioned	(162)

↑: Increased, ↓: Decreased, →: Not changed.

**The proportion of Tregs in peripheral blood and peritoneal fluid in patients with endometriosis is compared with patients without endometriosis. #The proportion of Tregs in the ectopic peritoneal lesions in patients with endometriosis is compared with eutopic endometrium in patients without endometriosis.*

the implantation of endometrial tissues outside the uterus (144). Chronic pelvic pain, dysmenorrhea and infertility are the common symptoms occurring in patients with endometriosis (145, 146). As multiple factors, including genetic and environmental factors, contribute to the development of endometriosis, the pathogenesis of endometriosis remains uncertain. Many theories have been proposed to explain how endometriosis develops, and one of the most widely accepted is the retrograde menstruation theory. This theory hypothesizes that fragments of endometrial tissue reflux to the peritoneum through the fallopian tubes during menstruation (147). However, this theory fails to explain why only a few women develop endometriosis even though retrograde menstruation is a common phenomenon occurring in most women of childbearing age (148). Therefore, other studies have postulated that a disturbed local and systemic immune response may be responsible for the development and progression of endometriosis (149–151).

An aberrant immune environment that includes alternative activation of peritoneal macrophages (152), production of various cytokines (153), and reduction in natural killer cell cytotoxicity (154), all contribute to the survival and invasion of ectopic endometrial tissue. Dysregulation in T lymphocyte homeostasis is associated with the pathogenesis of endometriosis. The Th1/Th2 balance is altered in local and systemic immune conditions, such that there is skewing toward Th2 cells in endometriotic lesions, but skewing toward Th1 cells in peripheral blood (155).

A disturbance in Tregs activity may be a more prominent mechanism involved in the etiology of endometriosis due to their immune-suppressive function, derangement of which could potentially promote the survival of ectopic endometrial lesions. However, evidence regarding the change in the proportion of Tregs in peripheral blood, peritoneal fluid, eutopic endometrium, and ectopic endometrial tissues among patients with endometriosis is inconsistent (**Table 2**). The discrepancy may result from differences in patient selection, namely the patients with early or advanced endometriosis. Most studies suggest the proportion of Tregs is significantly increased in peritoneal fluid from women with endometriosis compared with control women (157, 158, 161). One study reported that the number of Tregs was increased in the peritoneal fluid and decreased in the peripheral blood, and another study found the number of Tregs was higher in peritoneal fluid than in peripheral blood, both indicating that active translocation of Tregs occurs from circulation to the local peritoneal cavity (158, 162). However, some studies failed to find any difference in the proportion of Tregs in patients with endometriosis when compared with women without endometriosis (159). To bypass the confounding influence of interpatient variability, research has been carried out in an established animal model with endometriosis to identify abnormalities in the proportion of Tregs. In a study of baboons with induced endometriosis, the proportion of Tregs was decreased in peripheral circulation and eutopic endometrium but increased in ectopic tissue, which is consistent with Tregs' local immunosuppressive activity Tregs played (163). Tanaka et al. focused on the variation in resting and activated Tregs and put forth a new concept that the proportion of activated Tregs in the endometrioma rather than in the peritoneal fluid or peripheral blood is decreased, which may be temporal and associated with the angiogenesis and progression of endometriosis (160). However, a study showed the proportion of Tregs in ectopic endometrium was increased in patients with endometriosis compared with eutopic endometrium (156). Further research is required with an expanded sample size and more detailed subgroup analysis to better determine the role Tregs play in the pathogenesis of endometriosis.

Change in the proportion of Tregs appears to contribute to the suppressed immune response against ectopic endometrial tissue, permitting implantation of endometrial tissue in the peritoneal cavity. Therefore, understanding the origin of local Tregs production may be provide new insights that will aid

in the development of targeted therapies for women with endometriosis. The accumulation of Tregs in the peritoneal cavity may not only be a result of active translocation from the peripheral blood but may also be due to their local induction (153). Higher levels of IL-10 and TGF-β, two key cytokines responsible for regulating the proliferation and activity of Tregs, were found in the peritoneal fluid and serum of patients with endometriosis than in normal controls (164, 165). Compared with serum levels, the level of cytokines in peritoneal fluid was significantly higher (165). Furthermore, IL-10 and TGF-β mRNA expression were significantly higher in ectopic lesions than eutopic endometrium from women with or without endometriosis, particularly in cases of advanced endometriosis (166). These results suggest Tregs and related cytokines maintain the local anti-inflammatory environment and play a crucial role in the development of endometriosis.

PREECLAMPSIA

Preeclampsia is a common pregnancy-related complication that occurrs in 3–5% of pregnant women and can lead to iatrogenic preterm birth and fetal growth restriction (167). The precise etiology of preeclampsia remains unknown, although insufficient formation of uterine spiral arteries, over-activated inflammation, injured endothelial cells, and genetic factors have all been implicated (168–171). Interestingly, preeclampsia seems to be more common in primiparous than multiparous women, whereas the protective effect is abrogated with the change of partner. A meta-analysis compared the difference in the risk of preeclampsia in women who were impregnated by donor or partner sperm and found the risk was significantly increased in conceptions resulting from donor sperm (172). Furthermore, another study reported that prior and prolonged partner sperm exposure before pregnancy is associated with a significant reduction of the risk of preeclampsia (173). Taken together, these observations suggest that paternal antigens and sperm exposure induce an immune tolerance during the first pregnancy and offer effective protection against the development of preeclampsia with subsequent pregnancies, implying the adaptive immune response with alloantigen specificity and immunological memory is involved in the pathogenesis of preeclampsia (174).

An increasing body of evidence suggests that an inadequate immune tolerance induced by Tregs-associated abnormalities play a pivotal role in the etiology of preeclampsia. Several studies have reported that, compared with normal pregnancy, both the number of Tregs and the ratio of Tregs to Th17 cells in peripheral blood are significantly reduced in preeclampsia (175–177). The increased ratio of Th17/Treg cells has also been confirmed by an analysis of Th17/Treg expression of related transcription factors and the secretion of Th17/Treg-related cytokines. Compared with healthy pregnant women, a reduction in the expression of Treg-specific transcription factor Foxp3 and an elevation in Th17-specific transcription factor RORγt in patients with preeclampsia has been reported (178). Furthermore, analysis of cytokine profiles have revealed a significant decrease in IL-10, and a significant increase in IL-17 levels in patients with preeclampsia (178, 179). Taken together, these studies suggest that a shift occurs from Tregs to Th17 cells in the development of preeclampsia, leading to an abnormal immune state that triggers inflammation and an impairment of immune tolerance. The mechanism underlying the imbalance of Th17/Treg cells remains unclear. Eghbal-Fard et al. suggested the upregulation of miRNA in patients with preeclampsia may affect the differentiation and expansion of Th17/Treg cells by regulating the expression of specific transcription factors (178). In addition to the alteration in the proportion of Tregs, the immunosuppressive activity of Tregs is also altered in patients with preeclampsia. Darmochwal-Kolarz et al. reported the proliferation of effector T lymphocytes in patients with preeclampsia was significantly inhibited by Tregs isolated from healthy pregnant women. However, the suppressive response was lost if replaced with Tregs from patients with preeclampsia (180).

The recruitment of Tregs from peripheral blood into decidua and the local expansion of decidual Tregs are important for maintaining fetal-maternal immune tolerance at the fetal-maternal interface. It has been well-established that the proportion of Tregs in decidua is decreased in preeclampsia (181). Though the reduction of decidual Tregs may be associated with an imbalance in systemic Tregs, local expansion may also play an important role. TCR repertoire analysis of decidual Tregs showed an insufficient clonal expansion of decidual Tregs in preeclampsia compared with healthy pregnancy (182). In normal pregnancy, induced rather than native Tregs are the dominant Tregs subset located in the decidua and are clonally expanded, while the expansive and suppressive capacity of iTregs is significantly impaired in preeclampsia (183). The local induction of Tregs depends on specific APCs within the decidual microenvironment. A significant reduction in the expression of HLA-G and ILT4 on decidual APCs is observed in preeclampsia compared with normal pregnancy, providing a possible clue to the lack of iTregs in preeclampsia (183). An aberrant proportion and type of Tregs in the decidua disturb the immune homeostasis during pregnancy and promote the development of preeclampsia.

Tregs and Immune Therapy During Pregnancy

Taken together, the above studies suggest that Tregs play a prominent role in regulating fetal-maternal immune tolerance, and a defect in the proportion and activity of Tregs is involved in the development of RSA, endometriosis, and preeclampsia. Thus, approaches designed to boost the proportion of Tregs or strengthen their suppressive function may lead to promising strategies for treating pregnancy-related diseases. Several Tregs-based target therapies are entering into clinical trials, including adoptive Treg cell therapy, Tregs-enhancing drugs, and low dose IL-2 administration (184).

Administration of purified Tregs was firstly applied as Tregs-based target therapy. With the development of immune cell therapy, antigen-specific Tregs therapy was also proposed

for treating autoimmune and graft-versus-host diseases. Phase I/II clinical trials aimed to explore the curative effect, and some have reported that Tregs administration alleviates clinical symptoms induced by autoimmunity (184). Some research has attempted to determine whether Tregs administration improves pregnancy outcomes. Yin et al. and Wang et al. examined the effectiveness of adoptive transfer of Tregs in preventing spontaneous abortion in mice models (136, 185). Yin et al. established an abortion-prone pregnancy mice model with DBA/2J-mated pregnant CBA/J mice and performed adoptive transfer of freshly isolated and *in vitro* expanded Tregs from non-pregnant CBA/J mice. Wang et al. induced spontaneous abortions by administration of IL-17 in a CBA/J × BALB/c mouse model of normal pregnancy and performed adoptive transfer of *in vitro* expanded Tregs purified from pregnant CBA/J mice. These two studies demonstrated transfusion with *in vitro* expanded Tregs promotes immune suppressive activity, increases the secretion of suppressive cytokines and significantly reduces the rate of spontaneous abortion.

Although Treg cell therapy has not been widely used in clinical practice, clinical research has initiated several non-specific immunotherapies partially regulating the proportion and activity of Tregs for the treatment of pregnancy-related diseases. Intravenous immunoglobulin G (IVIG) and paternal or third-party lymphocyte immunization therapy have been proposed for the treatment of patients with RSA due to the potential immunomodulatory effects. Although the benefit for these immunotherapies is controversial, a growing body of evidence suggests that they may increase rates of live birth and decrease rates of miscarriage (186–188). A variety of studies and clinical trials have reported both IVIG and lymphocyte immunization therapy correct the Tregs defect and rebalances the Th17/Treg paradigm in peripheral blood. Compared with a control group, the treatment triggers a shift toward Tregs in the Th17/Treg balance by enhancing the expansion of Tregs, promoting the secretion of suppressive cytokines, and inhibiting Th17 cells proliferation (186, 188–192).

Tregs-enhancing drugs are another type of Tregs-based target therapy. Rapamycin (Sirolimus) is an mTOR inhibitor, which acts as an immunosuppressive drug by selectively promoting the expansion of Tregs and inducing differentiation of T helper cells into Tregs. Royster et al. established a murine model with conditional knockdown of Tregs induced by diphtheria toxin. They found the deletion of Tregs decreased litter sizes and triggered embryo implantation failure, effects that were reversed after the treatment with rapamycin (193). A multicenter, double-blind, phase II randomized clinical trial administrated 2 mg/day of sirolimus for 2 days before embryo transfer to patients receiving IVF-ET therapy and who had a history of recurrent implantation failure. The study collected blood samples and assessed the ratio of Th17/Treg cells by flow cytometry 5–10 days prior to the initiation of an IVF cycle. Only patients with a high ratio of Th17/Treg cells were included in this trial. The trial reported that the administration of sirolimus reversed the imbalance in the ratio of Th17/Treg cells and significantly increased the rate of clinical pregnancy and live birth compared with those in the control group (194).

Taken together, some studies have demonstrated the effectiveness of Tregs-based therapy in treating several autoimmune diseases and cases of organ transplantation. However, the methods cannot be directly applied for pregnancy-related diseases because the dynamic change in the immune state during pregnancy and the possibility of fetal drug toxicity must be taken into account. Most of the current treatments for pregnancy-related diseases focus on a reduction in an overactive immune response with the use of non-specific immunosuppressive therapy. This triggers the simultaneous activation of numerous immune cells and makes it difficult to control the dose and to evaluate the curative effect because of individual heterogeneity. Therefore, more studies should be conducted to further explore the effectiveness and safety of Tregs-based target therapies for the treatment of pregnancy-related diseases.

CONCLUSION AND FUTURE PERSPECTIVE

Tregs are generally viewed as arising from a specific T cell lineage generated in the thymus or induced in peripheral organs. Being the most predominant immune-suppressive cells, a tremendous amount of research has focused on determining the molecular mechanisms responsible for inducing the expansion of Tregs and their activity in the periphery and in specific organs. This effort will provide new insights that will guide the improvement of Tregs-based targeted immune therapy. In recent years, increasing data has shown that the expansion of Tregs is triggered after exposure to the fetal alloantigens and changes dynamically over the course of pregnancy. Hormones such as estradiol and progesterone as well as HCG are significantly increased during pregnancy, and regulate the number and function of Tregs to sustain a proper pregnancy-related immune tolerance. Furthermore, various reproductive diseases such as recurrent miscarriage, endometriosis and preeclampsia result in part from the deficiency in the number and activity of Tregs. Therefore, modulating the immune response by boosting the number of Tregs and enhancing their activity may be a potential therapeutic strategy for managing these pregnancy-related complications.

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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Upregulated TRAIL and Reduced DcR2 Mediate Apoptosis of Decidual PMN-MDSC in Unexplained Recurrent Pregnancy Loss

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Myeloid-derived suppressor cells (MDSC), especially polymorphonuclear MDSC (PMN-MDSC), accumulate in maternal-fetal interface during pregnancy and are involved in the maintenance of immune tolerance. Decreased PMN-MDSC is associated with pregnancy complications such as unexplained recurrent pregnancy loss (URPL). In the present study we showed decreased PMN-MDSC in the URPL group compared with the normal pregnancy (NP) group, and PMN-MDSC was the major subset of MDSC in human decidua with potent immune suppression activity. We then performed gene expression profile and found that human decidual PMN-MDSC in the NP and URPL groups showed different gene and pathway signature, including apoptosis. Apoptosis of decidual PMN-MDSC was mediated by TNF-related apoptosis–induced ligand (TRAIL) in a Caspase 3 dependent manner. TRAIL was expressed in decidua and upregulated in decidua of the URPL group. Notably, of all the membrane TRAIL receptors, only DcR2 was down-regulated in PMN-MDSC in the URPL group. *In vitro* experiment demonstrated that DcR2 blockade sensitized PMN-MDSC to TRAIL-mediated apoptosis. Together, these data indicate that increased TRAIL and reduced DcR2 on PMN-MDSC sensitize PMN-MDSC response to TRAIL-induced apoptosis in the URPL group, which is responsible for decreased accumulation of PMN-MDSC in URPL.

Keywords: polymorphonuclear myeloid-derived suppressor cell, TRAIL, TRAIL receptor, apoptosis, unexplained recurrent pregnancy loss

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INTRODUCTION

Recurrent pregnancy loss (RPL), defined as two or more failed pregnancies, occurs in 5% pregnancies and about 50% of all RPL are idiopathic, which is defined as unexplained RPL (URPL) (1, 2). The pathogenesis of URPL is poorly understood and maternal-fetal immune dysfunction is considered to be one major cause. Maternal-fetal immune tolerance depends on intricate interactions of the immune system (3), and recent reports indicate a role of myeloid-derived suppressor cells (MDSC) in maintenance of maternal-fetal immune tolerance (4–6).

MDSC are recently identified as heterogeneous cell populations of myeloid origin with potent immunosuppressive activity (7, 8). In human, MDSC are defined as HLA-DR$^{-/low}$CD11b^{+}CD33^{+},

and can be further categorized into two major subsets: $CD33^{bright}CD14^{+}CD15^{-}$ monocytic MDSC (M-MDSC) and $CD33^{dim}CD14^{-}CD15^{+}$ polymorphonuclear MDSC (PMN-MDSC) (8–10). Accumulation of MDSC occurs in a lot of physiological and pathological conditions, such as cancer, infection, autoimmune disorders, obesity, and pregnancy (7). Immune suppressive activity is the hallmark of MDSC. The most prominent immune regulatory factors of MDSC include Arginase 1, reactive oxygen species (ROS), prostaglandin E2, nitric oxide synthase, and immune checkpoints (11, 12). In different contexts MDSC suppress the immune response via different mechanisms (7). MDSC are involved in the maintenance of immune tolerance of pregnancy by inhibiting cytotoxic T cells activation, suppressing NK cells killing activities and regulating regulatory T cells (5, 6, 13, 14). Decreased MDSC has been associated with URPL and depletion of MDSC in murine pregnancy model can lead to implantation failure or embryo loss (6, 14, 15). Several studies have reported potential mechanisms of MDSC expansion during pregnancy in healthy women. Estrogen or progesterone can facilitate expansion and activation of MDSC (15, 16). Fetal-derived factor HLA-G also plays a role in PMN-MDSC accumulation via STAT3 pathway stimulation (17). Moreover, CXCR2/CXCL1 axis, which is also related to PMN-MDSC infiltration into tumor, promotes PMN-MDSC recruitment to the decidual microenvironment (18). Nevertheless, little is known about transcription features and cell fate of MDSC in normal pregnancy (NP) and URPL. Reagents targeting MDSC survival have been demonstrated to be effective for cancer treatment in both murine models and human participants (19–21). Understanding the cell fate of decidual MDSC is critical for developing better therapeutic approaches for pregnancy complications such as URPL.

In this study, we showed PMN-MDSC was the most abundant MDSC subset in decidua and only PMN-MDSC, not M-MDSC, decreased in decidua isolated from patients with URPL. Furthermore, we found apoptosis of decidual PMN-MDSC was activated in the URPL group. Increased TRAIL expression, together with reduced DcR2 in decidual PMN-MDSC, played an important role in excessive apoptosis of PMN-MDSC in URPL.

MATERIALS AND METHODS

Study Participants

From June 2018 to December 2019, a total of 33 women with clinical NP and 23 women with URPL were enrolled in the study. The demographic characteristics of participants in the URPL group and the NP group are concluded in **Supplemental Table 1**. The fetal heartbeat of the NP group was verified by ultrasound before elective termination of pregnancy. Patients with URPL were enrolled in the URPL group if they fitted the following criteria: (1) two or more previous spontaneous abortions; (2) absence of uterine malformation; (3) normal karyotype of parents and abortus; and (4) absence of endocrine, metabolic, autoimmune diseases, thrombophilia, or infection; (5) Fetal heartbeat had ceased or never detected. Decidual tissues from 6 to 9 weeks were harvested immediately after surgery under sterile conditions and washed in cold PBS to remove blood and fetal tissues. Decidual tissues were frozen in liquid nitrogen for protein or RNA extraction. For immunohistochemistry, decidual tissues were fixed in formalin and embedded in paraffin for preservation. Peripheral blood was collected from healthy non-pregnant donors. The study was approved by the Human Research Ethics Committee of Renji Hospital and written informed consent was obtained from all participants.

Cell Isolation

Single-cell suspensions were obtained by homogenizing tissues in PBS in the gentleMACS Dissociator (Miltenyi Biotec, Germany) with gentleMACS program B and C. Cell suspensions were strained through a 70-μm strainer and subsequently through a 40-μm strainer. Afterwards, the cells were washed with PBS and isolated using Ficoll density gradient (GE healthcare, USA). Mononuclear cells were harvested from the interphase. Cells were used immediately after isolation for phenotypic characterization and functional analysis. For sorting of decidual PMN-MDSC, decidual mononuclear cells were labeled with: CD11b-APC (BioLegend, USA), HLA-DR-FITC (BioLegend, USA) and CD15-PE (BD Bioscience, USA). PMN-MDSC were sorted as $CD11b^{+}HLA\text{-}DR^{-}CD15^{+}$ using FACS Aria II (BD Bioscience, USA). In experiments involving survival analysis, CD15 MicroBeads (Miltenyi Biotec, Germany) and MACS sorting were used for separation of PMN-MDSC. To isolate $CD3^{+}$ T cells for T cell suppression assay, peripheral blood mononuclear cells (PBMCs) of healthy donors were labeled with CD3 MicroBeads (Miltenyi Biotec, Germany) and sorted. Purity was >90% as confirmed by flow cytometry.

Analytical Flow Cytometry

Fc receptor blocking solution (BioLegend, USA) was added prior to staining. The following antibodies were used in this study: CD45-APC-H7 (BD Bioscience, USA), CD45-Percp-Cy5.5 (BD Bioscience, USA), CD33-PE-Cy7 (BioLegend, USA), CD11b-FITC (BioLegend, USA), CD11b-BV421 (BD Bioscience, USA), CD11b-APC (BioLegend, USA), HLA-DR-BV421 (BD Bioscience, USA), HLA-DR-FITC (BioLegend, USA), CD14-PE (BD Bioscience, USA), CD15-APC (BD Bioscience, USA), CD15-BV510 (BioLegend, USA), CD3-FTIC (BioLegend, USA), IFN-γ-PE-Cy7 (BioLegend, USA), CD4-APC (BioLegend, USA), CD8-PE (BioLegend, USA), CD16-PE (BD Bioscience, USA), CD56-PE-Cy7 (BD Bioscience, USA), Fas-APC (BD Bioscience, USA), CD261-APC (BioLegend, USA), CD262-PE (BioLegend, USA), CD263-PE (eBioscience, USA), and CD264-PE (R&D Systems, USA). FVD eFluor 780 (eBioscience, USA) was used to identify dead cells for excluding them from the analysis. Flow cytometry data were acquired with LSRFortessa (BD Bioscience, USA) or Beckman Coulter FC500 (Beckman, USA), and were analyzed using FlowJo software (BD Bioscience, USA). Positive subpopulations were identified by comparing stained samples with Fluorescence minus one (FMO) controls.

T Cell Suppression Assay

$CD3^{+}$ cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) (BD Bioscience, USA) according to the manufacturer's instructions and cultured with PMN-MDSC

isolated from decidual tissues at a ratio of 2:1 or 6:1 in 96-well plates. $CD3^+$ cells were stimulated with 10 μg/mL pre-coated anti-CD3 (OKT3, BioLegend, USA) and 1 μg/mL soluble anti-CD28 (CD28.2, BioLegend, USA). After 3.5 days of incubation, cells were resuspended in PBS for flow cytometry. For T cell secretion suppression assay, unlabeled $CD3^+$ cells were used in the T: MDSC cell co-culture system. After 3.5 days, leukocyte activation cocktail with BD Golgiplug (BD Bioscience, USA) was added into the culture system for 5 h. Afterwards, the cells were harvested for the analysis of intracellular cytokine expression. Cells were cultured at 37°C in humidified air with 5% CO_2 in RPMI 1640 (HyClone, USA) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Gibco, USA) and 1% penicillin/streptomycin (Gibco, USA).

Gene Expression Profile Analysis

The Human Whole Genome OneArray HOA 7.1 (Phalanx Biotech Group, China) was used to examine the whole-genome expression profiles of sorted PMN-MDSC from three women of the NP group and three women of the URPL group. Total RNA was extracted using Trizol Reagent (Invitrogen, USA) according to the manufacturer's instructions. The RNA integrity number (RIN) was 7–10. Cy5-labeled aRNA was hybridized and scanned on a G2505C Agilent Microarray Scanner (Agilent Technologies, USA) with Agilent 0.1 XDR software. Heatmap analysis was performed using R and a fold change of >1.5 was considered to be significant. Gene set enrichment analysis (GSEA) including GO and KEGG pathway was carried out using GSEA 4.0.1 and gene sets were obtained from the MSigDB database v7.0 (22, 23). The complete data were deposited in NCBI Gene Expression Omnibus with accession number GSE139180.

Survival Assay

PMN-MDSC isolated from decidual tissues of normal pregnancy were cultured at 37°C in humidified air with 5% CO2 in RPMI 1640 (HyClone, USA) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Gibco, USA), 1% penicillin/streptomycin (Gibco, USA) and 5 ng/mL recombinant GM-CSF (R&D Systems, USA). Decidual PMN-MDSC were treated with FasL (10, 100 ng/mL, BioLegend, USA), TRAIL (10, 100 ng/mL, Gibco, USA) or DR5 agonist Bioymifi (10, 50 μM, Selleck, USA) for 24 h and then were collected. In some experiments, PMN-MDSC were preincubated with anti-human DcR2 Ab (10 μg/mL, R&D Systems, USA) for 1 h before exposed to TRAIL. Apoptosis of PMN-MDSC was tested using activated Caspase-3-PE (BD Bioscience, USA) staining or Annexin V Apoptosis Detection Kit (BD Bioscience, USA) according to the manufacturer's instructions.

Real-Time Quantitative RT-PCR

Total RNA was extracted from decidual tissues with TaKaRa MiniBEST Universal RNA Extraction Kit (Takara, Japan) according to the manufacturer's instructions. Concentration of RNA was measured by NanoDrop ND-1000 (Thermo Fisher Scientific, USA). mRNA was synthesized into cDNA using PrimeScript RT Reagent Kit (Takara, Japan). FasL, TRAIL and GAPDH were amplified through qRT-PCR using SYBR Premix Ex Taq II (Takara, Japan) with QuantStudio Dx Real-Time Instrument (Life Technologies, USA). For clinical samples, relative gene expression was calculated with $2^{-\Delta CT}$ method normalized to GAPDH. The sequences of primers were listed in **Supplemental Table 2**.

Western Blot

Decidual samples were homogenized, incubated with radio-immuno precipitation assay (RIPA) lysis buffer (Thermo Fisher Scientific, USA) with protease inhibitors (Sigma-Aldrich, USA) for 30 min on ice. Total protein extracts were obtained after centrifuging at 12,000 g for 15 min at 4°C. Protein concentrations were measured using a bicinchoninic acid assay (BCA) assay kit (Beyotime Biotechnology, China). A hundred microgram protein were loaded on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Sigma-Aldrich, USA). Membranes were blocked in 5% w/v bovine serum albumin (BSA) for 1 h at room temperature. Then membranes were incubated at 4°C overnight with the following primary antibodies: anti-TRAIL (Cell Signaling Technology, USA, 1:1,000), anti-FasL (Absin, China, 1:500), and anti-β-actin (Santa Cruz Biotechnology, USA, 1:1,000). β-actin was used as the internal control. Then the blot was incubated with the corresponding IRDye 800CW-conjugated secondary antibody (LI-COR Biosciences, USA, 1:10,000) for 1 h at room temperature. Signals were detected using Odyssey Infrared Imaging System (LI-COR Biosciences, USA) and the blots were quantified using ImageJ (McMaster Biophotonics Facility, Canada).

Immunohistochemistry

Immunohistochemistry was performed as previously described (24). Four micrometer sections of formalin-fixed paraffin-embedded decidual samples were incubated overnight with anti-FasL antibody (1:200; Abcam, USA) and anti-TRAIL antibody (1:200; Cell Signaling Technology, USA, 1:500) at 4°C. Monoclonal or polyclonal rabbit IgG served as the negative control. Bright-field images were taken using Leica DM2500 (Leica, Germany). Images were randomly taken from each section, and the average optic density was identified with ImageJ (McMaster Biophotonics Facility, Canada).

Statistical Analysis

Results are presented as mean ± standard deviation (SD). Unpaired Student's *t*-test was used to analyze the differences between the two groups. When the variances of the two groups differed in F test, the Mann-Whitney *U*-test was used to compare the two groups. Comparison among multiple groups was carried out by one-way ANOVA followed by Tukey's *post-hoc* test. Correlations between parameters were evaluated using Pearson correlation analysis. P-value < 0.05 was considered to be statistically significant. All statistical analyses were performed using GraphPad Prism 7 Software (GraphPad Software, USA).

RESULTS

PMN-MDSC Was the Major Subset of Decidual MDSC and Decreased in the URPL Group

We used current consensual marker combinations for characterization of MDSC subsets. PMN-MDSC were defined as HLA-DR$^{-/low}$CD11b^{+}CD33^{+}CD15^{+}CD14^{-} and M-MDSC were defined as HLA-DR$^{-/low}$CD11b^{+}CD33^{+}CD15^{-}CD14^{+} (**Figure 1A**). We found that both CD33^{dim}CD15^{+} PMN-MDSC and CD33^{bright}CD14^{+} M-MDSC existed in decidua of early pregnancy. To determine the potential role of MDSC in pathogenesis of URPL, we analyzed percentage of MDSC subsets within the total CD45^{+} leukocytes of 23 patients with URPL and 33 women with normal pregnancy using flow cytometry. Compared with M-MDSC, more PMN-MDSC accumulated in decidua in both the NP group and the URPL group (**Figure 1B**; $P < 0.0001$). Notably, only decidual PMN-MDSC significantly decreased in the URPL group compared with normal pregnancy (**Figure 1B**; $P = 0.001$).

Decidual PMN-MDSC in Both the NP Group and the URPL Group Had Suppressive Activity

T cell suppression ability is a hallmark of MDSC and is indispensable when defining MDSC. PMN-MDSC were isolated from decidual tissues of the NP group and the URPL group and then cocultured with purified CD3^{+} T cells at ratio of 1:2 or 1:6 in the presentence of anti-CD3/CD28 stimulation for 3.5 days. PMN-MDSC in both groups remarkably suppressed proliferation of CD4^{+} T cells or CD8^{+} T cells (**Figures 2A,B**). IFN-γ production was also suppressed by PMN-MDSC in both the NP group (**Figure 2C**; $P = 0.02$) and the URPL group (**Figure 2C**; $P = 0.04$). Altogether, these data indicated that PMN-MDSC in both the NP group and URPL group exerted potent suppression ability.

Decidual PMN-MDSC in the URPL vs. the NP Groups Showed Different Gene and Pathway Signature

To evaluate the potential role of PMN-MDSC in maintaining the normal pregnancy, we performed whole-genome expression profile analysis of decidual PMN-MDSC in the NP group and the URPL group. The gene expression pattern of the decidual PMN-MDSC was significantly different between the NP group and the URPL group. Altogether, 423 differentially expressed genes (DEG) exhibited a fold change of >1.5 with an adjusted *P*-value of <0.05; 303 genes were upregulated and 120 genes were down-regulated in the URPL group compared with the NP group (**Figures 3A,B**). To elucidate functional features of PMN-MDSC in NP and URPL, GSEA were performed. KEGG gene sets and GO gene sets were used in the analysis. Of note, toll-like receptor signal pathway, apoptosis, leukocyte activation involved in inflammatory response and phagocytic vesicle were significantly enriched in the URPL group while extracellular matrix (ECM) receptor interaction, TGF-beta signaling pathway, cell adhesion mediator activity and growth factor binding were remarkably enriched in the NP group (**Figures 3C,D**).

Decidual PMN-MDSC in URPL Underwent More Apoptosis

To further examine whether decidual PMN-MDSC in the URPL group experienced more apoptosis than that in the NP group, we analyzed activated Caspase 3 expression in PMN-MDSC of the two groups. There was significant difference in the proportion of apoptotic cells in freshly isolated decidual PMN-MDSC of the NP group and the URPL group (**Figure 4A**; $P = 0.004$). After cultured *in vitro* for 24 h, activated Caspase3 expression was also higher in PMN-MDSC of the URPL group (**Figure 4A**; $P = 0.002$). Furthermore, the proportion of PMN-MDSC within the total decidual CD45^{+} leukocytes was negatively correlated with activated Caspase 3 expression in PMN-MDSC (**Figure 4B**; Pearson $r = -0.51$, $P = 0.031$). The apoptosis of decidual PMN-MDSC was also examined by Annexin V staining. For PMN-MDSC which were freshly isolated or cultured for 24 h, the percentage of Annexin V^{+} PMN-MDSC was higher in the URPL group compared with the NP group (**Figure 4C**; $P = 0.02$, $P = 0.006$). Activated Caspase 3 expression was of no difference between the NP group and the URPL group for freshly isolated decidual NK cells (**Supplemental Figure 1**) and decidual T cells (**Supplemental Figure 1**). These data indicated that decidual PMN-MDSC in the URPL group underwent more apoptosis than that in the NP group.

Decidual FasL and TRAIL Expression Were Increased in URPL

We determined the expression levels of FasL and TRAIL in decidual tissues of the URPL and the NP group. qRT-PCR results showed that mRNA level of FasL (**Figure 5A**; $P = 0.034$) and TRAIL (**Figure 5B**; $P = 0.008$) significantly increased in the URPL group. Western blot showed protein levels of FasL (**Figure 5C**; $P = 0.011$) and TRAIL (**Figure 5D**; $P = 0.022$) were remarkably upregulated in the URPL group. Immunohistochemistry staining showed that in the NP group, FasL expression was stronger in the glandular epithelial cells than that in the decidual stromal cells (**Figure 5**E1); however, in the URPL group, both glandular epithelial cells and decidual stromal cells showed moderate FasL expression (**Figure 5**E2). TRAIL was localized in both glandular epithelial cells and decidual stromal cells (**Figure 5**F1,F2), and epithelial cells showed stronger expression than stromal cells in the NP group (**Figure 5**F1). FasL and TRAIL staining were stronger in the URPL group (**Figures 5E,F**; $P = 0.005$, $P < 0.0001$). Localization of glandular epithelial cells and decidual stromal cells were determined by staining of cytokeratin 7 and vimentin (**Supplemental Figure 2**), respectively. The negative control stained with polyclonal or monoclonal rabbit IgG showed no positive staining (**Figure 5**E3,F3).

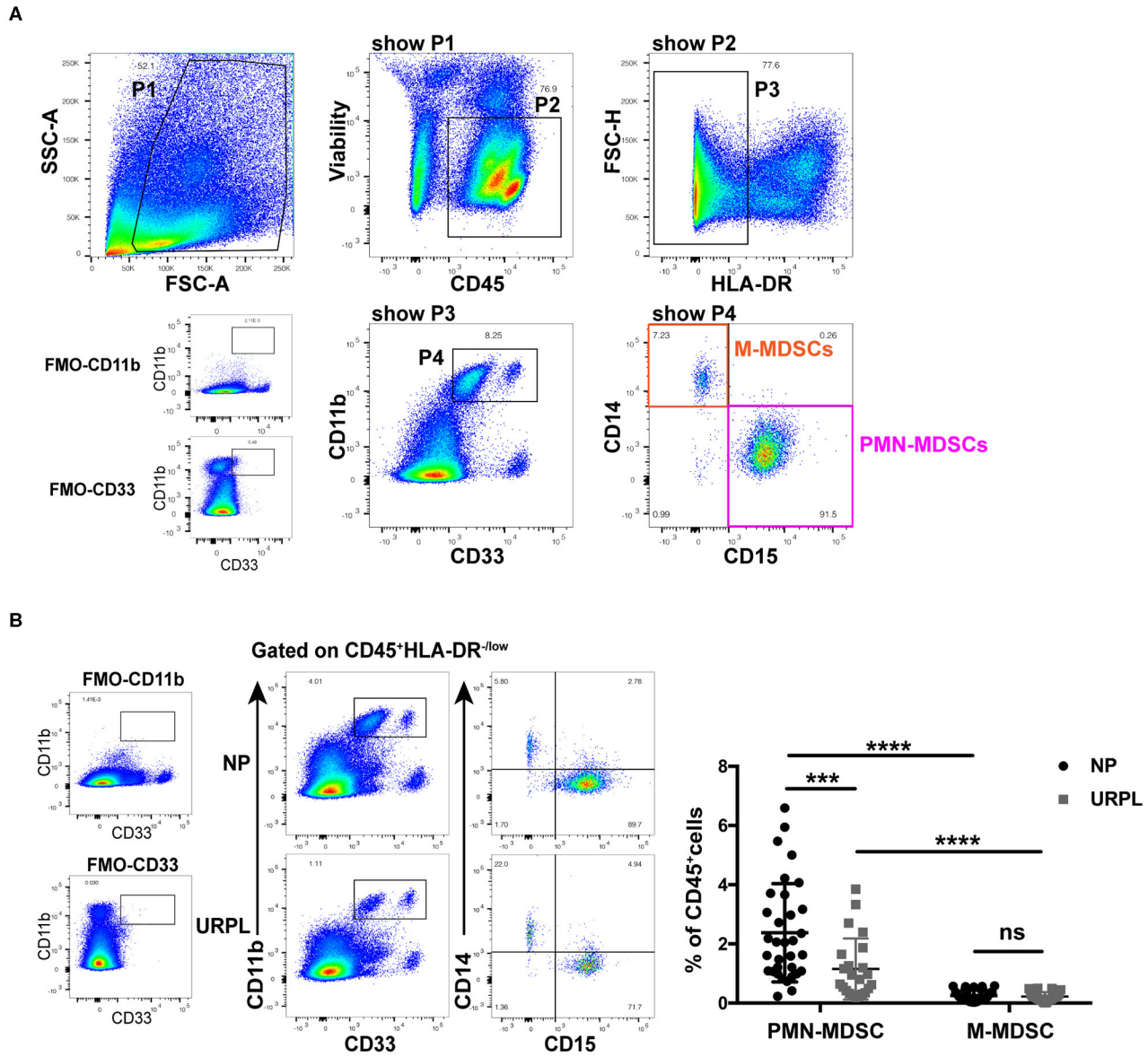

FIGURE 1 | Phenotypic characteristics and frequency of MDSC subsets of human decidual tissue in the normal pregnancy (NP) group and the unexplained recurrent spontaneous abortion (URPL) group. **(A)** Representative flow cytometry showing gating strategy of PMN-MDSC (gated on HLA-DR$^{-/low}$CD11b^{+}CD33^{+} CD15^{+}CD14^{-}) and M-MDSC (gated on HLA-DR$^{-/low}$CD11b^{+}CD33^{+}CD15^{-}CD14^{+}) in decidua. **(B)** Representative flow cytometry of decidual PMN-MDSC and M-MDSC of the NP and the URPL group (left). Percentage of decidual PMN-MDSC and M-MDSC of NP ($n = 33$) and URPL ($n = 23$) (right) were analyzed (mean ± SD, Mann-Whitney U-test). ***$P < 0.001$; ****$P < 0.0001$; ns, not significant. FMO, Fluorescent minus one.

Apoptosis of Decidual PMN-MDSC Was Regulated by TRAIL and TRAIL-Rs

We then analyzed the expression of membrane receptors of FasL and TRAIL on decidual PMN-MDSC. Decidual PMN-MDSC in both the NP group and the URPL group showed expression of Fas, DR4, DR5, DcR1, and DcR2 (**Figure 6A**). The expression level of DR4 was rather low since the fluorescence intensity was very close to the FMO control. PMN-MDSC of the two groups did not differ in the expression of Fas, DR4, DR5, or DcR1; however, DcR2 expression on PMN-MDSC was significantly down-regulated in the URPL group ($P = 0.016$). We next investigated whether decidual PMN-MDSC was sensitive to FasL or TRAIL induced apoptosis. PMN-MDSC from the NP group were treated with recombinant human FasL and TRAIL. FasL could not induce apoptosis of decidual PMN-MDSC at the concentration of both 10 and 100 ng/mL (**Figure 6B**). Notably, decidual PMN-MDSC showed significantly higher apoptosis (**Figure 6B**, $P = 0.0006$) after exposure to 100 ng/mL TRAIL.

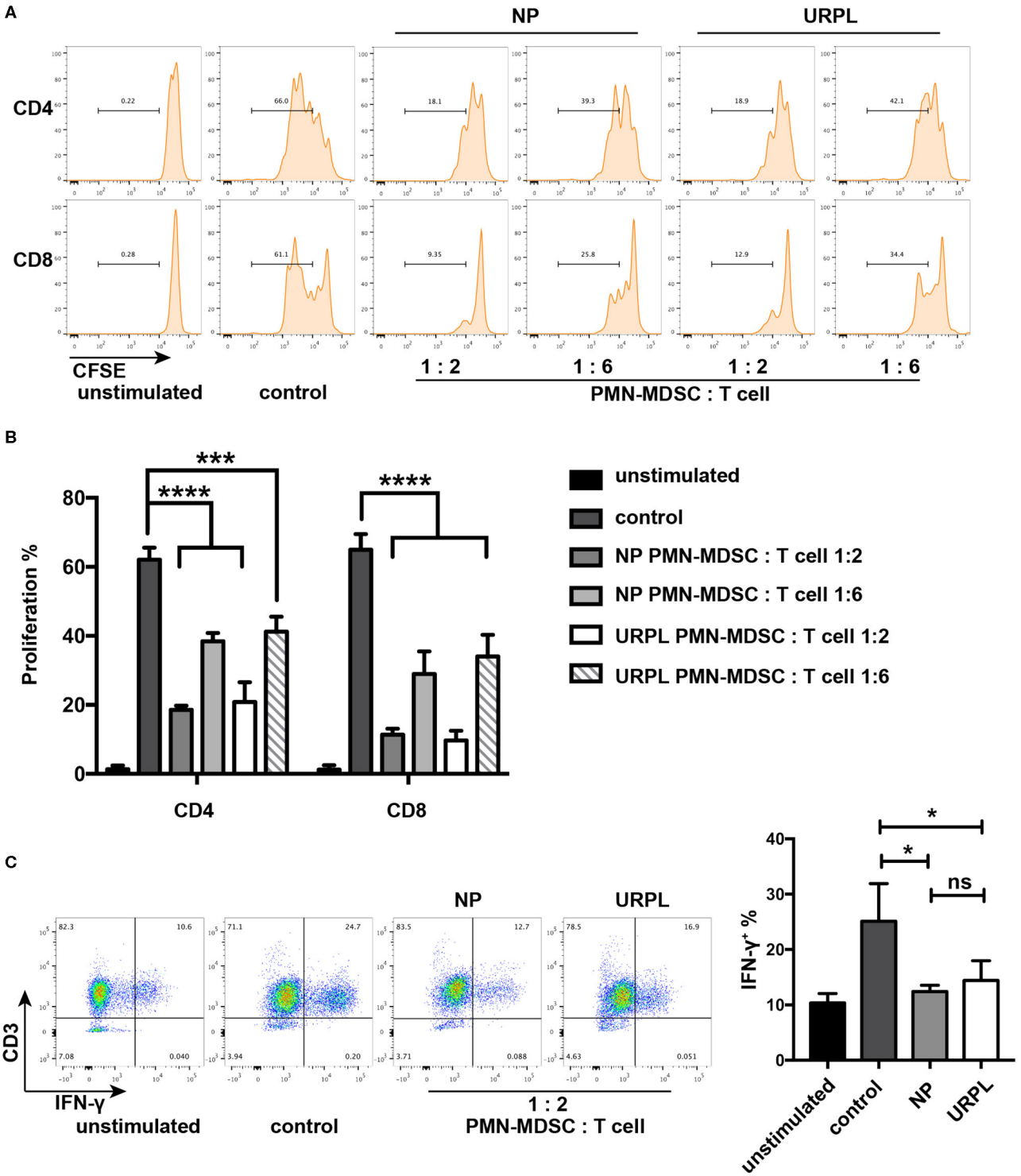

FIGURE 2 | Functional characteristics of decidual PMN-MDSC in the NP group and URPL group. **(A)** and **(B)** CD3/CD28-stimulated T cells were cocultured with purified PMN-MDSC of the NP group and the URPL group from decidua of pregnancy between 6 and 9 weeks at a ratio of 2:1 or 6:1 for 3.5 days. The percentage of proliferative $CD4^+$ T cells or $CD8^+$ T cells were analyzed (mean ± SD, one-way ANOVA, Tukey's *post-hoc* test). **(C)** CD3/CD28-stimulated T cells were cocultured with purified PMN-MDSC of the NP group and the URPL group from decidua of pregnancy between 6 and 9 weeks at a ratio of 2:1 for 3.5 days. Percentage of IFN-γ-expressing T cells were analyzed (mean ± SD, one-way ANOVA, Tukey's *post-hoc* test). $n = 3$; $^*P < 0.05$; $^{***}P < 0.001$; $^{****}P < 0.0001$; ns, not significant.

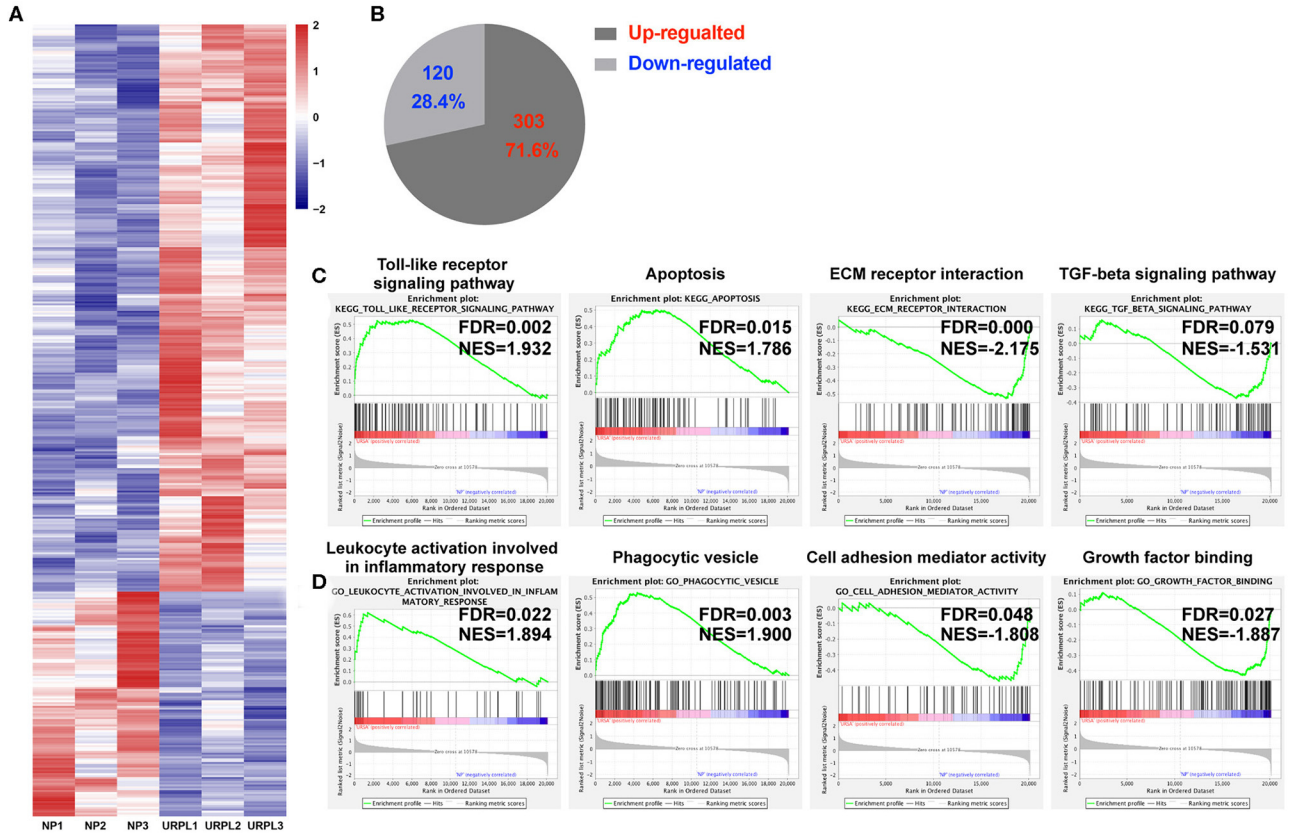

FIGURE 3 | Differential expression genes and pathways in human decidual PMN-MDSC of the NP and the URPL group. **(A)** Heatmap showing differentially expressed genes (DEGs). Rows in the heatmap represent DEGs. **(B)** The number and proportion of DEGs in PMN-MDSC that were upregulated or downregulated in the URPL group ($n = 3$) vs. the NP group ($n = 3$). **(C)** GSEA analysis of KEGG gene sets including toll-like receptor signal pathway, apoptosis, ECM receptor interaction and TGF-beta signaling pathway. **(D)** GSEA analysis of GO gene sets including leukocyte activation involved in leukocyte activation involved in inflammatory response, phagocytic vesicle, cell adhesion mediator activity, and growth factor binding. Normalized enrichment score (NES) reflects the degree to which a gene set was upregulated (positive NES) or downregulated (negative NES) in PMN-MDSC of URPL and false discovery rate (FDR) represents statistical significance of difference.

Moreover, exposure to 10 or 50 μM DR5 agonist Bioymifi induced a significantly high level of PMN-MDSC apoptosis than TRAIL ($P < 0.0001$, $P < 0.0001$). To examine whether the response to TRAIL-induced apoptosis was mediated by DcR2 expression, we detected the expression of activated Caspase 3 in decidual PMN-MDSC after exposure of anti-DcR2 Ab and TRAIL for 24 h (**Figure 6C**). Notably, compared with isotype control, DcR2 blockade significantly increased TRAIL-induced apoptosis ($P < 0.0001$).

DISCUSSION

As an important regulator of the immune system, the role of MDSC in pregnancy has been established in several studies (5, 6, 13, 14). A certain number of MDSC take part in maintaining immune tolerance during normal pregnancy and a lack of MDSC can lead to pregnancy failure (14, 15). Consistent with previous studies of our group and others (5, 13), we found that PMN-MDSC was the major subset of MDSC in human decidua since the percentage of PMN-MDSC remarkably exceeded that of paired M-MDSC in both the NP group and the URPL group. Also, only PMN-MDSC, but not M-MDSC, decreased significantly in decidua of patients with URPL compared with normal pregnancy. During pregnancy, PMN-MDSC can facilitate maternal-fetal immune tolerance via crosstalk with various immune cells. PMN-MDSC suppress T cell proliferation via ROS or Arginase I and polarize $CD4^+$ T cells toward a Th2 cytokine response (4, 14, 15). PMN-MDSC can also induce regulatory T cells in a TGF-beta dependent manner (5). Moreover, PMN-MDSC can inhibit NK cytotoxicity by inhibiting expression of perforin, granzyme B, and NKG2D (6). We found that PMN-MDSC of both the NP group and the URPL group potently suppressed T cell proliferation as well as cytokine production, validating the immune regulatory ability of decidual PMN-MDSC.

According to our whole-genome expression profile analysis, genes upregulated in PMN-MDSC of the URPL group significantly enriched in apoptosis compared with PMN-MDSC of the NP group. Flow cytometry analysis further validated that compared with PMN-MDSC in the NP group,

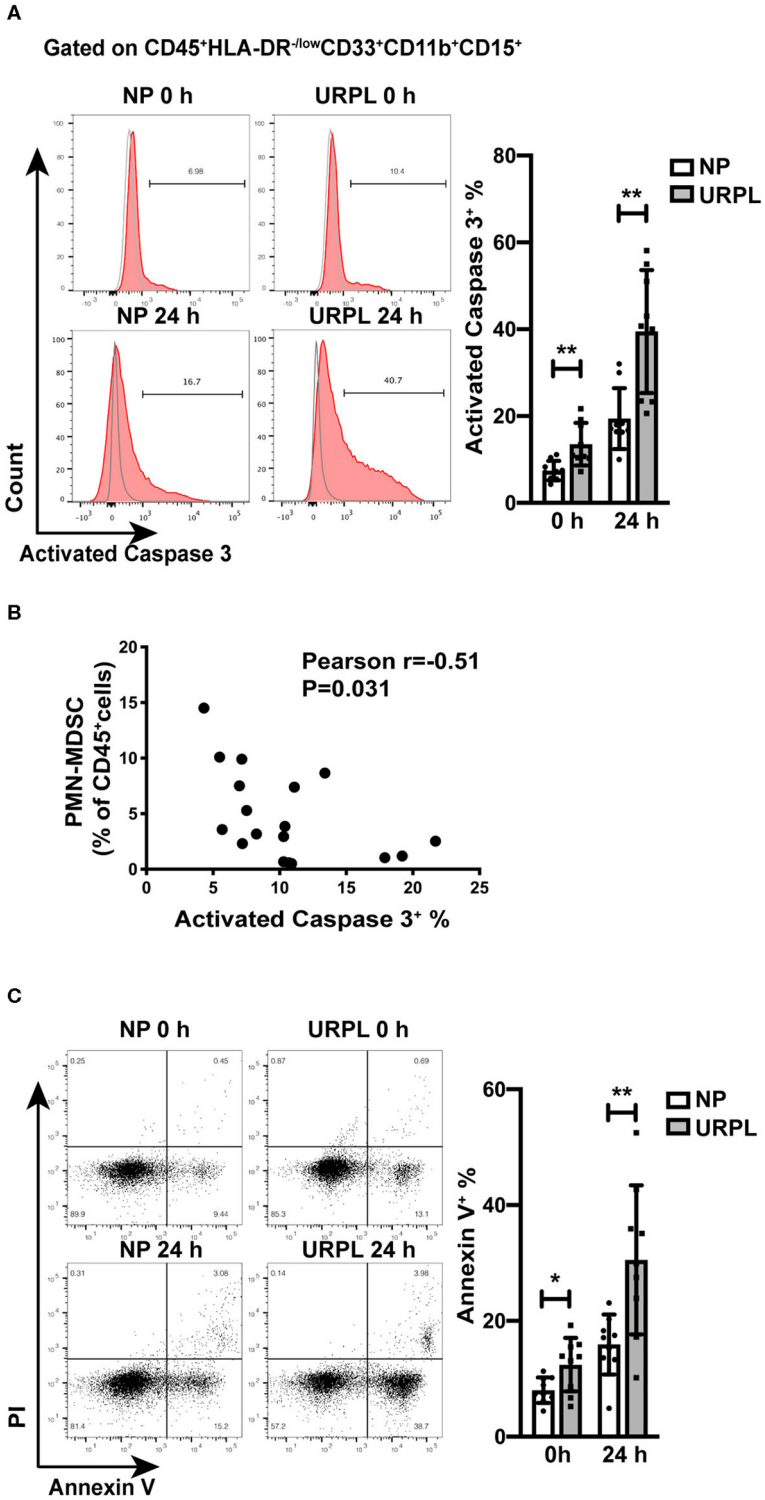

FIGURE 4 | Apoptosis of decidual PMN-MDSC of NP and URPL. **(A)** After isolated from decidual tissues or cultured *in vitro* for 24 h, expression of activated Caspase 3 of decidual PMN-MDSC between the NP group ($n = 9$) and the URPL group ($n = 9$) were determined. **(B)** The correlation of activated Caspase 3 expression in decidual PMN-MDSC with proportion of PMN-MDSC within the total decidual $CD45^+$ leukocytes was analyzed ($n = 18$). **(C)** After isolated from decidual tissues or cultured *in vitro* for 24 h, expression of Annexin V in PMN-MDSC between the NP group ($n = 9$) and the URPL group ($n = 9$) were analyzed. Mean ± SD, unpaired Student's *t*-test; $^*P < 0.05$; $^{**}P < 0.01$.

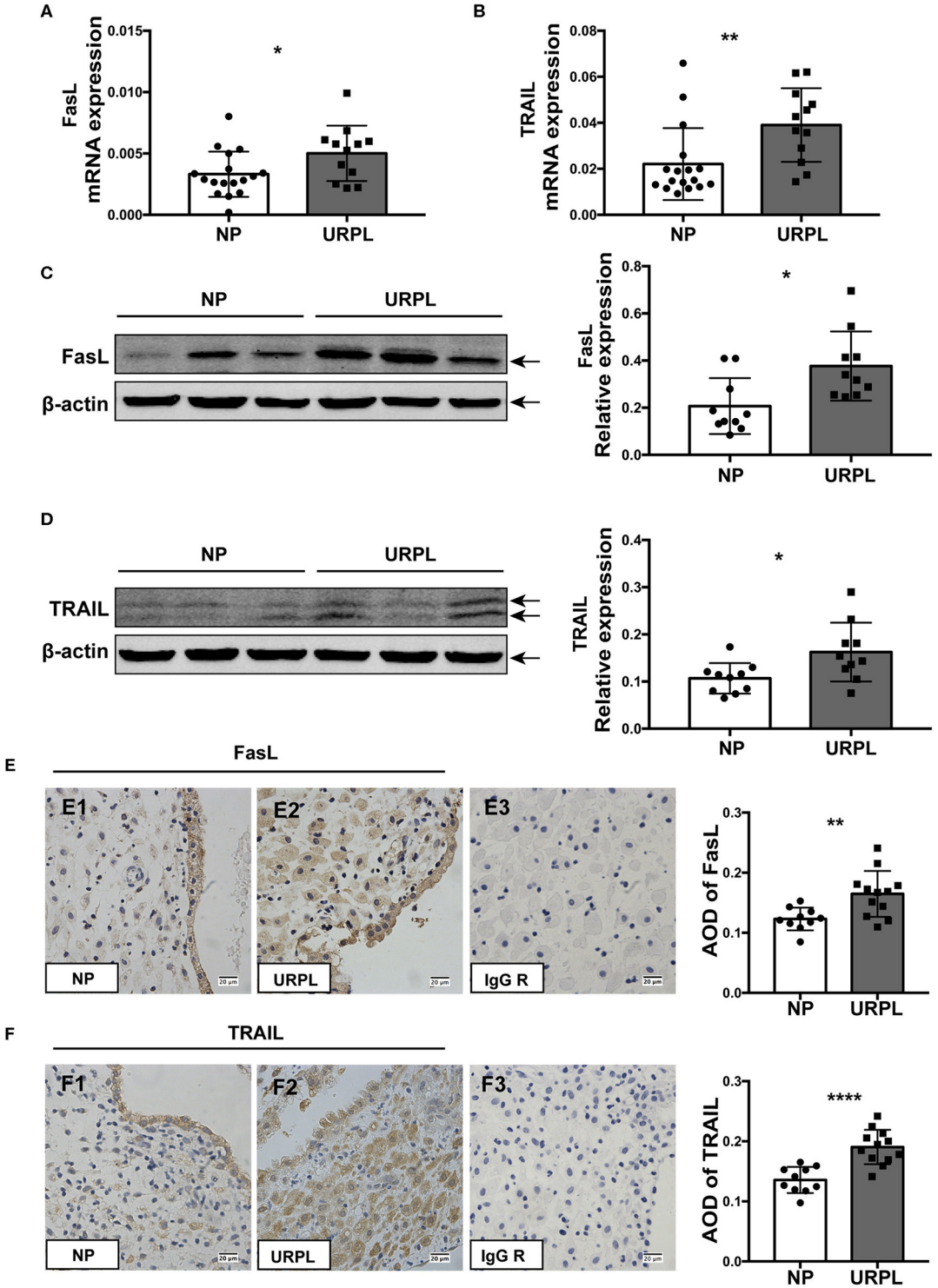

FIGURE 5 | FasL and TRAIL expression in human decidual tissue of the NP and the URPL group. **(A)** and **(B)** Decidual FasL and TRAIL mRNA expression in the NP group ($n = 17$) and the URPL group ($n = 12$) was quantified using $2^{-\Delta CT}$ method normalized to GAPDH. **(C,D)** Representative western blot results and statistical analysis of FasL and TRAIL in the NP ($n = 10$) group and URPL ($n = 10$) group were shown. Relative protein amount was normalized to β-actin. Arrows indicate the specific bands for each antibody. **(E,F)** Representative immunohistochemical staining image and quantification of the average optical density (AOD) of FasL and TRAIL in the NP group ($n = 10$) (E1, F1) and the URPL group ($n = 12$) (E2, F2). Polyclonal or monoclonal rabbit IgG substituted primary antibodies in the negative control (E3, F3) Original magnification, ×400; mean ± SD, unpaired Student's *t*-test; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{****}P < 0.0001$.

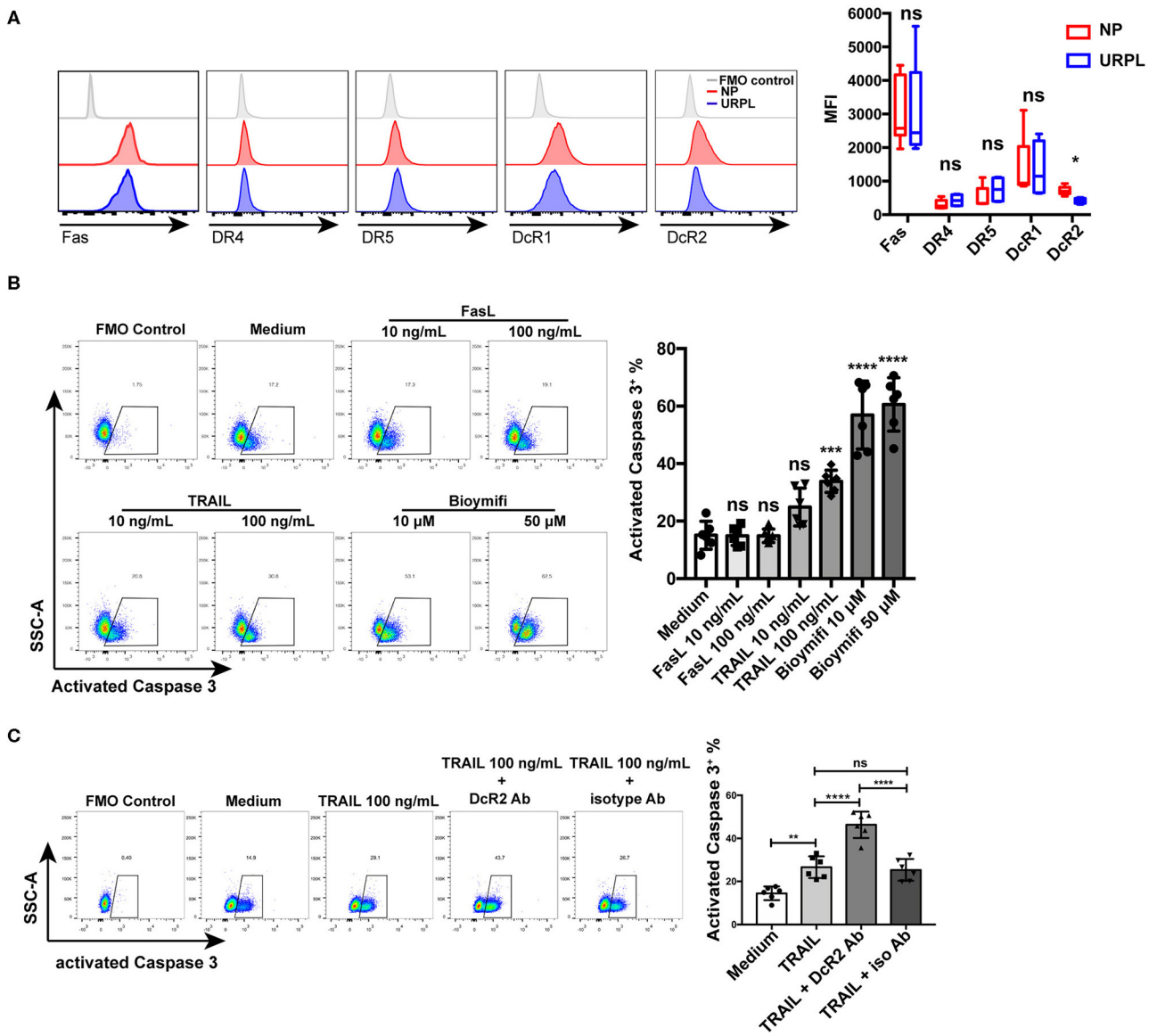

FIGURE 6 | TRAIL and TRAIL-Rs mediated decidual PMN-MDSC apoptosis. **(A)** Representative flow cytometry of Fas, DR4, DR5, DcR1, and DcR2 expression of decidual PMN-MDSC (left) and statistical analysis (right, $n = 4$–9, mean ± SD, Mann-Whitney *U*-test). **(B)** Decidual PMN-MDSC from the NP group were treated with FasL (10, 100 ng/mL), TRAIL (10, 100 ng/mL), or DR5 agonist Bioymifi (10, 50 μM) for 24 h ($n = 6$) and activated Caspase 3 expression was analyzed (mean ± SD, one-way ANOVA, Tukey's *post-hoc* test). **(C)** Decidual PMN-MDSC from the NP group were stimulated with TRAIL (100 ng/mL) in the presentence of 10 μg/mL anti-DcR2 Ab or isotype control for 24 h. Activated Caspase 3 was measured. Representative flow cytometry (left) and quantification (right) were shown ($n = 6$) (mean ± SD, one-way ANOVA, Tukey's *post-hoc* test). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$; ns, not significant. FMO, Fluorescent minus one.

PMN-MDSC in the URPL group underwent more apoptosis. Mechanisms on MDSC apoptosis and survival have been investigated in other microenvironments, however, the results remain controversial. Cytotoxic T cells can induce MDSC apoptosis via Fas/Fas ligand (FasL) signaling pathway while resistance to Fas-mediated apoptosis contributes to the presence of MDSC in tumor, and this effect is exclusive in MDSC since other myeloid cells also express a similar level of Fas but do not respond to FasL (25, 26). Interestingly, in another mouse model, FasL deficiency leads to reduced MDSC and skews MDSC toward M-MDSC, indicating that PMN-MDSC decreases more after FasL knockdown (27). Moreover, inflammation conditions can protect MDSC from extrinsic-induced apoptosis (28). Tumor necrosis factor (TNF)-related apoptosis induced ligand (TRAIL) is another regulator of MDSC apoptosis via interacting with membrane bound TRAIL receptors (TRAIL-Rs). TRAIL-R1 (DR4 or CD261) and TRAIL-R2 (DR5 or CD262) are two death receptors, and ligation of TRAIL with either of them can activate the apoptotic pathway. TRAIL-R3 (DcR1 or CD263) and TRAIL-R4 (DcR2 or CD264) are two decoy receptors which bind to TRAIL without further inducing apoptosis (29). In a murine model, high expression of DR5 mediated TRAIL-induced effects,

while in human, differently expressed DcR1 and DcR2 could regulate cell fate of PMN-MDSC, indicating different apoptosis mechanisms between species (19, 20). In primary HIV-infected individuals, high TRAIL level is also associated with decreased PMN-MDSC (30).

FasL and TRAIL are located in placenta as well as decidua, and are important for immune privilege and successful pregnancy since they can mediate apoptosis of cytotoxic T cells or other immune cells with cell toxicity (31, 32). We found that in human decidua both glandular epithelial cells and decidual stromal cells expressed FasL and TRAIL. FasL and TRAIL levels were elevated in the URPL group, which is in accordance with that excessive FasL as well as TRAIL could also be involved in URPL (33–36). Then we detected Fas and TRAIL-Rs levels of decidual PMN-MDSC between the URPL and the NP group and only DcR2 expression was differentially expressed. Decidual PMN-MDSC did not respond to FasL-mediated apoptosis. However, they were sensitive to TRAIL-mediated apoptosis via Caspase-3 dependent pathway. Interestingly, 100 ng/mL TRAIL only increased two times apoptosis while DR5 agonist Bioymifi increased up to four times apoptosis. Further *in vitro* blocking of DcR2 can facilitate TRAIL-induced apoptosis in human decidual PMN-MDSC, which is in line with a clinical trial showing that DR5 agonist selectively eliminated PMN-MDSC in cancer patients and the effect is reversely correlated with DcR2 expression level (20).

This is the first study focusing on decidual PMN-MDSC survival during early human pregnancy. We found that the apoptosis levels of two major decidual leukocytes, NK and T cells, are similar in the NP and the URPL groups, implying that PMN-MDSC apoptosis might not be the result of enhanced apoptosis state in the URPL group. In addition, previous studies mainly focused on disparity in MDSC number between the NP and URPL groups. In the present study, although we showed that PMN-MDSC in both NP and URPL groups exerted potent immune suppressive function, the gene expression profile indicated significantly different enriched biology pathways between PMN-MDSC of the two groups. Genes upregulated in the PMN-MDSC of the URPL group were enriched in toll-like receptor signal pathway, leukocyte activation involved in inflammatory response and phagocytic vesicle, indicating that these cells experienced more inflammation than PMN-MDSC in the NP group. The ECM receptor interaction, TGF-beta signaling pathway, cell adhesion mediator activity and growth factor binding were negatively enriched in PMN-MDSC of the URPL group, indicating impaired interaction with extracellular matrix and other cells, which is important for cell recruitment and immune crosstalk. These issues remain be further investigation.

In conclusion, we demonstrate that human decidual PMN-MDSC in URPL are more sensitive to TRAIL-mediated apoptosis signal pathway owing to elevated TRAIL and decreased DcR2 expression. This could be a mechanism of impaired viability of decidual PMN-MDSC in URPL, however, the underlying molecular pathways of decidual PMN-MDSC apoptosis needs to be further elucidated. The observations presented in this study provide a new insight into mechanisms of dysregulation of PMN-MDSC in URPL, and therapeutic targeting on TRAIL-induced apoptosis signaling may provide novel strategies for URPL treatment.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the Gene Expression Omnibus - GSE139180.

ETHICS STATEMENT

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

AUTHOR CONTRIBUTIONS

CL, XZ, and AZ conceived and designed this study. CL and XZ performed the experiments. CL, XZ, CC, FG, and QW collected the samples. CL, XZ, and XK analyzed the data. CL drafted the manuscript. AZ revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Altered Glycosylation Contributes to Placental Dysfunction Upon Early Disruption of the NK Cell-DC Dynamics

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Immune cells [e. g., dendritic cells (DC) and natural killer (NK) cells] are critical players during the pre-placentation stage for successful mammalian pregnancy. Proper placental and fetal development relies on balanced DC-NK cell interactions regulating immune cell homing, maternal vascular expansion, and trophoblast functions. Previously, we showed that *in vivo* disruption of the uterine NK cell-DC balance interferes with the decidualization process, with subsequent impact on placental and fetal development leading to fetal growth restriction. Glycans are essential determinants of reproductive health and the glycocode expressed in a particular compartment (e.g., placenta) is highly dependent on the cell type and its developmental and pathological state. Here, we aimed to investigate the maternal and placental glycovariation during the pre- and post-placentation period associated with disruption of the NK cell-DC dynamics during early pregnancy. We observed that depletion of NK cells was associated with significant increases of O- and N-linked glycosylation and sialylation in the decidual vascular zone during the pre-placental period, followed by downregulation of core 1 and poly-LacNAc extended O-glycans and increased expression of branched N-glycans affecting mainly the placental giant cells and spongiotrophoblasts of the junctional zone. On the other hand, expansion of DC induced a milder increase of Tn antigen (truncated form of mucin-type O-glycans) and branched N-glycan expression in the vascular zone, with only modest changes in the glycosylation pattern during the post-placentation period. In both groups, this spatiotemporal variation in the glycosylation pattern of the implantation site was accompanied by corresponding

changes in galectin-1 expression. Our results show that pre- and post- placentation implantation sites have a differential glycopattern upon disruption of the NK cell-DC dynamics, suggesting that immune imbalance early in gestation impacts placentation and fetal development by directly influencing the placental glycocode.

Keywords: dendritic cells, natural killer cells, implantation, glycoimmunology, placentation

INTRODUCTION

In hemochorial placentation, the placental trophoblasts have direct contact to maternal immune cells. Thus, trophoblast cells are exposed to allogenic immune responses by the mother. Uterine immune responses must be regulated in a way that allows access of the placenta to the maternal blood supply but also prevents excess invasion of fetal cells and infections (1). For successful pregnancy, maternal tolerance to the fetus needs to be established, otherwise failure of the maternal immune response to adapt correctly can lead to aberrant immune activation, which is associated with preeclampsia and miscarriage (2).

Highly specialized subpopulations of maternal leukocytes, such as uterine NK (uNK) cells, infiltrate the murine decidua in large numbers during the first half of pregnancy (3, 4). Through expression of different factors (e.g., VEGF and IFN-γ), uNK cells guide the remodeling of decidual spiral arteries increasing the availability of maternal blood at the implantation site and promoting trophoblast invasion (5–7). Another important subpopulation of maternal leukocytes key for modulation of local immunity and tolerance are uterine DC (uDC), which increase in number during the pre-placentation period, reaching a plateau in the post-placentation phase (8). These cells also support vascular adaptations during pregnancy including vessel permeability and blood flow to the implantation site through the CXCL12/CXCR4 pathway (9–11). Recruitment of NK cells, which is facilitated by DC, represents a mechanism to confine the immunogenic potential of uDC. Thus, healthy dynamics in the proportion of uNK cells and uDC during pregnancy play a critical role not only in the regulation of angiogenesis and decidualization (11, 12) but also in the placentation process. Immune cell imbalance during early pregnancy, such as expansion of DC or depletion of NK cells, has an effect on the pre-placentation period and also on the placental phenotype (13). For instance, implantation sites from NK cell depleted dams showed decidual growth defects during early pregnancy, indicated by a disrupted dynamics of decidua maturation (12). Additionally, these mice exhibited vascular defects (i.e., narrow lumens and cuffed appearance) in the central, proximal region of the decidua basalis during the post-placentation period together with increased accumulation of vascular- and tissue-associated NK cells in the mesometrial lymphoid aggregate of pregnancy (13). As a result from placental insufficiency, fetuses derived from NK cell depleted dams suffer from intrauterine growth restriction (IUGR) accompanied by an overall reduction of global DNA methylation levels and epigenetic changes in the methylation of specific hepatic gene promoters. Likewise, the expansion of DC during early pregnancy also provoked decidual growth defects on E5.5 (12) and changes in immune cell recruitment, with increased numbers of perivascular DC at the mesometrial decidua (MD) (11) and upregulation of IL-10 expressing NK cells on E7.5 (14). Expansion of DC also led to significant changes in placental morphology, with impaired vascular development of the labyrinth and an increased accumulation of glycogen cells in the junctional zone (13), but the effect on pregnancy outcome was milder as offspring derived from these pregnancies did not suffer from IUGR and exhibited slight gene-specific epigenetic changes.

Glycans are sequences of carbohydrates that are added to proteins and lipids to modulate their structure and function (15). Two major types of glycosylation are observed: N-linked glycosylation is the attachment of oligosaccharides to asparagine or arginine side-chains, whereas O-linked glycosylation occurs mainly at serine and threonine (**Figure 1A**). Glycans modify proteins required for trophoblast function, and alterations have been associated with pathological conditions. Thus, aberrant N-glycosylation of integrin β1 in villous tissues, which influences trophoblast invasion, was linked to early spontaneous miscarriage in humans (16). Lectin histochemistry analyses performed in human placentas revealed significant alterations of carbohydrate metabolism (i.e., dysregulation of α-D-mannose, GlcNAc, β-GalNAc, and α-Fucose) after the onset of different types of hypertensive disorders and fetal growth restriction (17, 18); showing for instance alterations in the trophoblast and/or endothelial cell glycophenotype of the pathological groups (17) and an altered distribution of α2,3 and α2,6-linked sialic acid in placentas from hypertensive disorders (18). More recently, Tannetta et al. showed that preeclampsia is associated with changes in the surface glycosylation of syncytiotrophoblast derived extracellular vesicles (STBEVs), which are released in increased numbers and exhibit a proinflammatory, anti-angiogenic, and procoagulant activity. Indeed, STBEVs derived from preeclamptic patients exhibited increased binding of *Sambucus nigra* lectin and *Ricinus communis* agglutinin I, which bind to α2,6-linked sialic acid and galactose or N-acetylgalactosamine residues (19), which may be a link to changes in vesicle-cell interactions affecting functions like cell targeting, clearance, and immune activity. However, further investigation is needed to determine whether and how different alterations in glycosylation contribute to inappropriate maternal-fetal immune responses and poor pregnancy outcomes. In this work, we analyzed the effect of temporary changes within the DC or NK cell pool during early pregnancy on the glycophenotype during the pre- and post-placentation process, before the onset of the IUGR disease phenotype. We show that pre- and post-placentation implantations have a differential glycopattern where

either NK cells were temporally ablated or DC were expanded. Our data confirm that immune dysregulations early in gestation have an impact on the placental glycocode, influencing the placentation process itself and subsequently fetal development.

MATERIALS AND METHODS

Animals

All animals tissues used in this work were collected for previous experiments assessing the role of NK cell—DC interactions in the modulation of early pregnancy maternal adaptations, placentation and fetal growth (11–13) in accordance with guidelines for the care and use of laboratory research animals promulgated by the Charité—Universitätsmedizin Berlin and Regional Office for Health and Social Affairs. Animals were purchased from Jaxmice® and maintained on a 12L/12D cycle. Five- to six-weeks old CD11c.DTR females were mated with Balb/c males. The presence of a vaginal plug after cohabitation was denoted as embryonic day (E) 0.5. Females were kept in groups of 4–5 animals and injected (i.p.) on E4.5 with anti asialo GM1 (WAKO, Cat.no. 986-10001, 2 mg/g BW) for transient ablation of NK cells (aNK group, **Figure 1B**). For the expansion of uterine DC (eDC group, **Figure 1B**), Balb/c-mated CD11c.DTR females were treated with one daily injection of human recombinant Fms-related tyrosine kinase 3 ligand (FL; BioX cell, Cat.no. BE0098, 10 mg/mouse/day) from E0.5 to E7.5. Control CD11c.DTR females received PBS supplemented with rabbit normal serum (2 mg/g body weight i.p.). On E7.5 and 13.5, mice from the respective groups were sacrificed and uterine tissue from the implantation sites ($n = 5$) was processed for histological sectioning according to standard procedures. Pregnancy outcomes for the different groups have been described in our previous studies (11–13).

Immunofluorescence

We used a panel of lectins that recognize specific O-glycan structures (Helix pomatia agglutinin (HPA; Tn-antigen), Arachis hypogaea lectin (PNA; core 1), and *L*ycopersicon esculentum lectin [LEA; core 2)]. In addition, we employed *Phaseolus vulgaris* lectin (PHA-L), which specifically recognizes β1,6GlcNAc-branched complex N-glycans. Finally, sialyation was determined using the Maackia amurensis lectin (MAA) and Sambucus nigra agglutinin (SNA-I) which bind to α2,3- and α2,6-linked sialic acid, respectively (**Figure 1A**). Serial cryosections of implantation sites were prepared at 8 µm. Briefly, slides were washed in TBS and blocked with Biotin Blocking system (X0590, DAKO Corporation) for 20 min in a humid chamber at RT. Afterwards, slides were blocked with Carbo-Free Blocking Solution (SP-5040, Vector Laboratories) for 30 min in a humid chamber at RT. Subsequently, slides were incubated with biotinylated lectin (EY Laboratories) diluted in Carbo-Free Blocking Solution for 16 h at 4°C in a humid chamber HPA (20 ng/ml; BA-3601-1), PHA-L (20 ng/ml; BA-1801-2), or SNA-I (10 ng/ml; BA-6802-1). Lectin-stained sections were then incubated with 2 µg/ml Streptavidin-Tetramethylrhodamine (S-870; Invitrogen) for 1 h in a humid chamber at RT. Subsequently, slides were incubated with FITC-labeled lectin (EY Laboratories) diluted in Carbo-Free Blocking Solution for 2 h at RT PNA (20 ng/ml; F-2301-1), LEA (20 ng/ml; F-7001-1), or MAA (20 ng/ml; F-7801-2). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) for 5 min at RT and mounted in Prolong Gold (P36930, Invitrogen). Stainings of whole implantation sites were digitally scanned by a high-resolution bright field and fluorescence slide scanner (Pannoramic MIDI BF/FL, 3DHISTECH Ltd.), and staining was evaluated on virtual slides using Pannoramic Viewer 1.15.4 (3DHISTECH Ltd.) by two examiners blinded to the experimental group.

Galectin-1 Staining

Staining of 8 µm cryo sections was performed by washing in TBS, followed by blocking with Duale Endogenous Enzyme Block (S2003, Dako) for 30 min in a humid chamber at RT. Afterwards, slides were blocked with Proteinblock (PHA-70873, Dianova) for 20 min. The primary antibody against galectin-1 (1:400; GTX 101566, GeneTex) was incubated over night at 4°C. The slides were than washed and incubated with HRP-conjugated secondary antibody (111-035-003; Jackson ImmunoResearch) for 1 h at RT. The signal was detected by incubation at RT with a 0.05% diaminobenzidine in 0.015% H_2O_2 substrate solution. After washing, nuclei were counterstained with 0.1% Mayer's hematoxylin followed by a standard dehydration procedure and mounting in Entellan (Merck Millipore).

Statistics

Data analysis was performed with GraphPad Prism 7 (GraphPad Software, Inc.). Data are presented as mean ± SEM and were analyzed with D'Agostino-Pearson omnibus normality test followed by unpaired *t*-test or Mann-Whitney test. A $p < 0.05$ was considered as significant.

RESULTS

Dysregulation of the NK Cell or DC Pool Changed the Distribution of O-Glycans, Complex N-Glycans, and Sialylation in the Mesometrial Decidua and Vascular Zone During the Pre-placentation Period

In order to determine whether temporary ablation of NK cell or expansion of DC during early pregnancy could influence the glycophenotype of the implantation sites, we analyzed implantation sites during the pre-placentation period (on E7.5) focusing on the quantification within the mesometrial decidua (MD) and vascular zone (VZ) (**Figure 1B**).

We first examined the O-glycans during the pre-placentation period (**Figure 1C**). During normal gestation abundant expression of core 1 O-glycans (PNA) compared to Tn antigen (HPA) and core 2 O-glycans (LEA) in the MD was observed (**Figure 1C**, left panel). Depletion of NK cells during early pregnancy caused a decrease in core 1 O-glycans (PNA) and an increase of Tn antigens (HPA) in this region. In contrast, the expansion of DC during the pre-placentation period caused a

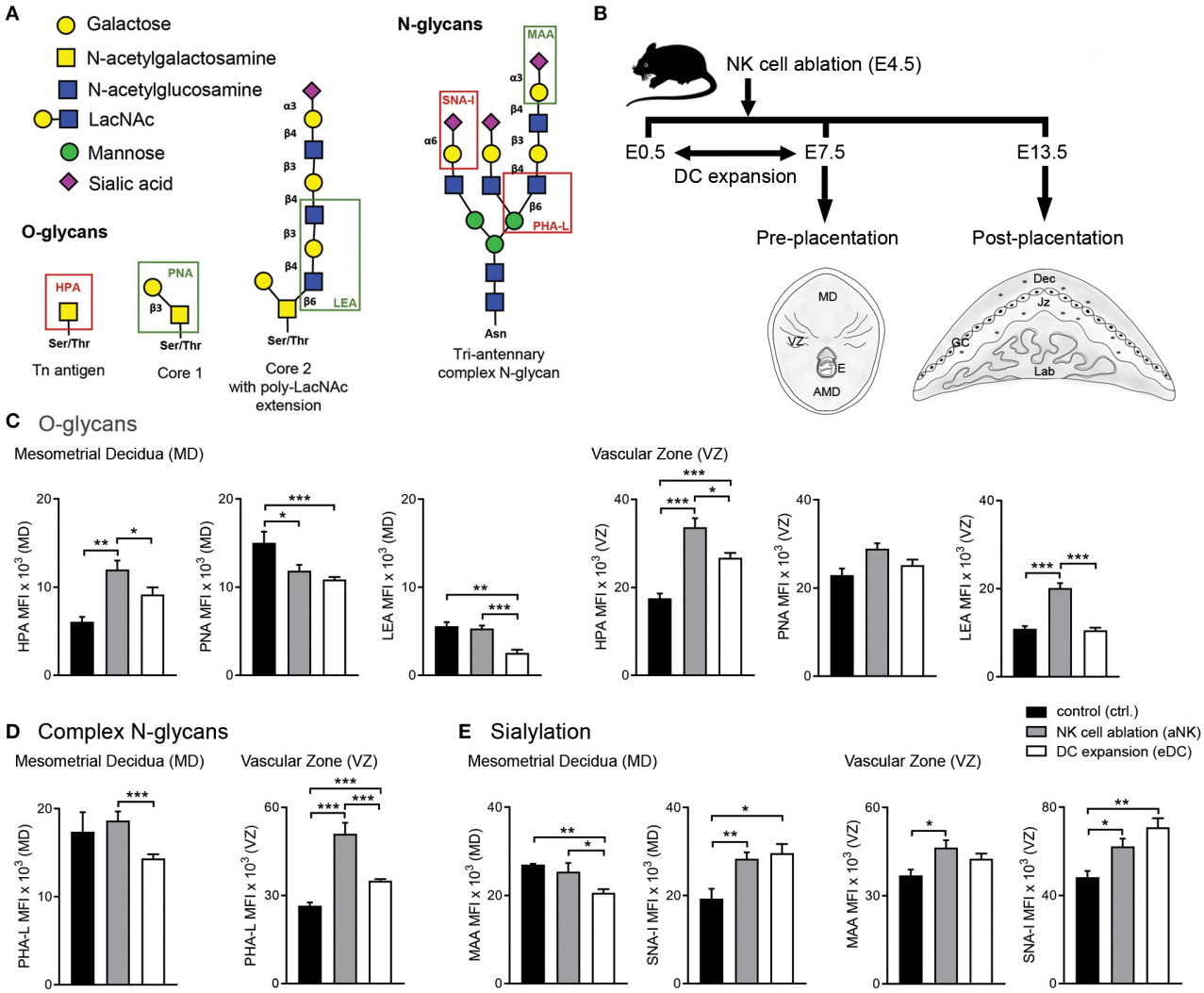

FIGURE 1 | Influence of NK cell depletion and DC expansion on the glycophenotype of implantation sites during the pre-placentation period. **(A)** For analysis of the glycophenotype, lectins were used to detect different types of glycosylation. O-glycan structures were recognized by Helix pomatia agglutinin (HPA; Tn-antigen), Arachis hypogaea lectin (PNA; core 1), and *L*ycopersicon esculentum lectin (LEA; core 2). In addition, we employed *Phaseolus vulgaris* lectin (PHA-L), which specifically recognizes β1-6GlcNAc-branched complex N-glycans. Finally, sialyation was determined using the Maackia amurensis lectin (MAA) and Sambucus nigra agglutinin (SNA-I) which bind to α2,3- and α2,6-linked sialic acid, respectively. **(B)** Experimental design: pregnant CD11c.DTR females were injected (i.p.) on E4.5 with anti asialo GM1 for transient ablation of NK cells. For the expansion of uterine DC, pregnant CD11c.DTR females were treated with one daily injection of FL (10 mg/mouse/day) from E0.5 to E7.5 as described in material and methods. Pre- (E7.5) and post-placentation (E13.5) period implantation sites were included in the glycodynamics analysis. **(C–E)** Quantification of O-glycan **(C)**, complex N-glycan **(D)**, and sialylated glycan **(E)** mean fluorescence intensity (MFI) in the mesometrial decidual (MD), and vascular zone (VZ) of implantation sites following NK cell ablation or DC expansion during the pre-placentation stage. In all figures, data shown are mean ± S.E.M. and differences are denoted as $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, as analyzed by *Mann-Whitney U-test*. AMD, antimesometrial decidua; Dec, decidua; MD, mesometrial decidua; VZ, vascular zone; GC, giant cells; Jz, junctional zone; Lab, labyrinth.

slight increase in Tn antigens (HPA) and decreased expression of core 1 (PNA) and core 2 O-glycans (LEA). Under normal conditions, HPA reactive O-glycans were observed in the VZ on E7.5. Of note, HPA-reactivity was significantly increased in the VZ of the aNK and the eDC group compared to the control group, with the aNK group showing the highest MFI. No changes were observed in PNA reactive glycans. LEA staining was increased in the VZ of the aNK group but not in the eDC group compared to the control group (**Figure 1C**, right panel). Next, we examined the distribution of complex branched N-glycans (specifically MGAT5-modified) during the pre-placentation period (**Figure 1D**). Glycans bound by PHA-L were observed in the MD of all groups (**Figure 1D**, left panel), with comparable mean fluorescence intensities (MFIs) of the control and the aNK group. Notably, a significantly lower MFI in the MD of the eDC group was observed compared to the aNK group. Regarding the distribution of complex branched N-glycans within the VZ, binding of PHA-L showed that

staining intensity was significantly increased in the aNK and the eDC group compared to the control group (**Figure 1D**, right panel). As for the distribution pattern of sialylated glycans in the MD, the control group and the aNK group showed comparable MFIs but the eDC group displayed a lower MAA MFI (**Figure 1E**, left panel). For SNA-I reactive glycans, similar MFIs in the aNK and the eDC group were observed at the MD during the pre-placentation period. Staining intensity for α2,3-linked sialic acid (MAA) was significantly increased in the VZ of the aNK group compared to the control group, whereas SNA-I reactive glycans showed a significant increase in the aNK and eDC dams (**Figure 1E**, right panel).

Imbalance on NK or DC Cell Subsets During Early Gestation Provokes Altered O- and N-Glycosylation Patterns in the Post-placentation Period

Taking into account that alterations of NK cell and DC relative abundance were shown to influence the placentation process and epigenetic programming in the offspring (13), we next examined changes in the glycophenotype during the post-placentation period (E13.5). **Figure 2A** (upper panel) shows the distribution of O-glycans within the decidua and placenta. During normal gestation Tn antigen (HPA) was only observable in the decidua and on giant cells (GC). In contrast, core 1 (PNA, middle panels), and core 2 O-glycans (LEA, bottom panels) were observed in all layers of the implantation site (including decidua, GC, junctional zone (Jz), and labyrinth). More Tn antigen (HPA) was observed on GC trophoblast than in the decidua. Core 1 O-glycans (PNA) were abundantly expressed in all layers but core 2 O-glycans (LEA) were more abundant on GC than in the decidua. Depletion of NK cells during early pregnancy was associated with decreased levels of core 1 O-glycans (PNA) on GC and Jz and reduced expression of core 2 O-glycans (LEA) on GC (**Figure 2A**, middle and bottom panels). In contrast, expansion of DC provoked an increase of Tn antigen (HPA) in the decidua (**Figure 2A**, upper panel), accompanied by increased expression of core 1 O-glycans (PNA) on GC but reduced expression in the Jz (middle panels). When analyzing the complex branched N-glycans (PHA-L, **Figure 2B**), we observed that during the post-placentation period reactivity in the decidua is stronger than in the placenta in undisturbed pregnancy. Expression of branched, complex N-glycans (PHA-L) was increased in the decidua and the Jz of the aNK group, but only in the labyrinth of eDC placentas. Finally, analysis of sialyation showed that MAA-reactive α2,3-linked sialic acid was detected on giant cells and in the labyrinth under normal placentation (**Figure 2C**, upper panel), accompanied with a strong expression of α2,6-linked sialic acid (SNA-I, bottom panel) in the decidua. Compared to controls, depletion of NK cells during early pregnancy provoked a decrease of α2,3-sialylation in the decidua and the Jz and an increase of α2,6-sialylation in the Jz, whereas placentas derived from DC expanded dams showed a decrease of α2,6-sialylation in the Jz.

Alteration of the Glycosylation Signature During the Pre- and Post-placentation Period is Accompanied by Changes on Gal-1 Expression

Given its well-established role in the modulation of pregnancy associated processes (20, 21), our next aim was to characterize galectin-1 (gal-1) expression during the pre- (E7.5) and post- (E13.5) placentation period. During the pre-placental period, we observed reduced gal-1 expression on the mesometrial decidua upon NK cell depletion compared to untreated dams (**Figure 2D**, left panel), whereas MD expression of this lectin was not sensitive to DC expansion. In contrast, both treated groups (aNK and eDC) exhibited decreased levels of gal-1 expression in the VZ, especially on endothelial cells during the pre-placentation period. As pregnancy progressed to the post-placentation period, aNK dams showed increased gal-1 expression within the decidua and placental layers (including GC, Jz, and labyrinth) compared to controls (**Figure 2D**, right panel). However, eDC placentas showed decreased gal-1 levels on the GC and the labyrinth on E13.5, suggesting that changes of gal-1 expression together with an altered glycosylation signature could interfere with the pregnancy protective functions of this lectin.

DISCUSSION

Changes in local immune cells dynamics (e.g., uNK cells and DC) during early gestation lead to the development of placental abnormalities and particularly upon NK cell depletion, fetal growth restriction (11–13). Our study on gal-1-glycan circuits in mice shows that changes in immune cell subset frequencies during the pre-placentation period differentially alter the placental glycophenotypes: placenta derived from NK depleted dams displayed reduced expression of O-glycans and α2,3-sialylation in placental layers accompanied by upregulation of complex N-glycans (**Figure 3**). This does not seem to be the case in placenta derived from expanded DC dams, which by contrast displayed milder changes in the placental glycophenotype with a modest reduction of core 1 O-glycosylation and α2,6-sialylation specially in the junctional zone and only a slight increase of N-glycans in the labyrinth.

Our study has limitations regarding the challenges of studying diversity on glycopatterns and the lack of *in vitro* experimentation, with specific consideration for the technical difficulty to preserve glycan structure and mimic the complex glycovariations in an *in vitro* setting. Nevertheless, the results reported herein highlight the notion that balanced innate immune cell dynamics at the maternal fetal interface have a strong impact on the glycophenotype, thereby influencing galectin-glycan interactions driving decidual and placental functions.

Pre-placentation Impaired NK Cell-DC Dynamic Alters Glycopatterns Within the Maternal Vascular Decidua

During early gestation, NK cells and DC shape decidual adaption to the developing embryo regulating angiogenesis and vascular

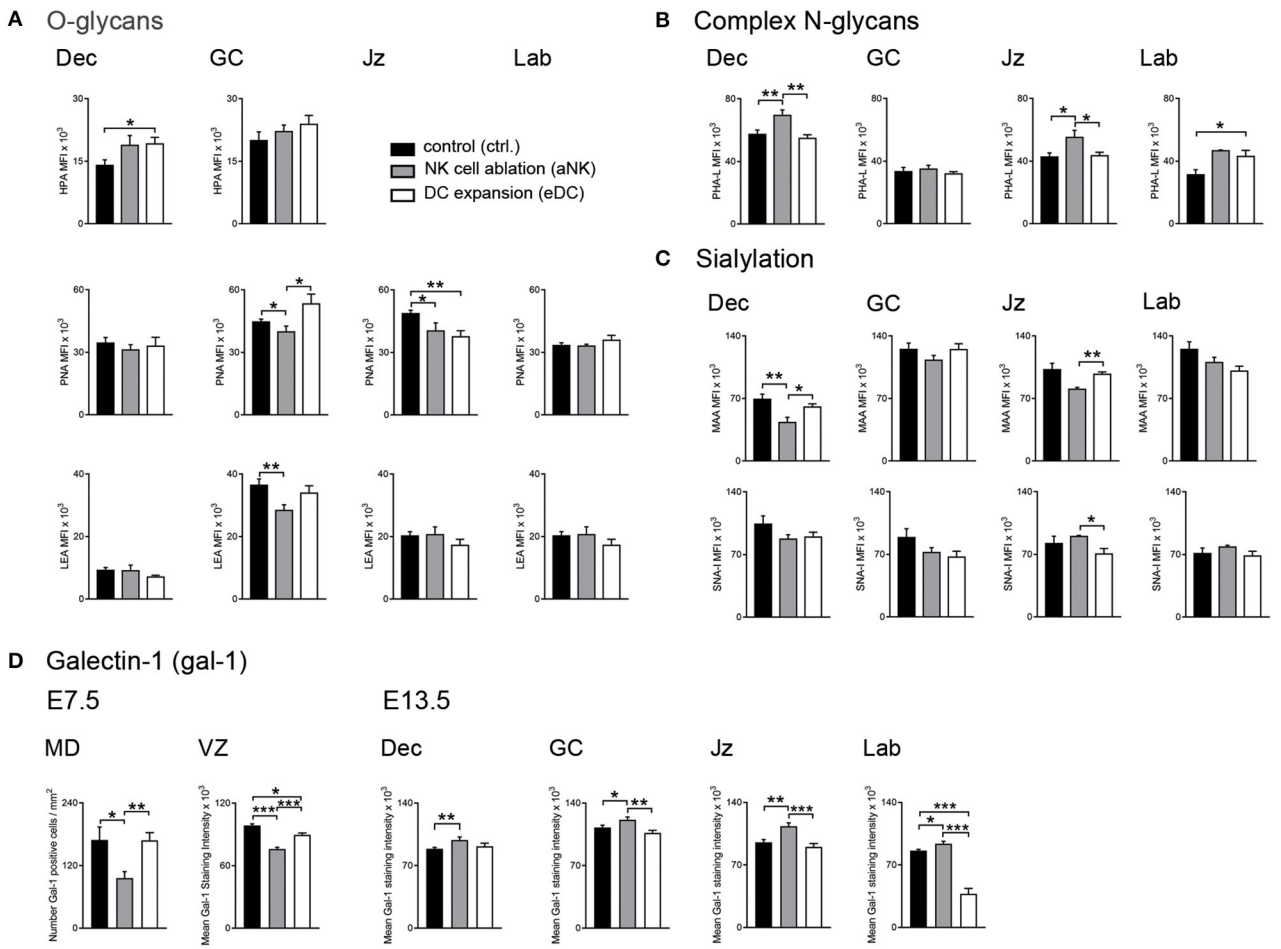

FIGURE 2 | Placental glycocode dynamics upon NK cell depletion and DC expansion during early gestation. **(A)** Quantification of O-glycan distribution across decidua and placenta layers on E13.5. Tn antigen was identified using the Helix pomatia agglutinin (HPA), core 1 and core 2 O-glycans were detected by Arachis hypogaea lectin (PNA) and *L*ycopersicon esculentum lectin (LEA), respectively. **(B)** Expression patterns of complex N-glycans were detected by *Phaseolus vulgaris* lectin (PHA-L) on E13.5. **(C)** Sialylation in the post-placentation period was characterized using Maackia amurensis lectin (MAA) and Sambucus nigra agglutinin (SNA-I) which bind to α2,3- and α2,6-linked sialic acid, respectively. **(D)** Analysis of galectin-1 (gal-1) expression during the pre- (E7.5) and post-placentation (E13.5) period. In all panels, bars show the MFI mean values and the corresponding S.E.M. Differences are noted as $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ according to *Mann-Whitney U-test*. MFI, mean fluorescence intensity; MD, mesometrial decidua; VZ, vascular zone; GC, giant cells; Jz, junctional zone; Lab, labyrinth.

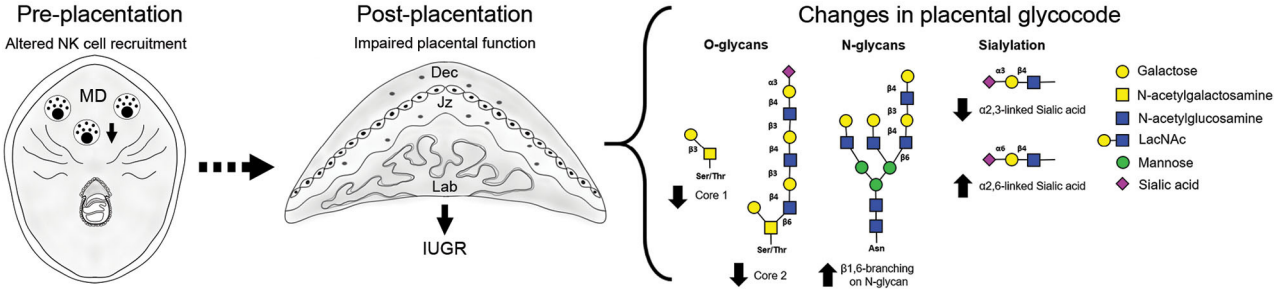

FIGURE 3 | Overview of the placenta glycocode dynamics in poor pregnancy outcome caused by disrupted NK cell recruitment. Fetal growth restriction as a consequence of NK cell depletion is associated with changes in O-glycan expression (↓ core 1 and core 2) in giant cells (GC). Junctional zone is characterized by increased expression of branched N-glycans and changes in sialylation (↓ α 2, 3- and ↑ α 2, 6- linked sialic acid). Placentas derived from NK ablated dams are characterized by an increased gal-1 expression.

growth (11–13). We have previously shown that DC found associated with the decidual vasculature co-express CXCR4 and impaired homing of $CXCR4^{+}$DC altered decidual vascular organization with impaired spiral artery remodeling later in gestation (11). In this study, we further reveal that alterations on the NK cell and DC pool dynamics during the pre-placentation period affect the glycopattern of the vasculature at the feto-maternal interface. In this regard, the VEGF system plays a paramount role in uterine vascular permeability and angiogenesis during implantation and decidualization (22, 23) and several findings have highlighted the importance of glycosylation for VEGFR2 functionality. For instance, VEGF-dependent proliferation is influenced by heparan sulfate (24) and complex branched N-glycans on the VEGFR2 are responsible for gal-1/VEGF-like signaling to sustain angiogenesis (25). Sialylation on VEGFR2 can also determine the signaling capacity of this receptor through gal-1. Thus, α2,6- linked, but not α2,3-terminal sialic acid inhibits binding of gal-1, which can also bind to the VEGFR2 to activate alternative pro-angiogenic signaling (25, 26). Additionally, exposure of endothelial cells to hypoxic conditions leads to increased branching of β1,6 branched N-glycan structures, and elongation of poly-LacNAc residues on core 2 O-glycans (25). These examples highlight the versatility of the endothelial glycome and its ability to adapt to cellular physiology. Indeed, several of these changes in the glycosylation pattern of the vascular zone during the pre-placentation period were observed in the present study upon DC expansion or NK cell ablation. Ablation of NK cells provoked an increase of core 2 O-glycans, branched N-glycans, and α2,3-sialyation compared to the control group, indicating the possibility of hypoxic or inflammatory conditions and increased gal-1 binding. These changes may occur to compensate the low gal-1 levels due to reduced NK cell abundance in these implantation sites. Expansion of DC, on the other hand, led to increased expression of branched N-glycans and α2,6-linked sialic acid compared to the control group; which despite not affecting the normal VEGF/VEGFR2 signaling pathway may lead to lower gal-1 sensitivity of cells in the vascular zone of this group. The corollary to these observations is that the decidual vascular glycocode appears to be dependent on the concerted actions of NK cells and DC, by virtue of their effect as modulators of VEGF/ gal-1 signaling pathways.

Thickness of the glycocalyx covering endothelial cells can influence the access of leukocytes to adhesion receptors on the endothelial cell surface. Pro-inflammatory cytokines, such as TNF-α, can lead to disruption of the endothelial glycocalyx and thus to an increase in leukocyte recruitment (27, 28). In this context, immune cell imbalance (i.e., DC expansion or NK cell depletion) during early pregnancy may influence the cytokine profile at the implantation site, leading to altered properties of the endothelial glycocalyx by directly influencing the expression of glycosyltransferases. Indeed, our previous studies have shown that expansion of DC was associated with a significant upregulation of the CXCL12/CXCR4 pathway; which has recently been shown to enhance megakaryocyte expression of *B4GalT1* (29), one of the main galactosyltransferases involved in the synthesis of the LacNAc moieties present in core 2 O-glycans and complex N-glycans. In turn, since B4GalT1-dependent galactosylation modulates β1 integrin function (29), such cytokine-mediated changes in the endothelial glycocalyx may further contribute to immune disbalance by provoking a differential recruitment of leukocytes due to altered cell adhesion properties. Indeed, DC expansion or NK cell depletion induced several changes in the glycosylation pattern in the vascular zone during the pre-placentation period, particularly in the expression of Tn antigen. In addition, endothelial gal-1 has been shown to reduce lymphocyte recruitment (30), further indicating that in the aNK group, which showed reduced gal-1 staining of endothelial cells, lymphocyte trafficking might be enhanced compared to the control group.

Pre-placentation Manipulation of the Relative NK Cell-DC Abundance Modifies Gal-1 Binding Placental Glycophenotypes

Trophoblast glycodiversity is part of the trophoblast lineage identity (31). Several pregnancy complications including preeclampsia, IUGR, and miscarriages were associated with specific differential glycosylation patterns after the onset of the disease (16–19, 32). In a first effort to identify early glycosignals that influence placental development upon disruption of the NK cell-DC dynamics, we show here that changes in trophoblast glycosylation patterns precede poor pregnancy outcomes (e.g., IUGR). For instance, Tn antigen O-glycans are exclusively expressed on the giant cell layer of the placenta and to a lesser extent in the decidua during unchallenged pregnancy. Both depletion of NK cells or expansion of DC in absence of dangers signals increased Tn antigen expression in the decidua. Since Tn antigen expression has been linked to enhanced growth and invasion ability in cancer cells (33–35), it is possible that increased decidual Tn antigen expression would act to facilitate trophoblast invasion. In this regard, trophoblast giant cells showed intense staining with LEA, indicating increased expression of LacNAc core 2 O-glycans during normal pregnancy. Giant cells in particular need to acquire an invasive character to make contact to the maternal arteries and replace the endothelial cell lining of the maternal blood vessels to funnel blood into the placenta. Importantly, our results further showed a down-regulation of core 2 O-glycans on giant cells derived from NK ablated dams. As cell surface mucin 1 (MUC1) carrying core 2 O-glycans is involved in trophoblast migration and adhesion to uterine endothelial cells (36–39), data suggests that changes in MUC1 core 2 O-glycans pattern would interfere with the invasive properties of giant cells in NK ablated placentas. This is in agreement with our previous work showing that aNK mice had impaired spiral artery remodeling and IUGR (13), indicating that a differential glycosylation pattern in the post-placentation period results in poor spiral artery remodeling. Moreover, expression of core 1 O-glycans has also been detected on MUC1 in the human placenta (40). In our study, staining of core 1 O-glycans by PNA also revealed reduced expression on trophoblast giant cells (aNK group) and trophoblasts in the junctional zone (aNK and eDC group), which could further indicate

alterations in mucin expression or glycosylation. Considering that gal-1 is able to bind mucins on trophoblast cells and is involved in the trophoblast invasion machinery (41, 42), the increased gal-1 expression in aNK placenta may represent an attempt to compensate reduced abundance of MUC1 binding partners.

Enhanced expression of N-acetylglucosaminyl transferase V (GnTV) characterizes first trimester placentas in normal gestation (43). GnTV generates β1-6-N-acetylglucosamine branches in complex N-glycans, which are recognized by gal-1. In this context, LacNAc motives are a glycan signature of invasive trophoblast cells not only on their surface but also on their secretion product HLA-G (31, 44, 51). The significantly higher expression of complex, branched N-glycans detected in the junctional zone of the aNK group indicates that the middle connecting layer of the placenta efficiently glycoadapts to the maternal environment giving rise to trophoblast giant cells and glycogen cells that invade and anchor the placenta to the decidua (45). In addition, we observed a switch on sialylation from α 2,3-linked to α2,6-linked sialic acid in the labyrinth of the aNK group. This finding correlates with the reduced fetal vascular density in the labyrinth upon NK depletion and with the inflammatory status due to the increased NK cell density in the mesometrial lymphoid aggregate of pregnancy (13). Interestingly, changes in the glycosylation status predominantly affecting the placental labyrinth and junctional zone have been reported in a rat model of hyperglycemic placental dysfunction (46, 47); suggesting that glycovariations in these layers induced by adverse maternal environments may have direct impact on placental function.

Our results further showed that increased α2,6 sialylation can reduce gal-1 mediated angiogenesis (48), which is critical for healthy placentation (49). Moreover, the inhibition of gal-1 binding by sialylation at the position six of galactose has been suggested to make T cells resistant to apoptosis (50) and might contribute to uncontrolled maternal inflammation during pregnancy complications (20, 49). Indeed, increased α2,6 sialylation in STBEV surface has been associated with human PE syndrome (19). Taken together, the results reported here highlight the relevance of glycodynamics during the pre- and post-placentation period that could be helpful to the understanding of the pathogenesis of poor pregnancy outcomes.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Charité—Universitätsmedizin Berlin and Regional Office for Health and Social Affairs.

AUTHOR CONTRIBUTIONS

SMB designed the study and secured grant funding. SB, IT-G, GB, NF, SMB, and MG performed experiments and/or analyzed data. SB, GB, and SMB wrote the manuscript. All authors gave approval for publication.

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Decidualization Process Induces Maternal Monocytes to Tolerogenic IL-10-Producing Dendritic Cells (DC-10)

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Decidualization is a process that involves phenotypic and functional changes of endometrial stromal cells to sustain endometrial receptivity and the participation of immunoregulatory factors to maintain immune homeostasis. In this context, tolerogenic dendritic cells (DCs) can induce regulatory T cells, which are essential to manage the pro- to anti-inflammatory transition during embryo implantation. Recently, Myeloid Regulatory Cells (MRCs) were proposed as immunosuppressants and tolerance-inducer cells, including the DC-10 subset. This novel and distinctive subset has the ability to produce IL-10 and to induce type 1 regulatory T cells (Tr1) through an HLA-G pathway. Here we focus on the impact of the decidualization process in conditioning peripheral monocytes to MRCs and the DC-10 subset, and their ability to induce regulatory T cells. An *in vitro* model of decidualization with the human endometrial stromal cell line (HESC), decidualized by medroxyprogesterone and dibutyryl-cAMP was used. Monocytes isolated from peripheral blood mononuclear cells from healthy women were cultured with rhGM-CSF + rhIL-4 and then, the effect of conditioned media from decidualized (Dec-CM) and non-decidualized cells (Non-dec-CM) was tested on monocyte cultures. We found that Dec-CM inhibited the differentiation to the $CD1a^{+}CD14^{-}$ immature DC profile in a concentration-dependent manner. Dec-CM also significantly increased the frequency of $CD83^{+}CD86^{low}$ and $HLA\text{-}DR^{+}$ cells in the monocyte-derived culture. These markers, associated with the increased production of IL-10, are consistent with a MRCs tolerogenic profile. Interestingly, Dec-CM treatment displayed a higher expression of the characteristic markers of the tolerogenic DC-10 subset, HLA-G and ILT2/CD85j; while this modulation was not observed in cultures treated with Non-dec-CM. Moreover, when monocyte cultures with Dec-CM were challenged with LPS, they sustained a higher IL-10 production and prevented the increase of CD83, CD86, IL-12p70, and TNF-α expression. Finally, the DC-10 subset

was able to induce a $CD4^+HLA\text{-}G^+$ regulatory T cells subset. These results suggest that the decidualization process might induce different subsets of MRCs, like DC-10, able to induce regulatory T cells as a novel $CD4^+HLA\text{-}G^+$ subset which might play an immunoregulatory role in embryo implantation.

Keywords: decidualization, DC-10, dendritic cells, immunomodulation, HLA-G, myeloid regulatory cells

INTRODUCTION

The maternal immune system was subjected to opposing selective pressures over millions of years of evolution: on the one hand it recognizes microbial pathogens and responds to eliminate them, whereas on the other hand, it accepts semi-allogeneic fetuses without ignoring its existence. Hence, the embryo has an "immunoprivileged status" that allows the establishment of early pregnancy by instructing immune tolerance induction in the maternal immune system. Therefore, the feto-maternal interface is characterized by dynamism: the microenvironment changes as pregnancy progresses accompanied by immunological phases with different profiles (1). The changes of the maternal immune profile are strictly controlled by complex regulatory mechanisms at decidualization, implantation, and placentation.

Particularly, the decidualization program involves phenotypic and functional changes of endometrial stromal cells and not only sustains the endometrial receptivity, but also allows the secretion of immunoregulatory factors which may condition maternal leukocytes to a regulatory profile (2). This process is unique and characteristic of endometrium and, in humans, it is activated independently of the presence of the blastocyst (2, 3). In this context, even though myeloid dendritic cells (DCs) are only 1–2% of decidual leukocytes, they initiate the adaptive immunity and, therefore, they are crucial for the establishment of immunological tolerance (1, 4). DCs in human decidua represent a complex population and their number fluctuates through different phases of the menstrual cycle and during pregnancy (5–8). Interestingly, in the last few years, Myeloid Regulatory Cells (MRCs) have been proposed as immune-suppressors and tolerance-inducers including the DC-10 (9, 10). This novel subset represents tolerogenic DCs (Tol-DCs) which notably spontaneously secrete large amounts of IL-10 and express different tolerogenic markers such as membrane-HLA-G and its receptors immunoglobulin-like transcript (ILT) 2, ILT-3, and ILT-4 (11). In fact, DC-10 are able to induce T cells anergy and type 1 regulatory T cells (Tr1) through the IL-10-dependent ILT4/HLA-G pathway *in vitro* (11). Remarkably, even though a single stimulation of allogeneic naïve T cells with DC-10 is sufficient to generate allo-specific Tr1 cells, the chronicity of allogeneic stimulation reinforces Tr1 induction (12). Previous reports indicate a higher percentage of DC-10 into the human decidua compared to peripheral blood during the first trimester of pregnancy; but, it is still unknown if these cells are recruited to the decidua or induced *in situ* (6). Tol-DCs also have the ability to induce regulatory T cells (Tregs, $CD4^+FOXP3^+$), a critical role in pregnancy that was proven using several *in vivo* and *in vitro* approaches in murine models as well as in humans (13–17). Recently, T cell subsets, which do not express FOXP3, with immunosuppressive ability based on the increase in HLA-G expression and IL-10 production were reported (8). The expression of HLA-G on T cells could be induced by DCs (6, 18, 19). The frequency of $CD4^+HLA\text{-}G^+$ cells in peripheral blood increases in healthy pregnant women, being even more pronounced within the decidua (6, 18); however, it is still unclear whether the decidualization program modulates their induction.

Since T cells and DCs are critical to sustain homeostasis in pregnancy, here we focused on the impact of the decidualization process in conditioning peripheral monocytes to MRCs and, particularly to the DC-10 subset. Finally, we investigated the ability of DC-10 to induce different regulatory T cell subsets.

MATERIALS AND METHODS

Reagents

Endotoxin-free reagents and plastic materials were used in all experiments. RPMI-1640, phosphate-buffered saline (PBS), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin/streptomycin were purchased from Gibco (Invitrogen, Argentina). Twenty-four-well flat bottom polystyrene plates were purchased from Jet-biofil (AP Biotech, Buenos Aires, Argentina) while 96-well U-bottom plates and half-area 96-well ELISA were obtained from Greiner Bio One (GBO, Buenos Aires, Argentina). Ficoll-Paque PLUS and Percoll were obtained from GE Healthcare Life Sciences (Embiotec, Buenos Aires, Argentina). Recombinant human IL-4 and recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) were obtained from Miltenyi Biotec (Lab Systems, Buenos Aires, Argentina). Lipopolysaccharide (LPS) from *Escherichia coli* was purchased from Sigma-Aldrich (Merck, Argentina).

Blood Samples

Buffy coats were obtained from fertile female volunteers, defined as women who had two or more previous normal pregnancies without any miscarriage in their clinical history, were non-smokers, and who were not under pharmacological treatment for at least 10 days before the day of sampling. The Investigation and Ethics Committees of 'Academia Nacional de Medicina' from CABA, Argentina have approved this study. All research was performed in accordance with relevant guidelines and regulations, and written informed consent for the collection of samples and subsequent analyses was obtained from all blood donors recruited by "Fundación Hemocentro

Buenos Aires," CABA, Argentina in accordance with the Declaration of Helsinki.

Human Endometrial Stromal Cell Line Culture

The human endometrial stromal cell (HESC) line was maintained in DMEM-F12 supplemented with 10% FBS, 50 U/ml penicillin, 50 μg/ml streptomycin, and 2 mM glutamine (20, 21) (complete medium). This cell line was kindly provided by Dr. Gil Mor of Medical School, Yale University, United States.

Decidualization: HESC cells were cultured in 24-well plates until they reached 70% confluence with complete medium. Then, they were treated with medroxyprogesterone (MPA) (10^{-7}M) and dibutyryl cAMP (db-cAMP) (2.5×10^{-3}M) for 8 days (Dec), changing half of the culture media and renewing the stimuli every 48 h. The decidualization process was confirmed by the evaluation of decidual markers and cell viability, as previously described (22). Non-decidualized (Non-dec) cells were cultured simultaneously in similar conditions in absence of decidualization stimuli.

After 8 days of culture, Non-dec and Dec-HESC cells were washed three times and cultured in RPMI 1640 medium supplemented with 10% of heat inactivated FBS, 50 U/ml penicillin, and 50 μg/ml streptomycin (DC complete medium) for an additional 48 h and Conditioned Media (CM) were collected.

Dendritic Cells Differentiation

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats by Ficoll-Paque PLUS density gradient centrifugation (1.077 g/mL). Monocytes were isolated by centrifugation on a discontinuous Percoll gradient with modifications of a previously described method (23, 24). Briefly, PBMC were suspended in $Ca2^{+}$, $Mg2^{+}$-free Tyrode's solution supplemented with 0.2% EDTA and incubated for 45 min at 37°C. During this incubation, the osmolarity of the medium was gradually increased from 290 to 360 osmol/l by addition of NaCl. Two different Percoll fractions were layered in polypropylene tubes: 50% at the bottom followed by 40%. PBMC (40×10^6/ml) were layered at the top and they were centrifuged at 620 g for 50 min at 4°C. Monocytes were recovered at the interface, washed, and the purity and viability were checked by flow cytometry analysis and trypan blue exclusion, respectively. The purity and the viability accepted in all cases were >85% and >95%, respectively.

To obtain immature DC (Media-treated cells), monocytes (1×10^6/ml) were cultured in DC complete medium with 30 ng/ml IL-4 and 30 ng/ml GM-CSF in 96-well U-bottom plates for at least 5 days. The expression of CD1a/CD14 was measured to confirm the differentiation to immature DC as previously described (25). In parallel, monocytes were also cultured in DC complete medium with IL-4 + GM-CSF in presence of HESC-CM (Non-dec-CM or Dec-CM). On the last day, cell supernatants were collected, and the phenotype was analyzed by flow cytometry. In some cases, on day 5, the cells were treated with LPS 0.2 μg/ml for 18 h if was required for the assays.

All experiments were performed independently using different donor monocytes (N is indicated in the legend of each figure).

Endocytosis Assay of FITC-OVA

At day 6 of differentiation, monocyte-derived cells were suspended at 2×10^6 cells/ml in fresh medium. FITC-Ovalbumin (FITC-OVA) was added at a final concentration of 100 μg/ml and cells were incubated for 25 min at 37°C. In parallel, a control was incubated on ice to determine unspecific binding. Cells were washed two times with ice-cold 2% FBS/PBS and fixed with 1% paraformaldehyde. The FITC-OVA uptake was then evaluated by flow cytometry as we have previously described (26).

Mixed Lymphocyte Reaction

Monocytes (5×10^4 cells/100 μl) were differentiated in presence or absence of 1:2 dilution HESC-CM for 6 days. The obtained monocyte-derived cells were then suspended in DC complete medium with 2.5×10^5 freshly isolated allogeneic lymphocytes (DC/lymphocyte ratio = 1/5) and cultured for 5 days more as we have previously described (25). The monocytes and lymphocytes used for mixed lymphocyte reaction (MLR) were isolated by centrifugation on a discontinuous Percoll gradient described above, reaching a purity >90 and >95%, respectively. At the last day of MLR, we evaluated the expression of different markers on T cells by flow cytometry and their cytokine production profile was evaluated in cell supernatants by ELISA.

Flow Cytometry

Cells were washed with PBS supplemented with 2% FBS/PBS and FITC-, APC- and PE-conjugated mAbs directed to CD1a, CD14, CD86, HLA-DR, CD83, CD4, CD25 (BD Biosciences), ILT-2/CD85j, and HLA-G (BioLegend, San Diego, CA, United States) or the corresponding isotype controls were added at saturating concentrations for 30 min at 4°C. Then, two additional washes were performed, and cells were fixed with 1% paraformaldehyde. Stained cells were acquired using an FACS Calibur and FACSAria II cytometers and results were analyzed using FlowJo 7.6 Software.

Measurement of Cytokines by ELISA

Cytokines were evaluated in cell supernatants using commercial kits: IL-10, IL-12p70, TNF-α, and IFN-γ (BD Biosciences), according to the manufacturer's recommendations.

Statistical Analysis

GraphPad Prism (GraphPad Software Inc., San Diego, CA, United States) was used to perform all statistical tests. Statistical significance was determined using the non-parametric Friedman test with Dunn's multiple comparisons post-test. Statistical significance was defined as $p < 0.05$ and exact p-values and comparisons were indicated in each graph.

RESULTS

Decidualized Cells Inhibit Monocyte Differentiation to $CD1a^{+}CD14^{-}$ Immature DC Profile in a Concentration-Dependent Manner

Considering that endometrial stromal cells change their secretome during the decidualization process, including the production of immunoregulators, we evaluated the influence of conditioned media (CM) of decidualized (Dec) and non-decidualized (Non-dec) HESC cells on immature DC differentiation. Monocytes were cultured to differentiate into immature DC with GM-CSF + IL-4 in absence (Media) or presence of different dilutions of Non-dec or Dec-CM for 5 days. As **Figure 1A** shows, Dec-CM inhibited monocyte differentiation to $CD1a^{+}CD14^{-}$ immature DC profile in a concentration-dependent manner. On the other hand, this effect was also accompanied by a persistence of $CD1a^{-}CD14^{+}$ cells (**Figure 1B**). **Figure 1C** shows representative dotplots of the immunostaining of DC differentiated in the absence or presence of CM from endometrial cells before and after decidualization.

Altogether, the present results suggest that CM from endometrial cells, after decidualization, interfere with DC differentiation while it increases the frequency of $CD1a^{-}CD14^{+}$.

Decidualized Cells Induce a Myeloid Regulatory Cells-Profile on Monocyte-Derived Cultures

To characterize the phenotype of monocyte-derived cells acquired after the treatment with Dec-CM, we tested activation/maturation markers as HLA-DR, CD86, and CD83 expressions. We observed that monocyte-derived cells cultured with Dec-CM showed a higher expression of HLA-DR (**Figures 2A,B,D**) compared with culture medium. In fact, it also increased the expression of the maturation marker CD83 (**Figures 2C,E**). Surprisingly, Dec-CM increased the frequency of $CD83^{+}CD86^{low}$ while it diminished the frequency of the $CD86^{high}$ population (**Figures 2C,E**). In line with its mature

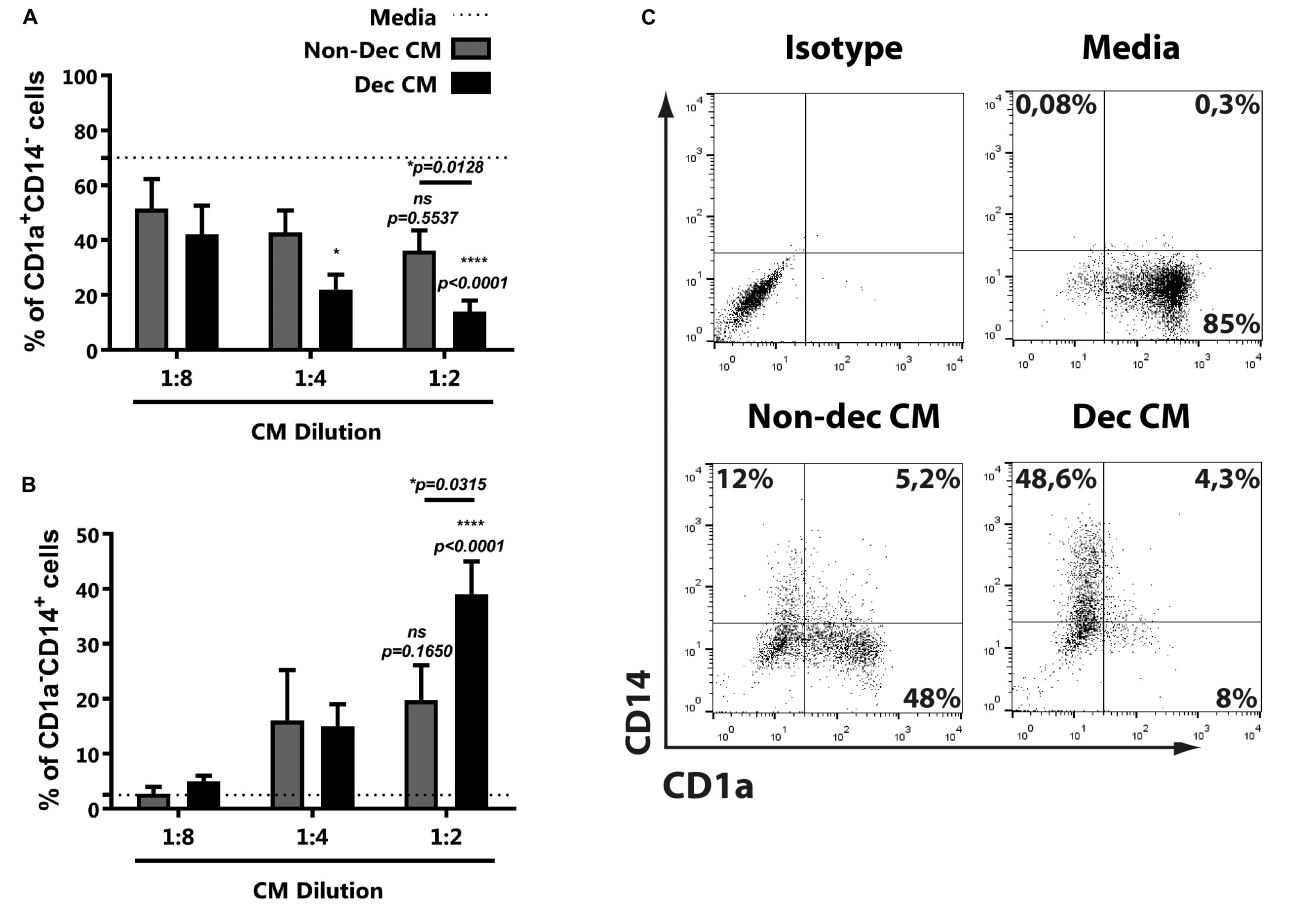

FIGURE 1 | Decidualized cells inhibit monocyte differentiation to immature DC in a concentration-dependent manner. Monocytes were cultured to differentiate to immature DC in absence (Media) or presence of different dilutions of Non-dec or Dec-CM for 5 days. Then, the CD1a/CD14 expression was evaluated by flow cytometry. **(A,B)** Mean ± SEM of positive cells from at least eight experiments are shown. Dotted lines represent the mean of positive cells of Media-treated culture. **(C)** Representative experiment of 1:2 dilution of CM is shown. The statistical test used is the Friedman test with Dunn's multiple comparisons post-test. *p*-values and comparisons were indicated in graph; *p*-values without lines indicate comparisons with dotted lines (Media).

phenotype, monocyte-derived cells differentiated with Dec-CM displayed significantly lower endocytic ability in an ovalbumin (OVA)-FITC uptake assay (**Figures 2F,G**).

When the cytokine secretion profile was evaluated, we observed that monocyte-derived cells differentiated in the presence of CM from HESC cells, either decidualized or not, secreted significantly higher levels of IL-10 compared to the culture medium, while IL-12 secretion was not modulated (**Figures 3A,B**). Notably, Dec-CM did not induce the production of TNF-α by monocyte-derived cells as Non-dec-CM did, highlighting the ability of Dec-CM to induce a different cytokine profile in these cultures (**Figure 3C**).

Altogether, the present results suggest that endometrial stromal cells, after decidualization, might induce an immunosuppressive regulatory phenotype on monocytes like the MRCs.

Decidualized Cells Prevent LPS-Induced Maturation of Monocyte-Derived Cells

Next, to confirm the maturation state and the activation of monocyte-derived cells differentiated in the presence of Dec-CM, we challenged them with LPS for 16 h and cytokine profile production and the activation/maturation marker's expression were assessed. As shown in **Figure 3D**, upon activation with LPS, Dec-CM cultures sustained higher IL-10 production while it prevented the increase of IL-12p70 and TNF-α secretion compared to culture medium-treated cells (**Figures 3E,F**). Moreover, in the presence of Dec-CM, significant increase of IL-10 and decrease of TNF-α expression in comparison with Non-dec-CM were observed, highlighting the effect of the decidualization treatment. On the other hand, the expression of activation/maturation markers in monocyte-derived cells, cultured or not, with HESC-CM and challenged with LPS was determined. Dec-CM treatment significantly prevented the increase in the frequency of HLA-DR^{high}, CD83^{+}CD86^{+}, and CD86^{high} subsets observed with LPS treatment (**Figures 4A–E**). Notably, a tendency to prevent the increase in the frequency of these subsets was also observed in Non-dec-CM cultures compared to the culture medium, reaching significance in the CD86^{high} subset (**Figure 4E**).

The present results indicate that, once decidualized, endometrial stromal cells might not only induce a phenotype like MRCs on monocyte-derived cells but also condition their functional status.

Decidualized Cells Favor a Higher Expression of the Characteristic Tolerogenic DC-10 Subset Markers on Myeloid Cells, HLA-G and ILT-2/CD85j

Based on the results shown above and considering that DC-10 spontaneously produce high amounts of IL-10 and increase tolerogenic markers, we next evaluated the ability of Dec-CM to induce tolerogenic markers on monocyte-derived cells. HLA-G expression was significantly increased in Dec-CM-treated cells compared to Non-dec-CM-treated cells. The increase of HLA-G expression was observed in both frequency and MFI parameters (**Figures 5A,B,D**). As expected, the expression of the HLA-G receptor, ILT-2/CD85j, was increased on monocyte-derived cells cultured in the presence of Dec-CM, compared to culture medium-treated cells (**Figures 5C,E**) suggesting that endometrial stromal cells might induce differentiation into the DC-10 subset compatible with a tolerogenic microenvironment only after decidualization. Interestingly, both DC-10-tolerogenic markers were not increased in those cultures treated with Non-dec-CM, highlighting the specificity of the decidualization process.

Decidualized Cells Condition Monocyte-Derived Cells to an Immunosuppressive and Tolerogenic Profile After Allogeneic Stimulation: CD4^{+}HLA-G^{+} T Cells Induction

As described above, the DC-10 subset induced regulatory T cells with suppressor function through the IL-10-dependent HLA-G pathway. Taking into account the higher expression of HLA-G and ILT-2 markers in Dec-CM cultures, we evaluated the ability of these conditioned monocyte-derived cells to induce a tolerogenic and suppressor response after allogeneic stimulation in the mixed lymphocyte reaction (MLR). Hence, monocyte-derived cells that had been differentiated in the presence or absence of HESC-CM for 6 days were cultured with allogeneic lymphocytes for 5 days more. On the last day of MLR, we evaluated the expression of different markers on T cells by flow cytometry and their cytokine production profile by ELISA. An anti-inflammatory microenvironment characterized by higher IL-10 and lower IFN-γ production was observed in cultures treated with either HESC-CM, compared to medium cultures (**Figures 6A,B**). These results suggest the induction of suppressor and regulatory profiles on T cells in both MLR cultures, although the IL-10:IFN-γ ratio was significantly higher only in Dec-CM cultures (**Figure 6C**). In parallel, we evaluated the expression of the activation marker CD25, on allogeneic lymphocytes in these MLR cultures. We observed a significant decrease in the frequency of CD4^{+}CD25^{+} cells in Dec-CM-cultures (**Figures 7A,B**), suggesting that monocyte-derived cells differentiated with CM of decidualized cells inhibited allogeneic CD4^{+} T cells activation. Finally, a significant increase in the frequency of CD4^{+}HLA-G^{+} cells was observed in Dec-CM-cultures compared with Non-dec-CM-cultures, indicating a specific effect of decidualization (**Figures 7C,D**).

The present results suggest that HESC cells condition the monocyte-derived cells to an immunosuppressive profile accompanied by a decrease in the frequency of allo-activated T cells. Particularly Dec-CM-treated cells displayed a higher IL-10:IFN-γ ratio production and an increase in the CD4^{+}HLAG^{+} T cells subset.

DISCUSSION

For many years, it was assumed that decidualized cells had a structural, passive role during embryo implantation, only associated with morphological changes of stromal cells.

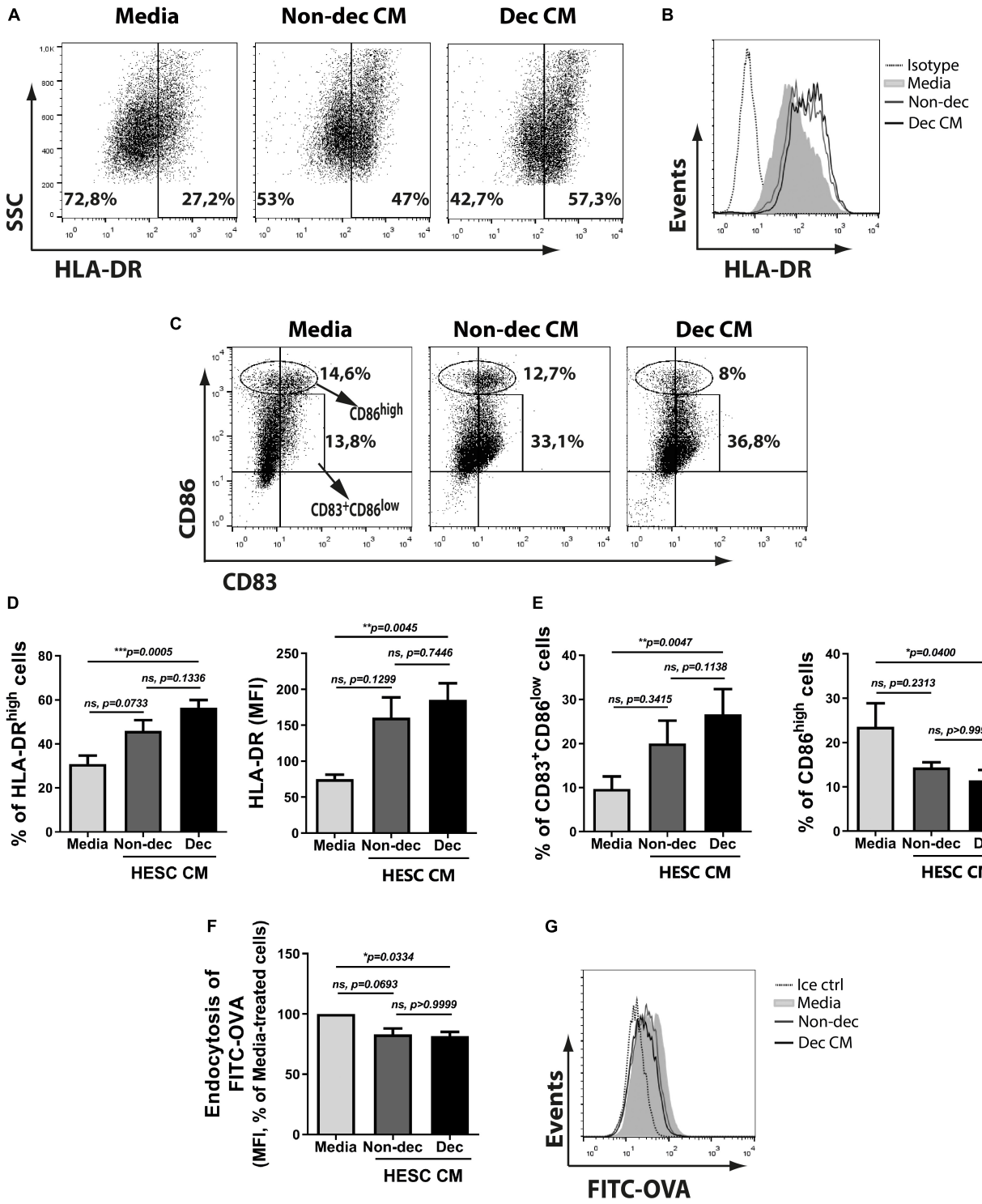

FIGURE 2 | Decidualized cells induce an MRC-profile on monocyte-derived cultures with higher frequency of $CD83^{+}CD86^{low}$ and $HLA\text{-}DR^{high}$ cells. Monocytes were cultured to differentiate to immature DC in absence (Media) or presence of 1:2 dilution of Non-dec or Dec-CM for 5 days. **(A–E)** After differentiation, the expression of HLA-DR, CD86, and CD83 was measured by flow cytometry. Representative experiments are shown in panels **(A–C)** and the mean ± SEM of positive cells or MFI from five to eight experiments is shown in panels **(D,E)**. **(F,G)** On day 6, cells were washed and stimulated with OVA-FITC in fresh medium for 25 min at 37°C and the endocytic ability was evaluated by flow cytometry. Cells incubated with FITC-OVA in ice were used as negative control. The mean ± SEM of MFI from seven experiments is shown in panel **(F)** and the representative experiment is shown in panel **(G)**. The statistical test used is the Friedman test with Dunn's multiple comparisons post-test. Exact *p*-values and comparisons are indicated in the graph.

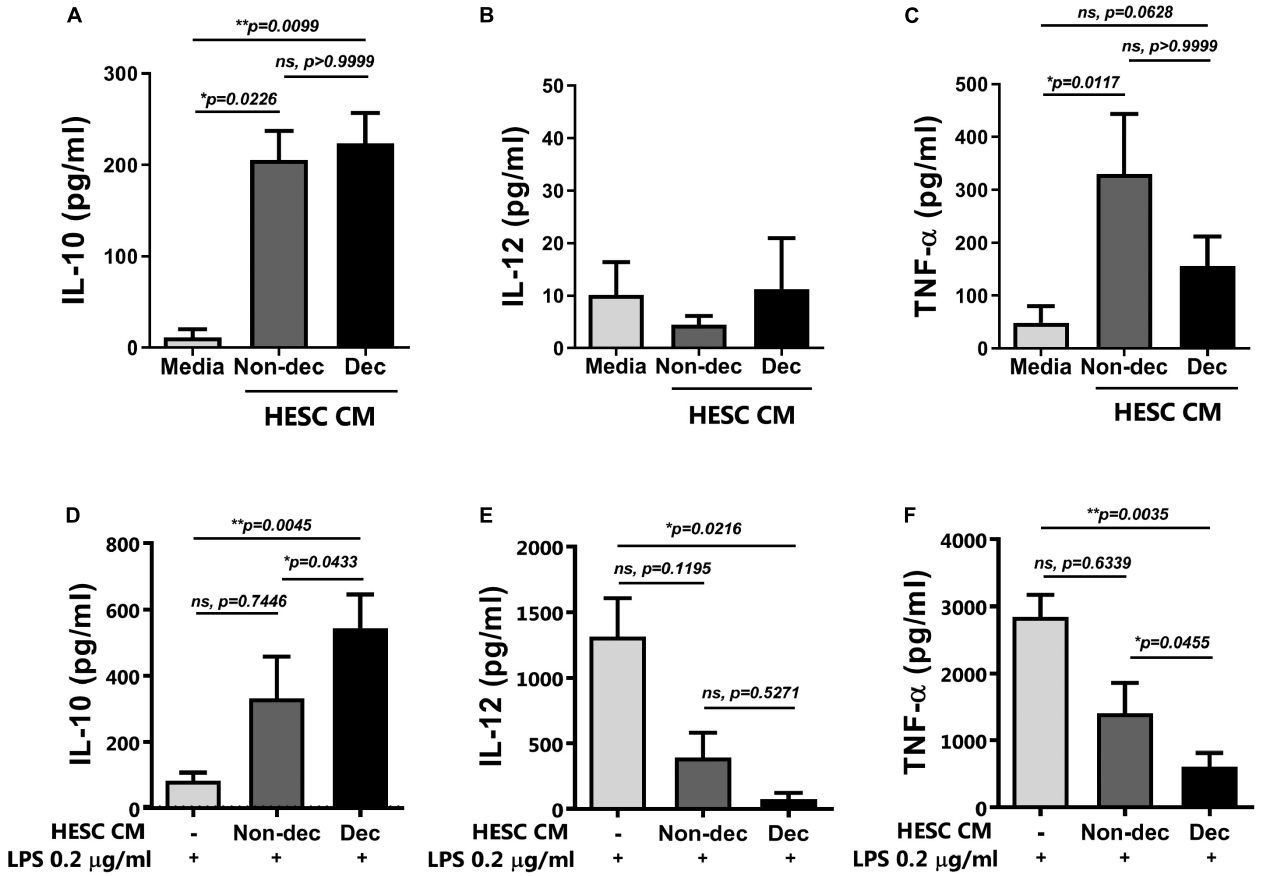

FIGURE 3 | Decidualized cells induce IL-10^{++} secretion on monocyte-derived cells and prevent the increase of IL-12p70 and TNF-α secretion upon maturation with LPS. Monocytes were cultured to differentiate to immature DC in absence (Media) or presence of 1:2 dilution of Non-dec or Dec-CM for 5 days and then, cells were stimulated **(D–F)** or not **(A–C)** with LPS 0.2 μg/ml for 18 h. The secretion of IL-10, IL-12 and TNF-α was evaluated by ELISA. Bars represent the mean ± SEM of four to eight experiments. Dotted lines represent the mean of Media-treatment without LPS. The statistical test used is the Friedman test with Dunn's multiple comparisons post-test. Exact *p*-values and comparisons are indicated in the graph.

Nowadays, evidence indicates that the decidualization program conditions the endometrium for receptivity as well as for local leukocyte profiling (1, 27, 28). In fact, defects in decidualization could condition future pregnancies as observed in women with severe preeclampsia (29).

Here, we provide new experimental evidence on the decidualization program as a conditioning factor for the differentiation of maternal monocytes to a unique and special subset of Tol-DC, the DC-10, thus contributing to the establishment of tolerogenic and immune suppressor milieu by regulatory T cells induction. Our conclusions are based on several observations. First, Dec-CM inhibited monocyte differentiation to a classical CD1a^{+}CD14^{-} immature DC profile in a concentration-dependent manner. Instead, CM induced a particular MRC-profile with a mature state and a higher IL-10 production on monocyte-derived cells. Moreover, Dec-CM prevented the increase of co-stimulatory molecules expression and pro-inflammatory cytokines production induced by LPS-stimulation. Finally, monocyte-derived cells differentiated in the presence of Dec-CM, expressed a higher level of DC-10-tolerogenic markers, HLA-G and ILT-2/CD85j, resulting in an immune suppressor and tolerogenic response with a higher IL-10:IFN-γ ratio and an increased frequency of regulatory CD4^{+}HLAG^{+} T cells.

Stromal cells are non-hematopoietic cells; however, they have the ability to mediate anti-inflammatory effects through targeting natural killer cells, monocyte/macrophages, DCs and inducing Tregs (30–32). The mechanisms involve cell contact and the production of soluble factors, including Indoleamine 2,3-dioxygenase (IDO), TGF-β, IL-10, PGE2, and nitric oxide among other chemokines and cytokines (30–32). In this sense, the endometrium suffers regular cycles of menstruation, repair, proliferation, and differentiation under hormonal control. Endometrial leukocytes and derived-mediators play important roles not only in the decidualization and embryo implantation but also as local regulators in menstrual tissue breakdown and endometrial repair (33). It was reported that during the pre-decidualized phase the endometrium switches from a pro-inflammatory to an anti-inflammatory microenvironment (33). In this line, we observed that monocyte-derived cells

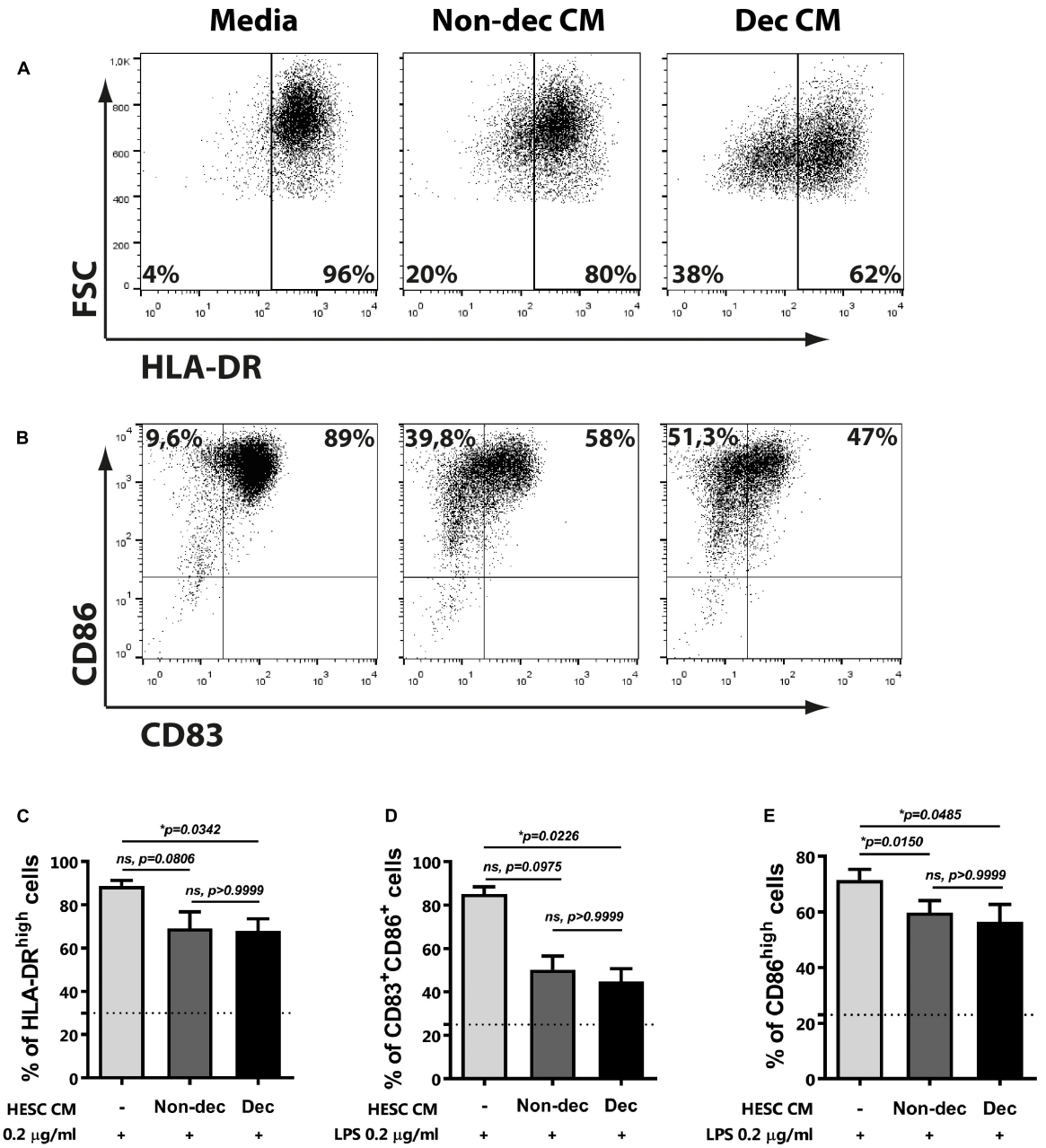

FIGURE 4 | Decidualized cells prevent LPS-induced maturation of monocyte-derived cells. Monocytes were cultured to differentiate to immature DC in absence (Media) or presence of 1:2 dilution of Non-dec or Dec-CM for 5 days and then, cells were stimulated with LPS 0.2 μg/ml for 18 h. The expression of HLA-DR **(A,C)**, CD83, and CD86 **(B,D,E)** was evaluated by flow cytometry. Representative experiments are shown in panels **(A,B)** and the mean ± SEM of positive cells from five to seven experiments is shown in panels **(C–E)**. Dotted lines represent the mean of positive cells of Media-treatment without LPS. The statistical test used is the Friedman test with Dunn's multiple comparisons post-test. Exact *p*-values and comparisons are indicated in the graph.

differentiated in presence of Non-dec-CM also acquired some features similar to those observed in Dec-CM cultures such as HLA-DR expression, IL-12 production, and $CD4^+CD25^+$ frequency with higher IL-10 and lower IFN-γ production after MLR. Therefore, Non-dec-CM might induce an anti-inflammatory profile on DC, though it is not as marked as the one induced by Dec-CM. Thus, we might infer that DC would begin to acquire characteristics associated with an anti-inflammatory profile during the pre-decidualization phase, reaching a more robust tolerogenic profile during the decidualization process.

Here, we demonstrated that monocyte-derived cells differentiated in the presence of Dec-CM exhibit a particular $CD83^+CD86^{low}$ mature status with high expression of HLA-DR and spontaneous production of high amounts of IL-10.

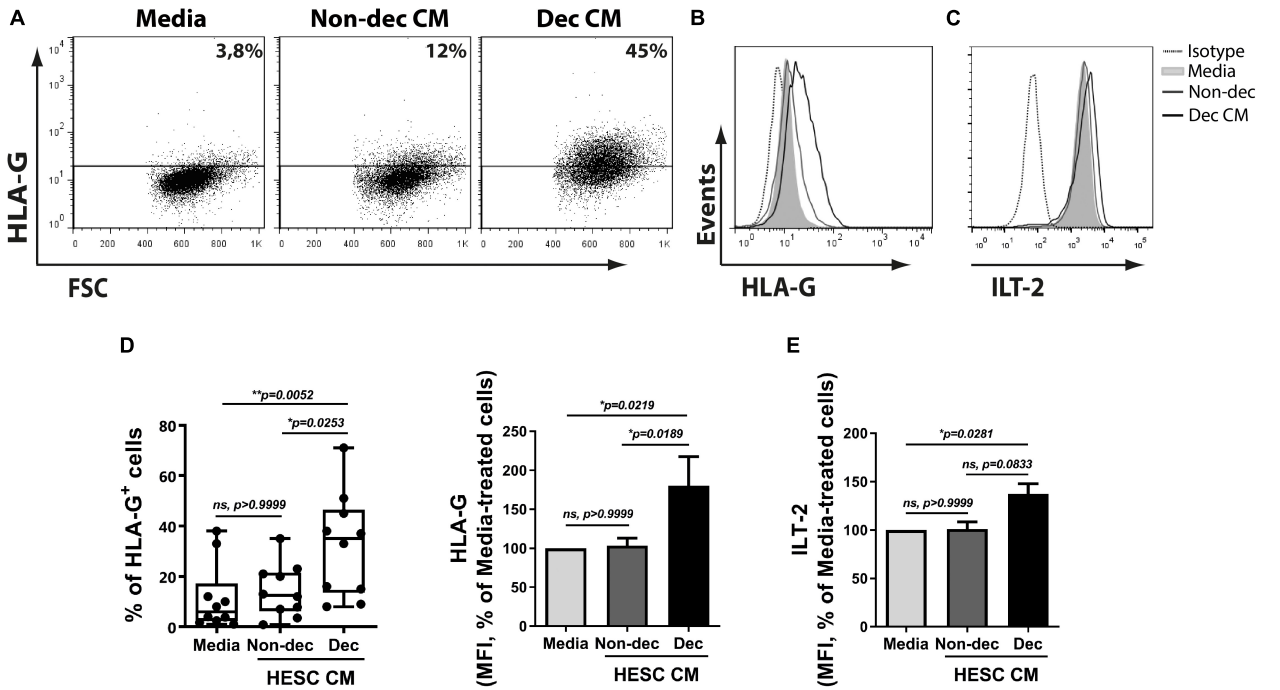

FIGURE 5 | Decidualized cells favor a higher expression of the characteristic tolerogenic DC-10 subset markers on myeloid cells, HLA-G and ILT-2/CD85j. Monocytes were cultured to differentiate to immature DC in absence (Media) or presence of 1:2 dilution of Non-dec or Dec-CM for 5 days and the expression of HLA-G and ILT2/CD85j was evaluated by flow cytometry. Representative experiments are shown in panels **(A–C)** and the mean ± SEM of positive cells or MFI from six to ten experiments is shown in panels **(D,E)**. The statistical test used is the Friedman test with Dunn's multiple comparisons post-test. Exact *p*-values and comparisons are indicated in the graph.

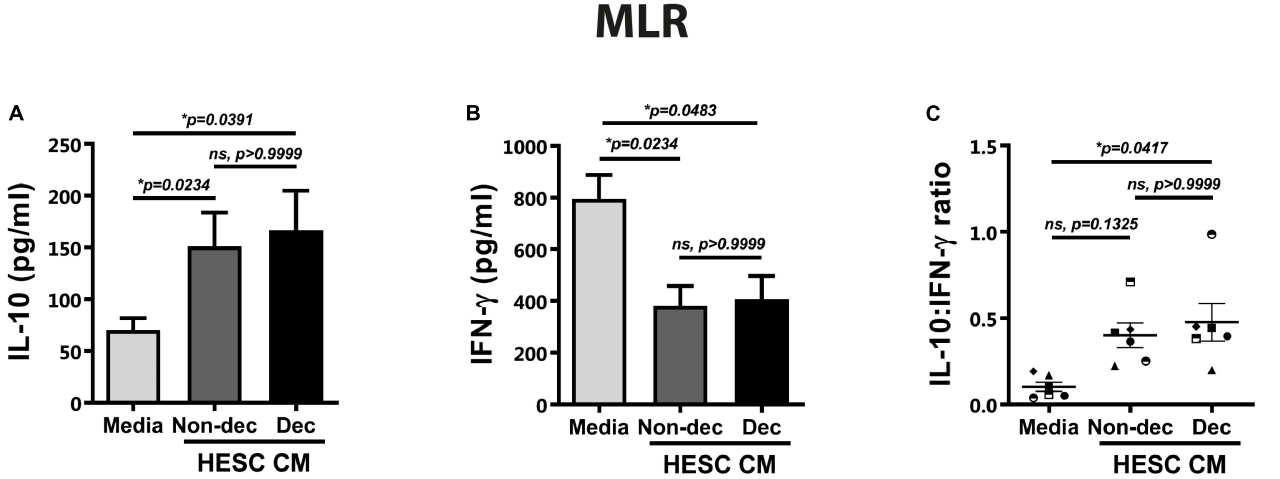

FIGURE 6 | Decidualized cells condition monocyte-derived cells to an immunosuppressive profile after allogeneic stimulation. Monocyte-derived cells (5×10^4 cells/100 μl) that had been differentiated in presence or absence of 1:2 dilution of Non-Dec or Dec-CM for 6 days were suspended in DC complete medium and co-cultured with allogeneic lymphocytes for 5 days more (DC/lymphocyte ratio = 1/5). On the last day of MLR, the IL-10 and IFN-γ secretion was evaluated by ELISA. Bars represent the mean ± SEM of six experiments **(A,B)** and scatter dot-plots represent the mean ± SEM of the IL-10:IFN-γ ratio production **(C)**. The statistical test used is the Friedman test with Dunn's multiple comparisons post-test. Exact *p*-values and comparisons are indicated in the graph.

Accordingly, in the last few years, Gregori et al. characterized a subset of Tol-DC, the DC-10 subset, which can be differentiated *in vitro* from monocytes with GM-CSF + IL-4 and IL-10 (11). DC-10 are $CD1a^{-}CD14^{+}$ and display a mature myeloid phenotype ($CD83^{+}CD86^{+}$ and $HLA\text{-}DR^{high}$) even in the absence of activation stimuli. Moreover, they secrete spontaneously high levels of IL-10 and express the tolerogenic markers HLA-G, ILT-2, ILT-3, and ILT-4. This phenotype turns them into

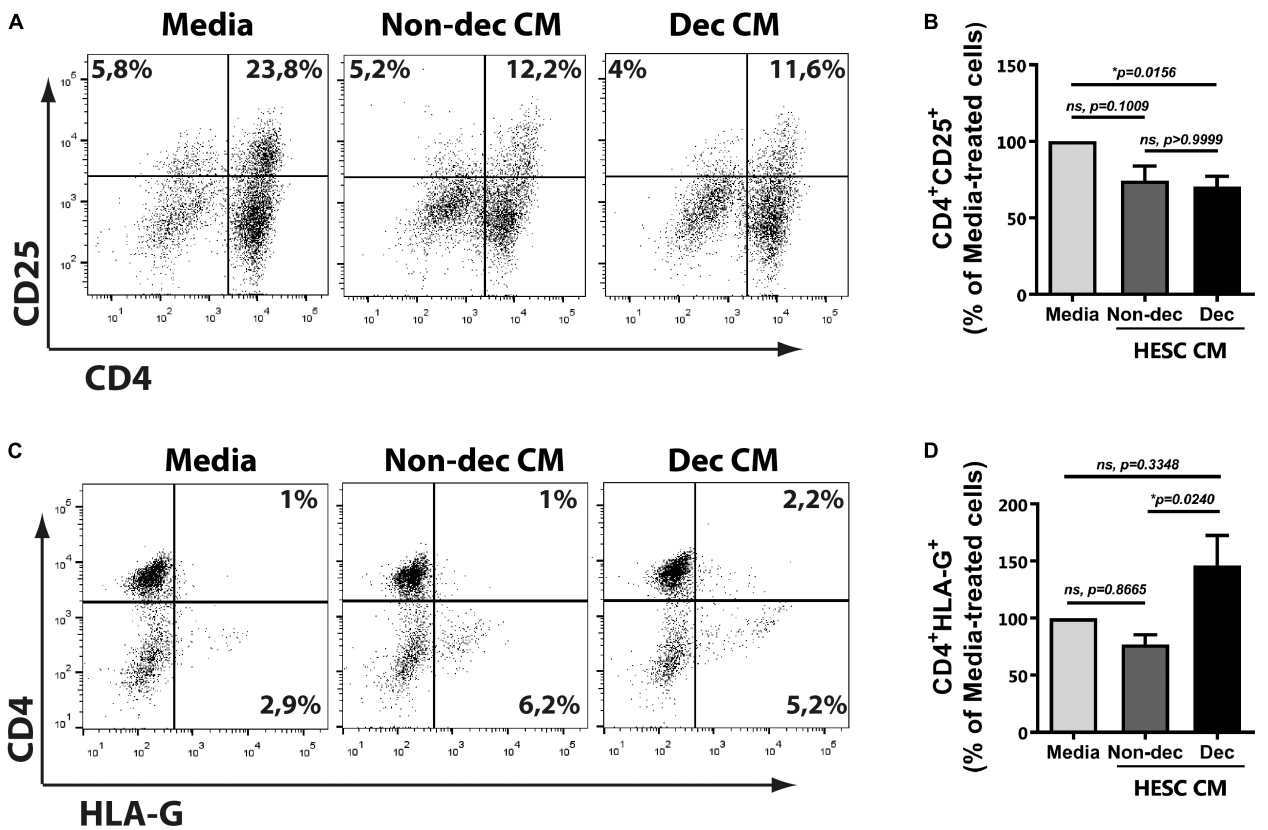

FIGURE 7 | Decidualized cells condition monocyte-derived cells to an immunosuppressive and tolerogenic profile with induction of $CD4^+HLA\text{-}G^+$ T cells after allogeneic stimulation. Monocyte-derived cells (5×10^4 cells/100 μl) that had been differentiated in presence or absence of 1:2 dilution of Non-Dec or Dec-CM for 6 days were suspended in DC complete medium and co-cultured with allogeneic lymphocytes for 5 days more (DC/lymphocyte ratio = 1/5). On the last day of MLR, the expression of CD4, CD25, and HLA-G was evaluated by flow cytometry, gating on lymphocytes. Representative dot plots are shown in panels **(A,C)**. Bars represent the mean ± SEM of positive cells from four experiments **(B,D)**. The statistical test used is the Friedman test with Dunn's multiple comparisons post-test. Exact *p*-values and comparisons are indicated in the graph.

potent inducers of Tr1 *in vitro* through the IL-10-dependent ILT-4/HLA-G pathway (11).

Although DC-10 share some similarities with other tolerogenic antigen-presenting cells, they represent a unique subset of Tol-DC that is phenotypically and functionally stable (34). Upon activation, DC-10 maintain their phenotype and their cytokine secretion profile with high IL-10 and low IL-12/TNF-α production. In accordance, here we showed that Dec-CM prevented the maturation of DC by LPS, inhibited IL-12 and TNF-α production, and increased even more the secretion of IL-10. The presence of DC-10 was recently reported in first trimester decidua and peripheral blood (6). However, it is still unclear if the increased frequency of DC-10 observed in the decidua is due to: (a) a higher recruitment from peripheral blood, (b) an increased conversion of resident decidual DCs into DC-10, or (c) if the decidual microenvironment promotes the *de novo* induction of DC-10 from monocytes recruited. Regarding the aforementioned frequency and based on the results presented here, we propose that *the novo* induction of DC-10 could be occurring within human decidua and independently of the blastocyst presence.

The frequency of DCs in human endometrium reaches its highest level during the implantation window (35) and it is associated to their ability to release soluble factors that improve the endometrial receptivity (36, 37). In this context, previous work performed in mice provided strong evidence of the indispensability of DCs in decidua formation and implantation (38, 39). IL-10 promotes the expression of several tolerogenic molecules in human DCs and in other antigen-presenting cells, including IL-10 itself, hemo-oxygenase (HO-1), ILT-3, and ILT-4 as well as another important mediator of immune tolerance in pregnancy, the HLA-G. This atypical MHC class I molecule, is one of the ILT-2/ILT-4 ligands with potent immunosuppressive properties (40).

In accordance with these observations, in our *in vitro* model of immune-decidual interaction, we demonstrated that Dec-CM

was able to induce HLA-G and increased ILT-2 expression on monocyte-derived cells. Interestingly, these DC-10-tolerogenic markers were not increased at all by Non-dec-CM treatment. It has been reported that the continuous ligation of ILT-2 on immature DC during differentiation maintains CD14 expression, inhibits the acquisition of CD1a expression and prevents the activation with LPS (41, 42). In fact, ligation through ILT-2 may preferentially induce and/or interact with Tregs that maintain T-cell unresponsiveness (42). Considering that the decidualization process increased IL-10 production by stromal cells (43), which induces the expression of IL-10, HLA-G and ILT-4 on DC-10 (11), and that HLA-G itself is able to up-regulate the expression of ILT-2 and ILT-4 (44), we suggest that Dec-CM induces a positive regulatory loop between IL-10 and these tolerogenic markers in monocyte-derived cultures.

In vitro experiments have demonstrated that DCs isolated from the decidua (45) are poor stimulators of allogeneic lymphocytes. Accordingly, here we showed for the first time that allogeneic lymphocytes co-cultured with monocytes-derived cells conditioned by Dec-CM treatment are hypo-responsive with significantly decreased $CD25^+$ expression, particularly on the $CD4^+$ subset. Indeed, a significantly higher IL-10/IFN-γ ratio was observed in these cultures, suggesting the induction of a regulatory T-cell profile. In this sense, new subsets of regulatory T cells have emerged, defined by the expression of the HLA-G cell surface; $CD4^+$ and $CD8^+$ HLA-G^+ T cells. They were identified in peripheral blood of healthy volunteers as small subsets but were able to suppress immune responses *in vitro* involving IL-10 and HLA-G as suppressive mechanisms (46–48). According to our results, high IL-10 and low IFN-γ secretion mediated by $CD4^+$HLA-G^+ T cells was previously reported by other authors and, therefore, the ability of this small regulatory T cell subset to promotion of an anti-inflammatory or antiproliferative cytokine milieu has been suggested (46–48). Here, we showed that Dec-CM induced the differentiation of regulatory HLA-G^+ T cells in monocytes-derived cell cultures while Non-dec CM was unable to induce this particular subset of regulatory $CD4^+$ T cells, highlighting characteristic properties of the decidualization process. It was demonstrated that $CD4^+$ T cells might acquire the HLA-G molecule from decidual DCs through the trogocytosis process (18). In fact, it was proposed that DC-10-derived extracellular vesicles also contain soluble HLA-G (sHLA-G) and T cells can acquire HLA-G (49). Although we demonstrated the presence of $CD4^+$HLA-G^+ T cells on MLR cultures performed with total lymphocytes, we observed a low frequency of this subpopulation, suggesting that their physiological relevance would be based on suppressive capacity through the production of high levels of IL-10 and sHLA-G. However, more functional studies should be performed to address this issue.

Here we also observed an increase in the non-$CD4^+$HLA^{-+} cells after the MLR cultures. In this sense, a higher frequency of $CD8^+$HLA-G^+ T cells cells in the peripheral blood of healthy pregnant compared to non-pregnant women, was recently reported (50). Taking into account that there are few studies on HLA-G^+ T cell subsets in the context of pregnancy, and are even less focused on $CD8^+$HLA-G^+, it would be interesting to perform functional studies to characterize this unexplored regulatory subset (6, 18, 50).

Considering the present results, we suggest that decidual regulatory HLA-G^+ T cells could be induced locally by DC-10 which were previously differentiated in the pre-implantation period by soluble factors released by decidualized cells. However, we cannot exclude that the HLA-G^+ T cells could also be recruited toward the decidua from the periphery. Finally, even though we demonstrated that, through soluble factors, the decidualized cells induce DC-10 and condition the T cell profile toward a tolerogenic one by the induction of regulatory T cells, it still remains to be defined whether these mechanisms operate in the human decidua *in vivo* and how they cooperate in promoting and maintaining feto-maternal tolerance.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Investigation and Ethics Committee from "Academia Nacional de Medicina." The participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

GS, CP, and RR designed the study, supervised the experimental work, and wrote the manuscript. SG carried out all the experiments using dendritic cells. ES, LF, and EG performed the HESC cells treatments. SG, EG, LG, FM, and LF did the flow cytometry analysis, ELISA assays, and interpretation analysis. SG, AC, and MB did the purification of monocytes and lymphocytes used in this work. GS and RR supervised the whole study. All authors read and approved the final manuscript.

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Visualizing Dynamic Changes at the Maternal-Fetal Interface Throughout Human Pregnancy by Mass Cytometry

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During healthy pregnancy, a balanced microenvironment at the maternal-fetal interface with coordinated interaction between various immune cells is necessary to maintain immunological tolerance. While specific decidual immune cell subsets have been investigated, a system-wide unbiased approach is lacking. Here, mass cytometry was applied for data-driven, in-depth immune profiling of the total leukocyte population isolated from first, second, and third trimester decidua, as well as maternal peripheral blood at time of delivery. The maternal-fetal interface showed a unique composition of immune cells, different from peripheral blood, with significant differences between early and term pregnancy samples. Profiling revealed substantial heterogeneity in the decidual lymphoid and myeloid cell lineages that shape gestational-specific immune networks and putative differentiation trajectories over time during gestation. Uncovering the overall complexity at the maternal-fetal interface throughout pregnancy resulted in a human atlas that may serve as a foundation upon which comprehension of the immune microenvironment and alterations thereof in pregnancy complications can be built.

Keywords: immune profiling, human atlas, pregnancy, placenta, decidua, peripheral blood

INTRODUCTION

Preserving immunological tolerance toward the semi-allogeneic fetus during pregnancy while providing protection against environmental pathogens relies on intricately regulated local and systemic immune adaptations. Direct contact between the mother and the fetus exists at the decidua basalis, located at the implantation site, and at the decidua parietalis that is part of the membranes which line the uterine cavity and surround the fetus. Fetal extravillous trophoblasts (EVT) migrate into the maternal decidua early during pregnancy (1), and express HLA-C, -G, -E, and -F but lack expression of the classical HLA-A and -B antigens, rendering them in part invisible to natural killer (NK) cells and the large majority of maternal allogeneic CD8+ T cells (2–4).

In concert, alterations in both the maternal innate and adaptive immune compartment occur, where NK and innate lymphoid cells (ILC) prevail in early pregnancy, while T cell proportions increase over the course of gestation (5, 6). Antigen-presenting cell (APC) numbers remain relatively constant throughout pregnancy while B cells have been described as a sparse population (5–8).

The fetus can be immunologically recognized as maternal NK cells may bind to fetal HLA-C and HLA-G, and fetal-specific CD8+ and CD4+ T cells have been observed in maternal peripheral blood and decidua (9–12). As such, aberrant regulation of the maternal immune system has been suggested to play a role in pregnancy complications, such as pre-eclampsia (13, 14), recurrent miscarriages (15, 16), preterm birth (17–19), and fetal growth restrictions (20).

A better understanding of the immune system at the maternal-fetal interface during a healthy pregnancy may drive the systematic investigation of major pregnancy complications. Most work in the field of reproductive immunology has focused on individual subsets of decidual immune cells while a comprehensive, system-wide approach that visualizes all decidual immune cell lineages at different time points during pregnancy is lacking. High-dimensional single-cell technologies such as mass cytometry (21) allow an in-depth and unbiased data-driven analysis of the composition of the immune system at the maternal-fetal interface. In the current study, we applied two mass cytometry antibody panels, one to detect heterogeneity within all major immune cell lineages while the other with a focus on T cell-specific markers, to determine the composition of the maternal immune compartment in first, second, and third (term) trimester decidual samples as well as maternal PBMC (mPBMC) at the time of delivery. Our results provide an immune atlas of the maternal-fetal interface in healthy pregnancy, which may serve as a foundation for improved understanding of pregnancy complications.

MATERIALS AND METHODS

Human Decidual and Blood Samples

De-identified 1st and 2nd trimester human decidual material (1st trimester, gestational age of 6–13 weeks, n = 12; 2nd trimester, 14–18 weeks, n = 6) was obtained from women undergoing elective pregnancy termination. The gestational age was determined by ultrasonography and the tissue obtained by vacuum aspiration. Paired 3rd trimester (term) decidua basalis, decidua parietalis, and heparinized mPBMC were obtained from healthy women after uncomplicated pregnancy (gestational age >38 weeks, n = 9) delivered by elective cesarean section or uncomplicated spontaneous vaginal delivery at Leiden University Medical Center (LUMC). Non-pregnant PBMC control samples were obtained from healthy females (n = 4). The clinical characteristics of the subjects are shown in **Table 1**. All samples were obtained after informed consent and the study was carried out in accordance with the guidelines issued by the Medical Ethics Committee of the LUMC (protocols P08.087 and P11.196), and in accordance with the Declaration of Helsinki.

Isolation of Lymphocytes From Decidual and PBMC Samples

Decidual leukocytes were isolated as previously described, with some adjustments (22). For isolation of 1st and 2nd trimester decidual leukocytes, villous and decidual tissues from elective pregnancy terminations were macroscopically identified and separated. Decidua basalis and parietalis from term pregnancy were macroscopically dissected by scraping the basalis membrane from the placenta and by removing the amnion and delicately scraping the decidua parietalis from the chorion. Decidual tissues were washed with PBS, minced, and resuspended in Accutase cell detachment solution (prewarmed to 37°C; Gibco Life technologies). Subsequently, tissues were transferred to a C tube, homogenized on a gentleMACS dissociator (Miltenyi Biotec Ltd.) and incubated for 60 min in a water bath (37°C, gently shaking), at 30 min spinning the C tubes once more. After digestion, released cell suspensions were filtered through 250 and 70 μm sieves (Sigma-Aldrich; Miltenyi Biotec Ltd.) and washed with RPMI 1640 (Life technologies). Next, the cell suspensions were dissolved in 20 ml of 1.023 g/ml Percoll (GE Healthcare) and layered on a Percoll gradient (10 ml 1.080 g/ml; 15 ml 1.053 g/ml) for density gradient centrifugation (25 min, 2000 rpm). Leukocytes were isolated from the 1.080–1.053 g/ml and the 1.053–1.023 g/ml interface, washed twice with RPMI, and left overnight at 4°C. Peripheral blood leukocytes were isolated from freshly drawn heparin anticoagulated blood using Ficoll (GE Healthcare) density gradient centrifugation (20 min, 2000 rpm) and left overnight at 4°C. The next day, cell suspensions were incubated with Benzonase Nuclease (Sigma-Aldrich; 20U/mL) for 5 min, washed, counted, and stained with antibodies for either mass cytometry or flow cytometry. To account for cell processing variation, the effects of enzymatic digestion and gentleMACS dissociation on cell surface protein markers in peripheral blood and decidual cell suspensions has extensively been validated in our laboratory and by others (23).

Mass Cytometry Antibody Staining and Data Acquisition

Antibodies used for mass cytometry are listed in **Supplementary Tables 1**, **2**. Primary metal-conjugated antibodies were purchased from Fluidigm or purified antibodies were conjugated with metal reporters by using a MaxPar X8 Antibody Labeling kit (Fluidigm) according to manufacturer's instructions. After conjugation, antibodies were diluted to 200 μl in antibody stabilization buffer (Candor Biosciences), supplemented with 0.05% sodium azide. Both antibody panels have previously been validated (24, 25), and in this study tested on both peripheral blood and decidual samples. Antibody staining and data acquisition were carried out as previously described (26, 27). In short, cells from decidual and peripheral blood samples were incubated with 1 mL of 1:500 diluted 500 μM Cell-ID Intercalator-^{103}Rh (Fluidigm) for 15 min at room temperature (RT), washed, and incubated with human Fc blocking antibody (Biolegend) for 10 min at RT. Cell suspensions were thereafter stained with a mix of metal-conjugated antibodies for 45 min at RT. After washing, cells were incubated with 125 nM Cell-ID Intercalator-Ir (Fluidigm) in MaxPar Fix and

TABLE 1 | Patient characteristics[1].

	1st trimester	2nd trimester	3rd trimester	NP PBMC
Demographics				
Maternal age (years; mean ± SD)	Unknown	Unknown	32.8 ± 4.3	30.5 ± 3.1
Body mass index (BMI; mean ± SD)	Unknown	Unknown	25.4 ± 3.9	22.1 ± 0.4
Gravity (median, IQR) [2]	Unknown	Unknown	2 (1, 2)	0
Parity (% nulliparous)	Unknown	Unknown	46	100
Pregnancy parameters				
Gestational age (weeks; mean ± SD)	9.2 ± 2.1	15.5 ± 1.2	39.1 ± 0.8	NA
Placenta weight (kg; mean ± SD)	NA	NA	564.5 ± 87.3	NA
Mode of delivery	Elective abortion	Elective abortion	Spontaneous + C-section	NA
Sex of child (%)	M 53.8%/ F 46.2%	M 9.1%/ F 90.9%	M 53.8%/ F 46.2%	NA
Experiment inclusions				
General CyTOF panel	*n* = 12	*n* = 6	*n* = 9	*n* = 4
T cell CyTOF panel	*n* = 11	*n* = 5	*n* = 8	*n* = 4
FACS panel	n = 3	n = 4	n = 4	NA

[1]All pregnancies were considered healthy as determined by demographics, pregnancy parameters, attending gynecologists/research nurses, and the absence of membrane discoloration and infarctions in the placenta. [2] IQR, interquartile range.

Perm buffer (Fluidigm) and left overnight at 4°C. Prior to data acquisition, cell pellets were diluted in distilled water containing 1:10 diluted EQ Four Element Calibration Beads (Fluidigm), and cells were acquired by a Helios mass cytometer (Fluidigm). After acquisition, data was normalized using the EQ beads with passport P13H2302 reference. To account for technical variation, a PBMC reference sample from a healthy donor was included for both the general and the T cell panel at ten intervals during 20 staining batches and 18 CyTOF acquisition runs over a time period of 7 months.

Mass Cytometry Data Analysis

For each data file, live single CD45+ immune cells were selected by gating in Cytobank (**Supplementary Figure S1**A). The gating strategy utilized the parameters residual, event length, width, and center to gate out debris and doublets. In addition, dead cells and normalization beads were excluded. Next, the files were subjected to sample-tagging, hyperbolic-arcsinh-transformation with cofactor 5 and dimensionality reduction in Cytosplore (28). Pair-wise Jensen-Shannon (JS) divergences were calculated for the individual samples within each tissue group, analyzed in a collective t-SNE, where low JS distances were indicative of high similarities between the samples within a group.

All data were pooled per panel and a five-level HSNE analysis was performed with default parameters (perplexity 30; iterations 1,000), where the major immune cell lineages were identified by automatic clustering (**Figure 1B** and **Supplementary Figures S1D, S2C**). No influence of the mode of delivery on clustering of term decidual samples was observed in our analyses and a previous report by Tilburgs et al. (29) similarly confirmed no influence of mode of delivery and other clinical variables on decidual cell types in term pregnancy. All HSNE, t-SNE, and Gaussian mean-shift clustering-derived cell clusters were generated in Cytosplore. A cluster is defined as a population of at least 100 cells with the same phenotype. Exported FCS files for all identified individual clusters were subjected to the CytoFast workflow in R (30). Hierarchical clustering of the heatmaps was created with Euclidean correction and average linkage, and the median intensity values of markers were visualized. The number of cells in each immune cluster were determined for each sample and cluster frequencies and sample frequencies were calculated. Sample frequencies were visualized in boxplots and sample t-SNE plots. Violin plots, PCA plots and correlation network analysis were generated in R. Diffusion maps were generated in R using the "destiny" package (31). Within the CD4+ T cell compartment, CD4+ T_N cells together with the CD4+ T_{RORA} cluster and Treg-like T cell clusters branched off completely and were omitted from the final CD4+ T cell diffusion map. Within the CD8+ T cell compartment, CD27- CD69-, CD27+CD69- T_N, and $CD27^{INT}CD69^{INT}$CD127+CCR6+ clusters branched off completely and were omitted from the final diffusion map. For the global test, incorporated within the Cytofast workflow, the absolute correlation distance with average linkage for hierarchical clustering was used. The branches colored in black show the significant multiplicity-corrected *p*-values.

Flow Cytometry

Antibodies for flow cytometric analysis are listed in **Supplementary Table 3**. For surface staining, cells were stained for 30 min at 4°C in PBS 1% FCS. For intracellular staining, cells were fixed and permeabilized using the FOXP3 staining buffer kit (eBioscience). Acquisition and analysis were performed on an LSR-II (BD Biosciences) using FACS Diva software. In addition, HSNE and t-SNE analysis of flow cytometric data was performed using Cytosplore. Co-expression of FOXP3, HELIOS, CTLA-4, CD39, ICOS, and TIGIT was confirmed by manual gating and HSNE analysis.

Statistical Analyses

Results are shown as median with interquartile range and the boxplots depict the 10–90 percentile. To determine

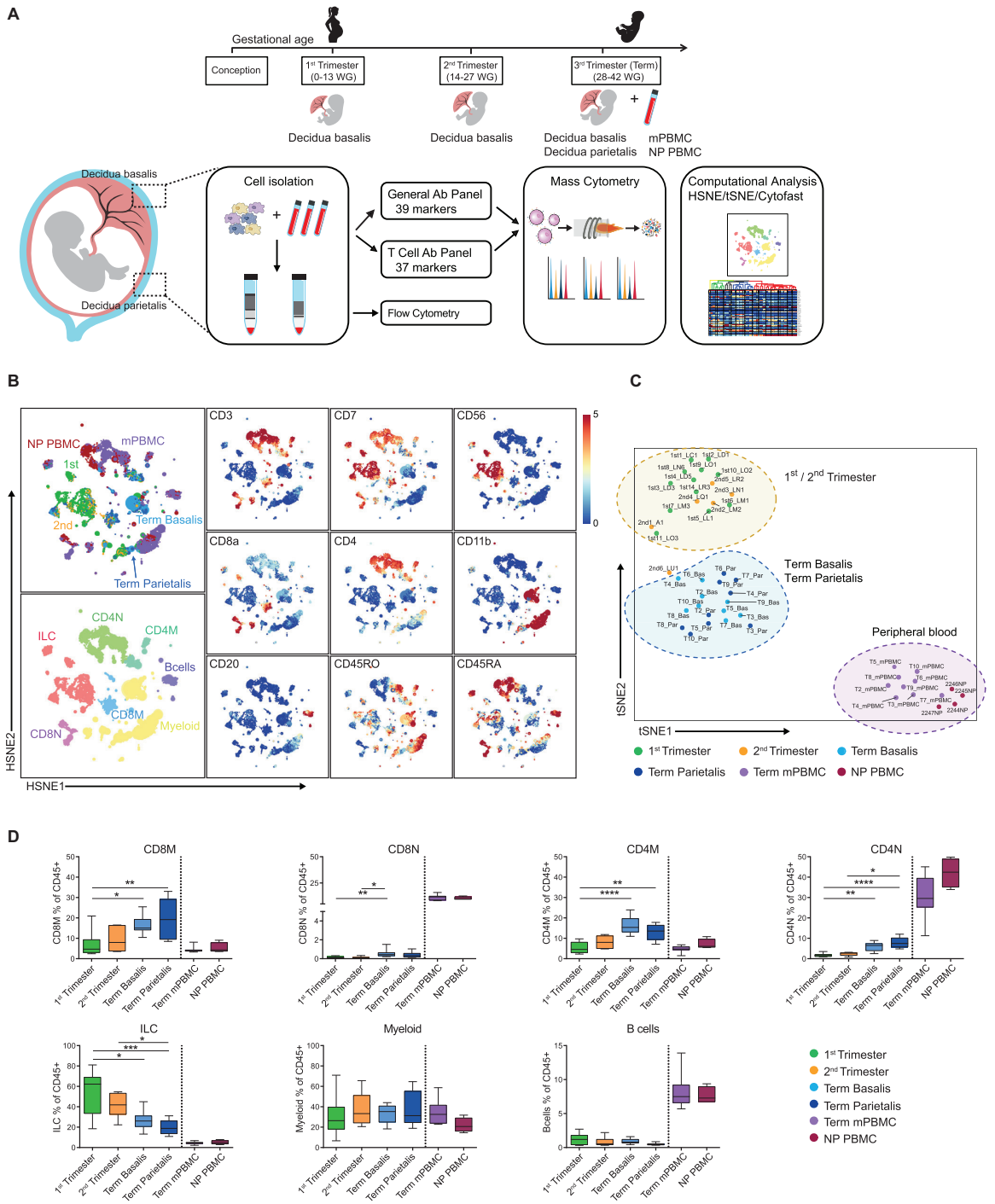

FIGURE 1 | Identification of major immune cell lineages at the maternal-fetal interface. **(A)** Experimental setup. First (6–13 weeks of gestation), second (14–18 weeks) and third trimester (term; >38 weeks; basalis and parietalis) decidual samples along with maternal peripheral blood mononuclear cells (mPBMC) and non-pregnant PBMC (NP PBMC) were analyzed. **(B)** First-level HSNE visualization of the major immune cell lineages derived from decidua and peripheral blood. Colors top left indicate tissue type (1st trimester $n = 12$; 2nd trimester $n = 6$; term basalis and parietalis $n = 9$; mPBMC $n = 9$; NP PBMC $n = 4$); colors bottom left indicate major immune cell types (CD8M, CD8 memory T cells; CD8N, CD8 naïve T cells; CD4M, CD4 memory T cells; CD4N, CD4 naïve T cells; ILC, innate lymphoid cells); colors for plots on the right indicate the arcSinh5-transformed expression values of the specified markers where every dot represents a landmark. Memory and naïve clusters were distinguished based on CD45RO and CD45RA expression. **(C)** t-SNE visualization of the separation between decidual and peripheral blood samples (as percentage of CD45+ cells). Every dot represents a single sample. **(D)** Major immune cell lineages (as percentage of CD45+ cells) throughout gestation and within mPBMC and NP PBMC. Boxplots depict the 10–90 percentile and the Kruskal-Wallis with Dunn's test for multiple comparisons was applied. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

TABLE 2 | Total number of cells and samples analyzed.

Panel	# of Decidual samples	CD4+ T cells	CD8+ T cells	B cells	Myeloid cells	ILC/NK cells	TCRγδ cells
General panel	36	1,136,799	1,082,234	72,414	2,390,451	3,841,125	114,875
T cell panel	32	818,800	707,147	73,579	2,087,932	3,556,369	96,640
Trimester	Total # of samples	General panel	T cell panel	Overlap (#, %)			
1st Basalis	14	12	11	9; 64%			
2nd Basalis	7	6	5	4; 57%			
3rd Basalis	9	9	8	8; 89%			
3rd Parietalis	9	9	8	8; 89%			
mPBMC	9	9	8	8; 89%			
NP PBMC	4	4	4	4; 100%			

differences among more than two unpaired groups, a non-parametric Kruskal-Wallis test with Dunn's multiple comparison post-test was applied where significance was assessed by controlling for false discovery at 5% (FDR). *P*-values < 0.05 were considered to denote statistically significant differences. Statistical analyses were performed in GraphPad Prism version 8.0 and R version 3.5.1.

RESULTS

The Maternal-Fetal Interface Harbors a Unique Immune Cell Composition

We analyzed first, second, and third trimester decidual samples along with mPBMC taken at the time of delivery and PBMC of non-pregnant age-matched women (NP PBMC) as a control (**Table 1**). A general mass cytometry panel comprising 39 antibodies (**Supplementary Table 1**) was used to provide a broad coverage of the myeloid and lymphoid immune compartments. For in-depth profiling of the T cell compartment, a second panel comprising 37 antibodies (**Supplementary Table 2**) was applied. After data acquisition (**Table 2**), live, single CD45+ cells were selected for downstream analysis (**Supplementary Figure S1A** and **Figure 1A**). Conventional cell populations were verified by manual gating and have previously been validated (27). To allow systematic comparison of samples, the data obtained with the general panel (49 samples; 19×10^6 CD45+ cells) and the data obtained with the T cell panel (44 samples; 17×10^6 CD45+ cells) were pooled separately and analyzed with hierarchical stochastic neighbor embedding (HSNE) and t-distributed stochastic neighbour embedding (t-SNE) in Cytosplore (28, 32). Comparison of the absolute numbers and percentages of CD45+ cells and correlations thereof showed a similar pattern in the general and T cell panel (**Supplementary Figures S1B,C**).

At several timepoints during the acquisition timeline a PBMC reference sample was included, which corroborated reproducible staining and acquisition among different sets of experiments (**Supplementary Figures S2A,B**). Clustering of technical PBMC reference samples together with the experimental decidual samples (for each panel separately) using Cytosplore revealed that reference samples clustered tightly together and that variation between decidual samples was much greater than between reference samples (**Supplementary Figures S3, S4**). This demonstrated that only a limited amount of variation is explained by staining inconsistencies between batches.

At the overview level, the HSNE landmarks depicted the global data heterogeneity and marker expression profiles in both panels and identified the major immune cell subsets of myeloid cells, ILC, CD4+ T cells, CD8+ T cells (including the TCRγδ lineage), and B cells (**Figure 1B** and **Supplementary Figure S1D**). Subsequently, t-SNE analysis based on cell frequencies separated the samples of 1st and 2nd trimester from samples of term basalis and parietalis, and peripheral blood, indicative of distinct immune profiles (**Figure 1C** and **Supplementary Figure S1E**). Cell frequencies of the major immune cell lineages confirmed ILC as being the predominant cell type in 1st trimester, decreasing toward the end of pregnancy, and contrasting the dynamics of T cells. This analysis also validated that the number of myeloid cells remains relatively constant throughout gestation, while B cells are hardly present (**Figure 1D** and **Supplementary Figure S1F**) (6).

Early Pregnancy Reveals a Heterogeneous Group of Myeloid Cells With High HLA-DR Expression

Next, for each antibody panel, the data from all decidual samples were pooled and HSNE analysis was performed on every lineage individually. Within the myeloid cell lineage (**Supplementary Figure S2C**), the second hierarchical level revealed six large subpopulations that could be discriminated based on differential expression of CD14, CD11c, CD11b, HLA-DR, CD16, and CD15 (**Supplementary Figures S2D,E**). Subsequently, Gaussian mean-shift clustering was applied and quantified with Cytofast (30), revealing 16 phenotypically distinct myeloid cell clusters (**Figure 2A**). Here, HSNE overview plots showed the individual markers that contributed to the separation into distinct clusters (**Figure 2B**). Next, we determined which myeloid cell clusters were differentially present in 1st and 2nd trimester, term basalis, and term parietalis samples to uncover dynamics throughout pregnancy (**Figure 2C**). Only cell clusters with significant differences (false discovery rate (FDR) <5%) between the groups are shown.

Notably, early pregnancy (1st and 2nd trimester) was characterized by the presence of a heterogeneous group of myeloid cells with high HLA-DR expression. CD163+HLA-DR+ cells, also expressing intermediate levels of CD56 and

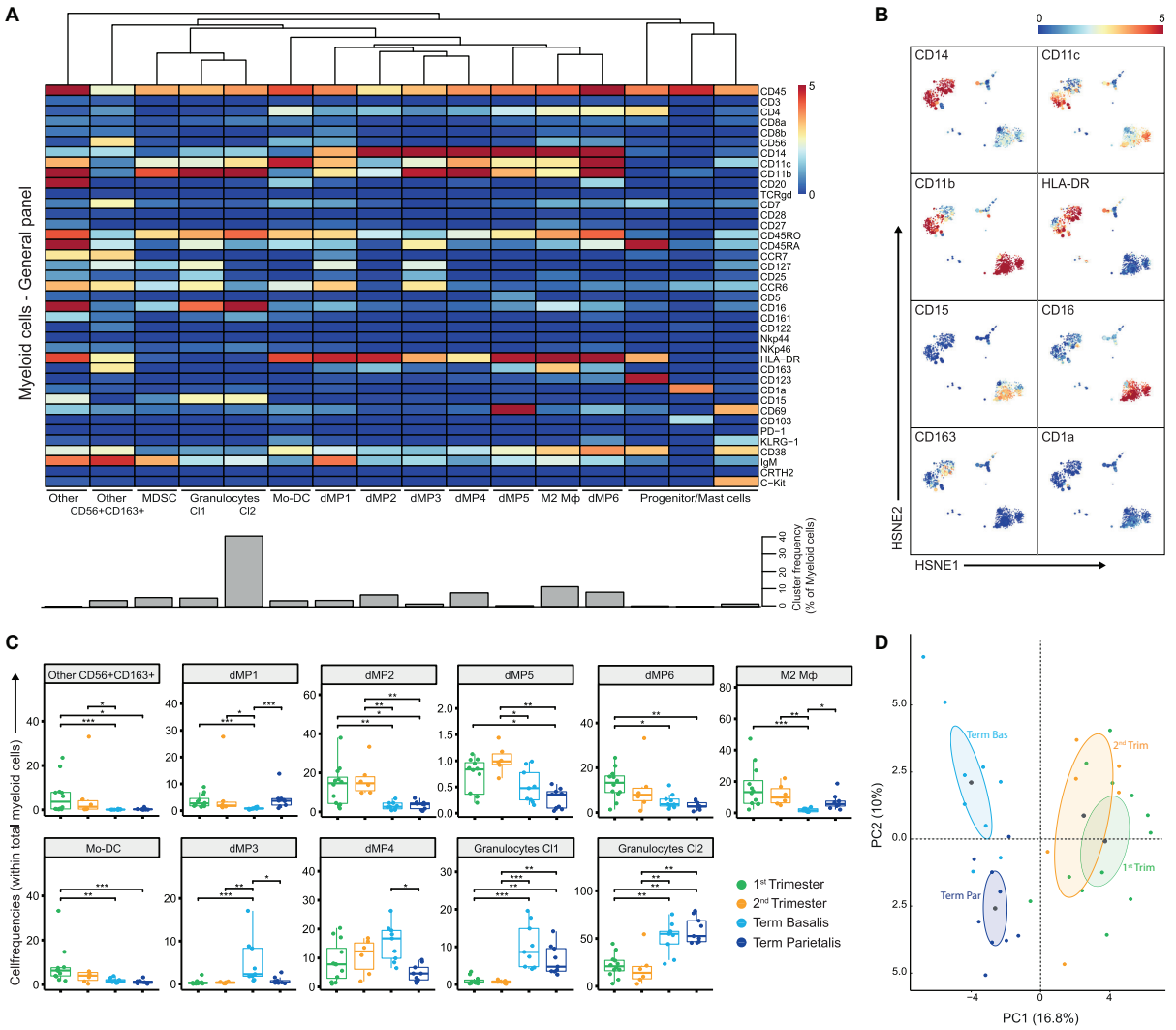

FIGURE 2 | The myeloid compartment is highly diverse in early pregnancy. **(A)** Heatmap showing the median arcSinh5-transformed marker expression values for the 16 identified myeloid clusters within the general panel (36 samples; 2,390,451 cells). Cluster IDs and cluster frequencies are displayed at the bottom of the heatmap. **(B)** First-level HSNE embedding of the arcSinh5-transformed expression values of the indicated markers (note these are the same plots as in **Supplementary Figure S2E**). **(C)** Boxplots of sample frequencies, divided per trimester, of the cell clusters plotted as a fraction of total myeloid cells. The Kruskal-Wallis with Dunn's test for multiple comparisons was performed and only clusters with significant differences (false discovery rate (FDR) < 5%) between the groups are shown. **(D)** Unsupervised principal component analysis (PCA) of the sample frequencies (as percentage of total myeloid cells), where the gestational age groups are depicted along the first two components. The centroid of each group is indicated in gray. MDSC, myeloid-derived suppressor cells; dMP, decidual mononuclear phagocytes; Mφ, macrophage. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

CD7 (cluster "Other CD56+CD163+"), were observed in the 1st and 2nd trimester (**Figure 2C**), and may represent myeloid-like NK cell progenitors or a distinct monocyte/dendritic cell population. Furthermore, cell clusters of decidual mononuclear phagocytes (dMP), namely dMP1, dMP2, dMP5, and dMP6, expressing various combinations of CD14, CD11b, CD11c, CCR6, CD38, and CD69 were more prominent in 1st and 2nd trimester decidua compared to term decidua. The immune-regulatory CD163+ M2 macrophage (Mφ) subtype was present in early pregnancy and term decidua parietalis, but hardly in term decidua basalis. In addition, CD11c^{high}CD14-CD16- Mo-DC were predominantly abundant in 1st trimester. Moreover, CCR6+CD45RA+CD38- dMP cell clusters with low HLA-DR expression (dMP3 and dMP4) were dominantly present in term decidua basalis while the largest population of CD15+CD16+ granulocytes was found in both term decidua basalis and parietalis (**Figure 2C**). Finally, a clear separation between early and late pregnancy samples in unsupervised principal component analysis (PCA; **Figure 2D**) was driven by an abundance of granulocytes in late pregnancy and supported by a previously unrecognized diverse composition of myeloid cells in early pregnancy. Together, these results reveal substantial changes in the composition of the myeloid compartment during gestation.

Dynamic Changes in the Composition of the ILC Compartment During Pregnancy

A similar analysis of the ILC compartment (CD3-CD7+) confirmed its well-described cellular composition in decidua (33–36). The general panel classified 14 clusters with high expression of CD56 and lack of CD16 (**Supplementary Figure S5A**). Early pregnancy was characterized by activated CD161+CD122+NKp46+CD69+ NK cells (NK2, NK3, NK5, NK7, NK8, NK13), tissue-resident CD69+CD103+ cell clusters (NK4, NK5, K7) and ILC3 (**Supplementary Figures S5A,B**), coupled to the expression of CD39 and TIM-3 (**Supplementary Figures S5C–E**) (37). Toward the end of pregnancy, NK cells displayed a less activated phenotype with lower expression of CD161, CD122, NKp46, and CD103, and higher expression of CD45RA and CD16 (mostly in term basalis; **Supplementary Figure S5B**). Tissue-resident-like ILC were not only observed in 1st trimester (dIC6; decidual ILC Cluster), but also in small numbers in term samples (dIC1) along with the expression of TIGIT (**Supplementary Figures S5C,D**). In addition, expression of the co-inhibitory receptors TIM-3 and CD39 was observed in both early and term parietalis samples. NK2, NK3, and NK5 clusters resembled a phenotype similar to the intermediate innate subset described in fetal intestine that can differentiate into ILC3 and NK cells (25).

In summary, high proportions of activated ILC are present early in pregnancy alternated by dissimilar, smaller proportions of ILC cell clusters in term pregnancy, where the largest separation was observed between 1st trimester and term basalis (**Supplementary Figure S5F**).

The Decidua Harbors NKT-Like TCRγδ T Cells

Substantial phenotypic diversity was observed within decidual TCRγδ cells where seven cell clusters were identified within the general panel (**Supplementary Figures S6A,B**). The most prominent cell clusters were CD161+KLRG1+ TCRγδ$_{EM}$, present throughout gestation, and CD69+ TCRγδ$_{EMRA}$ that were dominant in term basalis. Remarkably, NKT-like populations of TCRγδ cells expressing CD56 and CD11c were also observed. T$_{EMRA}$, with high expression of CD45RA, and T$_{EM}$ cell clusters persisted in early pregnancy while cells co-expressing CD45RA and CD45RO and positive for CD27, CD5, and CD69 increased in term parietalis (**Supplementary Figures S6A,C**). Even though differences throughout gestation were existent, close clustering between the three different trimesters was observed in a PCA (**Supplementary Figure S6D**). In summary, these results display heterogeneity and the presence of NKT-like populations within the TCRγδ compartment.

CD4+ T Cell Characterization Reveals Unexplored Diversity Within Memory and Regulatory Phenotypes

In the CD4+ T cell lineage 17 cell clusters were identified: one naïve (N; CD45RA+CCR7+), two terminally differentiated (TEMRA; CD45RA+CCR7−), one central-memory (CM; CD45RO+CCR7+), seven effector-memory (EM; CD45RO+CCR7−), one CD45RA+RO+ and five memory regulatory-like T cell (Treg-like; CD25+CD127−) clusters (**Figures 3A,B**). Early in pregnancy, natural-killer-like CD4+ T cells (NKT-like) exist that express CD56, CD11c, CD161, CD122, NKp46, and CD38 (**Supplementary Figure S7A** and **Figure 3C**). Expression of CD127 and CCR6 occurred toward the end of pregnancy (T2$_{EM}$), consistent with the early pregnancy-associated T4$_{EM}$ cluster that lacked expression of these markers. At term, CD4+CD7-CD161+ T$_{EM}$ cells expressing CD27 and CCR6 (T2$_{EM}$) were observed in term basalis, whereas CD4+CD7+CD161- T$_{EM}$ cells expressing CD38 and ICOS, and lacking CCR6 (T7$_{EM}$) were predominantly present in term parietalis (**Figure 3C**). Furthermore, CD4+ T$_{EM}$ cells showed co-expression of PD-1 and ICOS, at lower levels than the Treg-like population, and lack of TIGIT and CD39.

Considerable heterogeneity within the Treg-like compartment was uncovered, where CD25+CD127- cell clusters expressed high levels of co-inhibitory (PD-1, CD39, TIGIT) and stimulatory (ICOS, CD38, CD28, CD27) receptors, including co-expression thereof (**Figures 3A,B**). When investigating the Treg-like compartment in more detail, previously unrecognized heterogeneity was observed with respect to the expression of the Treg-associated markers TIM-3, CCR8, and CCR4 (**Supplementary Figure S7B**) (38–40). Tr1 cells, identified by co-expression of LAG-3 and CD49b (41), were absent in decidual CD4+ T cells (**Supplementary Figure S7B**), but were observed in mPBMC (data not shown). Quantification of the presence of these CD25+ cell clusters in the gestational age groups revealed that T4$_{REG}$ (HLA-DR-CD69-PD-1-) and T3$_{REG}$ (CCR4+CD38+) were more frequent in early pregnancy and lower in term basalis, whereas the largest Treg-like population, T2$_{REG}$ (ICOS+PD-1+TIGIT+CD39+), was significantly increased in term parietalis (**Figure 3C**). Furthermore, T5$_{REG}$ (CCR6+ICOS+TIGIT+PD-1-CD39-) was significantly increased in term decidua basalis and parietalis, while virtually absent in early pregnancy. By aligning cells from these five Treg-like clusters along a two-dimensional diffusion map (31), putative differentiation and/or plasticity trajectories were observed between cell clusters T2$_{REG}$, T3$_{REG}$, T4$_{REG}$, and T5$_{REG}$. T1$_{REG}$, the smallest Treg-like cluster, was distinct owing to the lack of CD7 and CD27 expression (**Supplementary Figure S7C**).

To further evaluate the Treg-like phenotypes, intracellular expression of FOXP3, HELIOS, and CTLA-4 in CD4+CD25+CD127- and CD127+ T cells was assessed by flow cytometry in decidual samples (**Supplementary Table 3**). Co-expression of FOXP3, HELIOS, CTLA-4, CD39, ICOS, and TIGIT was observed in HSNE analysis of flow cytometry data, confirming a valid regulatory T cell phenotype (**Figure 3D**). In addition, differential co-expression of these markers was observed in several cell clusters, where not all CD4+CD25+CD39+ICOS+ cells expressed FOXP3 and/or HELIOS. This indicates that the Treg-like CD25+CD127- populations detected by mass cytometry represent a heterogeneous group of Treg and Treg-like cells at the maternal-fetal interface (**Figure 3D**). Flow cytometry data revealed an increase in CD4+CD25+CD127+ T cells, known

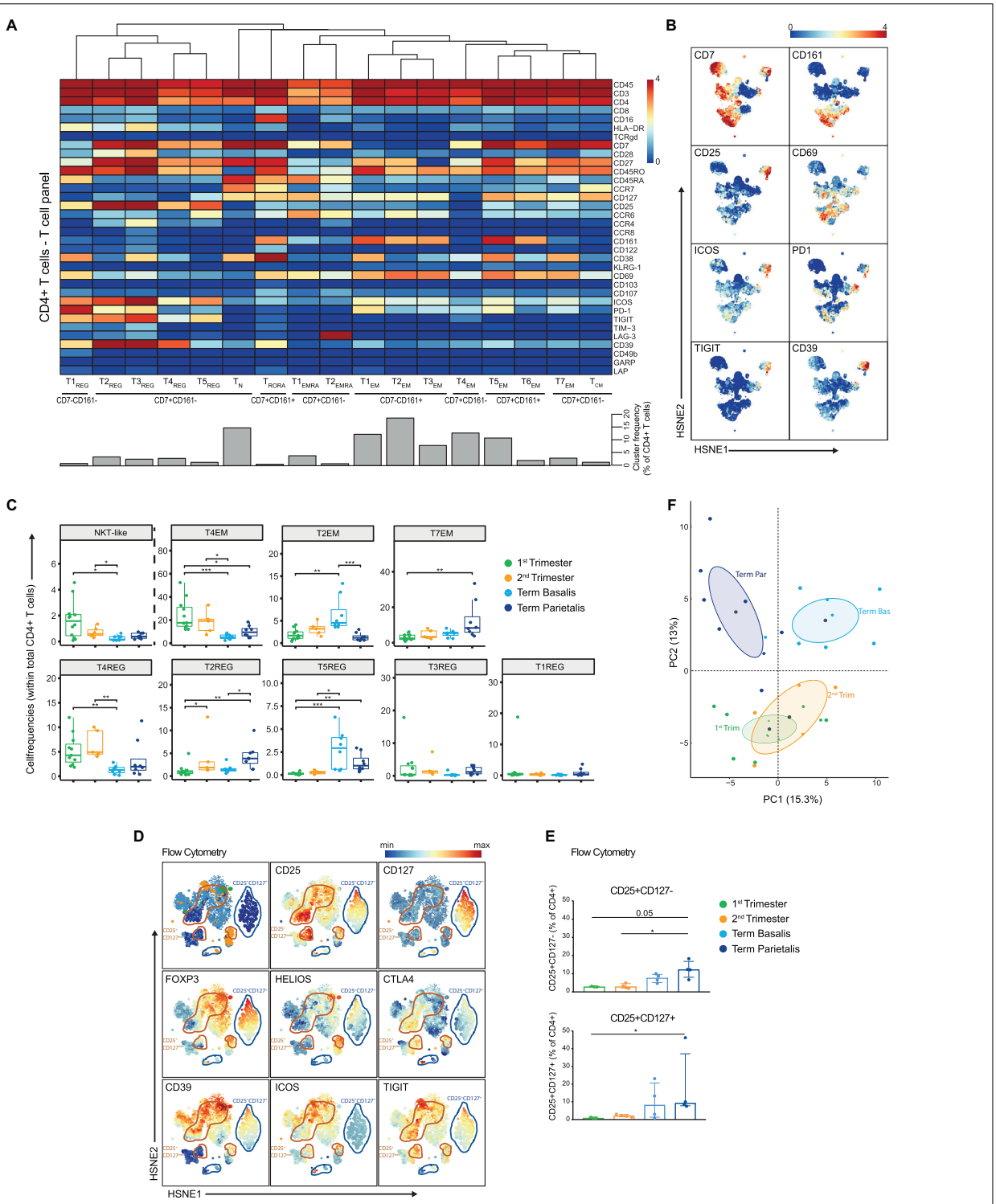

FIGURE 3 | In-depth characterization of the heterogeneity within the CD4+ T cell compartment. **(A)** Heatmap showing the marker expression values for the 17 identified CD4+ T cell clusters within the T cell panel (32 samples; 818,800 cells). Cluster IDs and cluster frequencies are displayed at the bottom of the heatmap. **(B)** First-level HSNE embedding of the expression values of the indicated markers. **(C)** Boxplots of sample frequencies, divided per trimester, of the clusters plotted as a fraction of total CD4+ T cells. The Kruskal-Wallis with Dunn's test for multiple comparisons was performed. **(D)** HSNE embedding of the expression values of the indicated markers, measured by flow cytometry and gated within CD3+CD4+ T cells. CD4+CD25+CD127- clusters are circled in orange; CD4+CD25+CD127+ clusters are circled in blue. 1st (n = 3), 2nd (n = 4) and term decidua (n = 4). **(E)** Boxplots depicting the CD25+CD127- (upper panel) and CD25+CD127+ (lower panel) populations as percentage of CD4+ T cells. **(F)** PCA of the sample frequencies (as percentage of total CD4+ T cells) where the gestational age groups are depicted along the first two components. The centroid of each group is indicated in gray. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.005$.

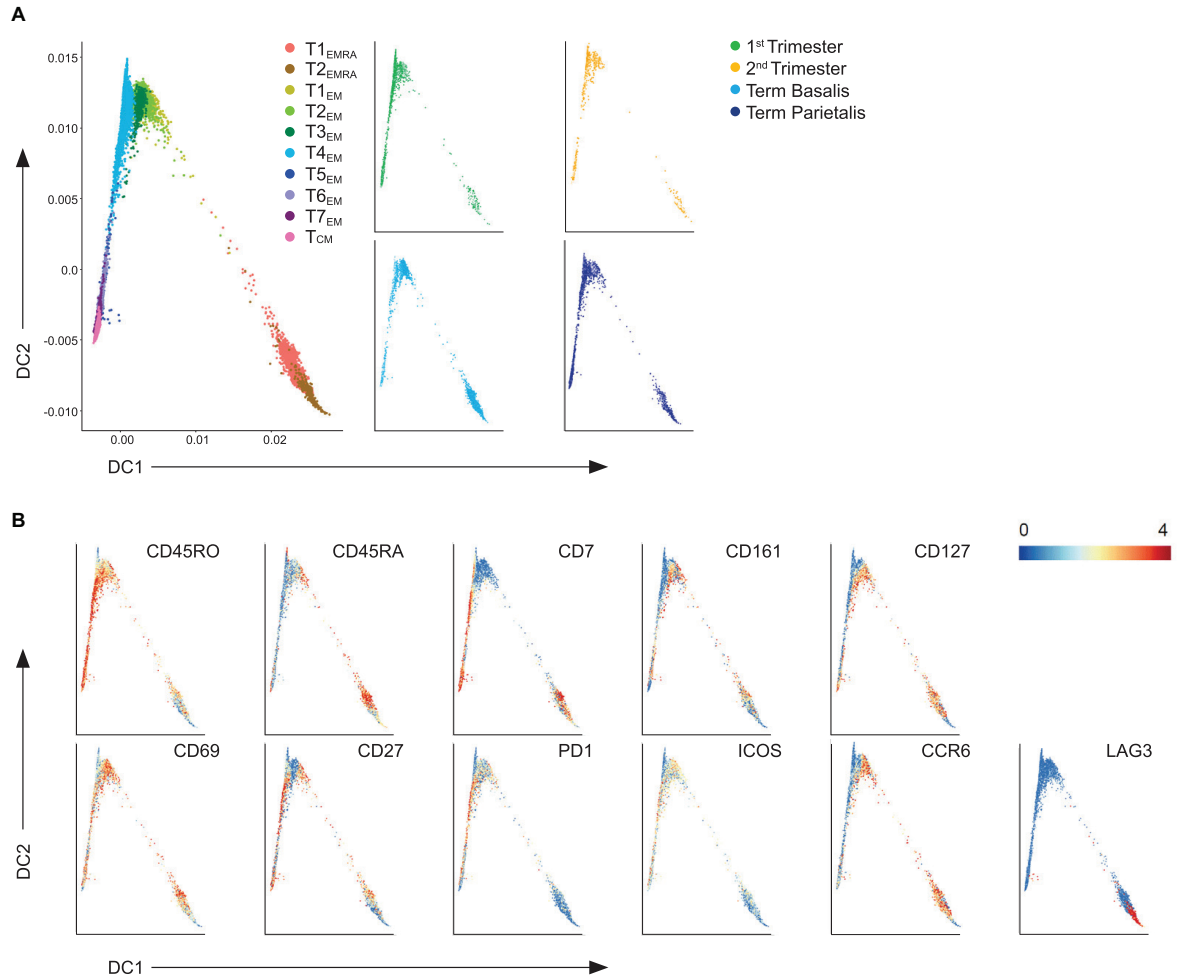

FIGURE 4 | Trajectory analysis of effector and memory decidual CD4+ T cells. **(A)** Visualization of terminally differentiated (EMRA) and effector-memory (EM) CD4+ T cell clusters in a diffusion map along two components. Each color in the left panel represents a cluster of cells. In the right panel, cells within the 1st trimester, 2nd trimester and term decidua basalis and parietalis are portrayed. **(B)** ArcSinh5-transformed expression values of the specified markers in the diffusion map.

to be activated effector CD4+ T cells (42), and regulatory-like CD4+CD25+CD127- T cells toward the end of pregnancy, with this increase being most apparent in term parietalis (**Figure 3E** and **Supplementary Figure S7D**). Overall, the data uncovered distinct memory and regulatory-like CD4+ T cell populations at different locations throughout pregnancy, where clear separation is revealed between early and term pregnancy, as well as between term basalis and parietalis (**Figure 3F**).

Next, diffusion mapping was used to distinguish prospective relationships among the different types of memory CD4+ T cell clusters. Two-dimensional diffusion plots revealed a split into two branches with $T4_{EM}$, lacking CD127 expression, at the center of the split (**Figure 4A**). Gradients of protein expression between cells were observed rather than discrete cell clusters (**Figure 4B**). The branch that expanded along diffusion component 2 (DC2) consisted of CD7+CD161+ and CD161- T_{EM} cells that were CD127+ and CD27+. T_{CM} was projected at the end of this trajectory branch. The second branch along DC1 consisted of the CD7-CD161+ clusters that showed CD127 expression, including one cluster ($T3_{EM}$) that lacked CD27 expression. The two EMRA clusters separated out from the EM clusters based on their expression of CD45RA and lack of CD27 expression. These results suggest putative differentiation states between the identified EM CD4+ T cell clusters throughout pregnancy.

Decidual CD8+ T Cells Co-express Inhibitory and Stimulatory Receptors

We next investigated the heterogeneity within the CD8+ T cell compartment where 20 CD8+ T cell clusters were characterized, namely one naïve, seven TEMRA, five EM, and seven clusters co-expressing CD45RA and CD45RO, a phenotype that is associated with proliferation (**Figures 5A,B**). Four of these clusters revealed significant differences between the decidual samples (**Figure 5C**). The tissue-resident memory (TRM) CD8+ T cell cluster $T4_{RORA}$ (CD69+CD103+CD38+CD161+PD-1+CD39+) was more frequent in early pregnancy, while $T5_{EMRA}$ ($CD69^{high}$) and $T6_{RORA}$ (CD127+CCR6+CD38+CD69+) were more abundant

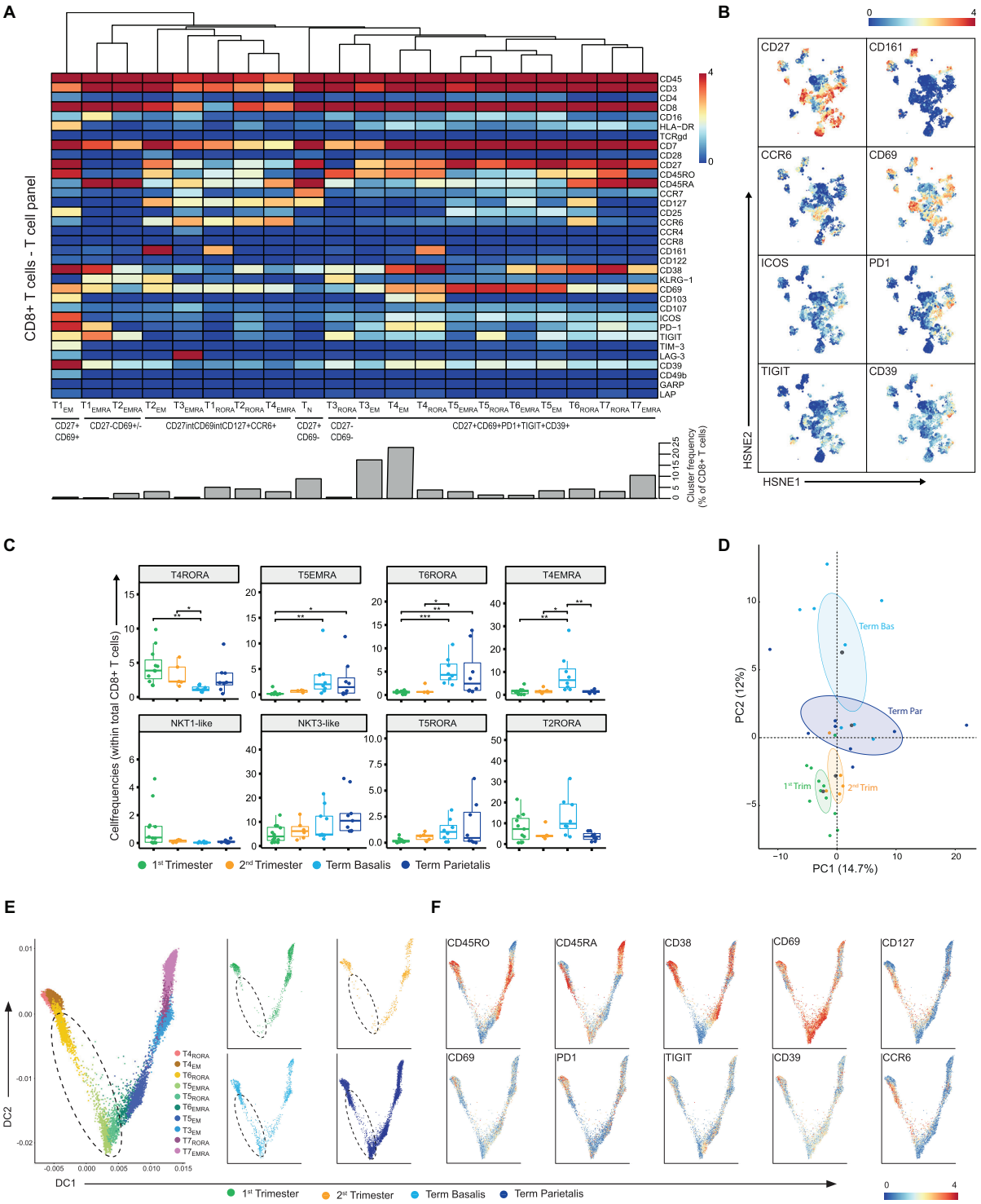

FIGURE 5 | In-depth characterization of the heterogeneity within the CD8+ T cell compartment. **(A)** Heatmap showing the marker expression values for the 20 identified CD8+ T cell clusters within the T cell panel (32 samples; 707,147 cells). Cluster IDs and cluster frequencies are displayed at the bottom of the heatmap. **(B)** First-level HSNE embedding of the expression values of the indicated markers. **(C)** Boxplots of sample frequencies, divided per trimester, of the clusters plotted as a fraction of total CD8+ T cells. The Kruskal-Wallis with Dunn's test for multiple comparisons was performed. **(D)** PCA of the sample frequencies (as percentage of total CD8+ T cells) where the gestational age groups are depicted along the first two components. The centroid of each group is indicated in gray. **(E)** Visualization of T_{EMRA}, T_{RORA}, and T_{EM} CD8+ T cell clusters in a diffusion map along two components. Each color in the left panel represents a cluster of cells. In the right panel, cells within the 1st trimester, 2nd trimester and term decidua basalis and parietalis are portrayed. **(F)** ArcSinh5-transformed expression values of the specified markers in the diffusion map. $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.005$.

in term samples. Also, $T4_{EMRA}$ (CD127+CCR6+) was increased in term basalis. In addition, a trend for a higher presence of NKT1-like cells in the 1st trimester, a gradual increase in NKT3-like and $T5_{RORA}$ cells from 1st trimester to term, and higher numbers of $T2_{RORA}$ in 1st trimester and term basalis were observed (**Figure 5C** and **Supplementary Figure S8A**). High levels of CD27 were observed in several effector and effector-memory cell clusters (e.g., $T5_{EMRA}$, $T5_{RORA}$, $T6_{RORA}$).

Where our recent work demonstrated a mixed gene expression signature of activation and dysfunction in bulk memory decidual CD8+ T cells (43), mass cytometry at the single-cell level revealed the expression of inhibitory and stimulatory receptors to be intertwined (**Figure 5A**). This co-expression of inhibitory (CD39, PD-1, TIGIT) and stimulatory (ICOS, CD69, CD27) receptors was verified by flow cytometry and mainly observed in term basalis and parietalis (**Supplementary Figure S8B**). Interestingly, the T_{EMRA} and T_{CM} clusters within the CD8+ T cell compartment contrasted the frequencies of these populations within the CD4+ T cell compartment with a higher percentage of T_{EMRA} and lower percentage of T_{CM} within the CD8+ T cells (**Supplementary Figure S8C**). In general, the differences in marker expression in the CD8+ T cell compartment were more subtle when compared to the CD4+ T cells. Consequently, the PCA showed a less clear separation between early and late pregnancy with term parietalis being more similar to 1st and 2nd trimester samples than term basalis (**Figure 5D**).

In a two-dimensional diffusion plot analysis, two branches were observed with the CD38- clusters ($T5_{EMRA}$, $T5_{RORA}$) at the center of the split. Here, the CD38+CD69+ T_{EMRA} and T_{EM} clusters expanded along DC1, while the TRM cells and CD127+CCR6+ T_{EM} cells expanded along DC2 (**Figures 5E,F**). Furthermore, along DC2 cell clusters $T5_{EMRA}$, $T5_{RORA}$, and $T6_{RORA}$ with lower expression of CD45RO and PD-1 and high expression of CD69, were absent in early pregnancy and appeared in term pregnancy, as observed in **Figure 5C** (**Figure 5E**; dashed circle). These potential differentiation trajectories suggest a phenotypic continuum and thereby possible plasticity between specific CD8+ T cell clusters.

In summary, these data show that a group of CD8+ T cells displays co-expression of inhibitory and stimulatory receptors at the protein level, and suggest that in this group several differentiation trajectories coupled to distinct functions throughout gestation may be at play. Whereas CD8+ NKT cells are present in early and late pregnancy, there are hardly any CM CD8+ T cells.

B Cells Are Mainly Present Early in Pregnancy

Although the number of B cells was low, nine CD20+ B cell clusters with variable expression of CD38, CD27, and IgM were identified within the general panel (**Supplementary Figure S9A**). Interestingly, most B cells were detected in the 1st trimester (**Supplementary Figure S9B**). CD20 was also included in the T cell panel (as exclusion marker) and showed to be useful in detecting CD39 expression on several B cell clusters (**Supplementary Figure S9C**).

Correlation Analysis Reveals Gestational-Specific Immune Networks

To conflate the 77 identified immune cell clusters within the general panel and visualize relationships between them, a correlation network analysis was performed using the sample frequencies. This analysis demonstrated that 73% of clusters were strongly correlated with each other (Spearman rank >0.7; **Figure 6A**). Subsequently, multivariate associations between individual and groups of clusters were detected by applying a multinomial logistic regression model with the global test (**Figure 6B**) (30, 44). Four networks were revealed in which colored nodes highlight the significance of individual cell clusters in one of the four gestational age groups. Cell clusters in network 1 consisted of myeloid cells, CD4+ T cells, CD8+ T cells, and B cells, and did not reveal significant gestational specificity. Network 2 revealed a correlation between NKT-like, B cell, and NK cell clusters (including tissue-resident-like phenotypes), most of which were significantly abundant in the 1st trimester. This may reflect unappreciated interactions between NK cells and NKT-like cells early in pregnancy. Network 3 is characterized by clusters predominantly present in term basalis and included a correlation between innate immune cells such as NK cell clusters, dMP3 and granulocytes, and adaptive immune cells including T_N, T_{EM} and Treg-like CD4+ clusters, CD8+ T_{RORA} cells, and $TCR\gamma\delta_{EMRA}$ cells. Interestingly, a different network of clusters was observed in term parietalis (network 4), where CD4+ and CD8+ T_N cells, CD4+CD127+CD161- T_{CM} cells, CD4+ Treg-like clusters, CD8+ T_{EM} and NKT-like cells, and TCRγδ cells were correlated. These results underline that distinct immune cell interactions in basalis versus parietalis contribute to the microenvironment in term pregnancy. Thus, three of the four networks correlated with either gestational age or tissue location.

DISCUSSION

To better understand the maternal immune landscape during healthy pregnancy, we performed mass cytometry analysis of immune cells isolated from decidua throughout the three trimesters of pregnancy and compared this to term mPBMC. This provided an unbiased, data-driven overview of all decidual immune cell populations throughout pregnancy. Previously described decidual immune cell subsets (5, 33, 45, 46) and the kinetics of the major immune cell lineages during gestation (5, 6, 47) were validated in the current study. Moreover, we observed unprecedented immune cell heterogeneity in the decidua.

By implementing replicate PBMC control samples along with the experimental decidual samples, we demonstrated that the identified decidual immune cell clusters described here displayed much greater phenotypic diversity than what could be explained by staining inconsistencies and that batch effects are therefore minimal. It should be noted that 11 (tissue-specific) out of 52 unique markers of both antibody panels combined displayed no or hardly any expression in the internal control PBMC reference samples and could, therefore, not be assessed for staining consistency during batch analysis.

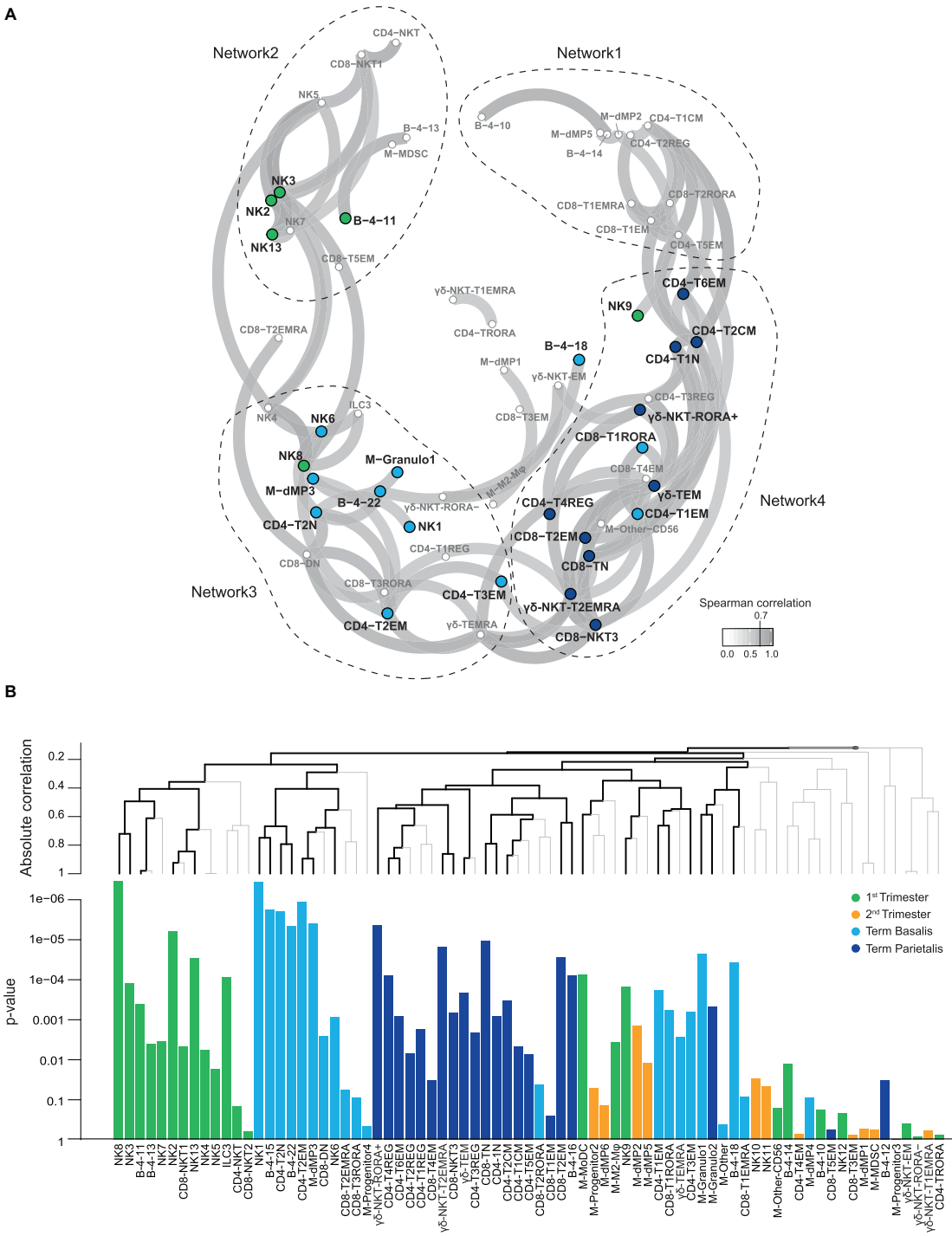

FIGURE 6 | Relationships between identified cell clusters from all major immune cell lineages. **(A)** Correlation network plot showing Spearman coefficients higher than 0.7 for relationships between the decidual immune cell clusters from the general panel. Every circle depicts an immune cell cluster, with colors corresponding to statistically significant contributions of clusters to 1st trimester, term decidua basalis or parietalis, as calculated by the global test (44). Higher correlation corresponds to greater proximity of cell clusters. **(B)** Multivariate associations between cell clusters detected by a multinomial logistic regression model with the global test. The top panel shows hierarchical clustering of absolute correlation distances, where the branches in black indicate significant multiplicity-corrected *p*-values. The bottom panel shows the corresponding *p*-values of immune cell clusters associated with 1st trimester (green), 2nd trimester (yellow), term decidua basalis (light blue), and parietalis (dark blue).

Distinct clusters of dMP were detected in early pregnancy, suggestive of an essential role for antigen presentation and thereby interaction with other immune cells at the initiation of pregnancy. Furthermore, the presence of different dMP cell clusters in term basalis and parietalis may reflect distinct local antigen presentation and function. Proportions of the dMP cells decrease over gestation accompanied by an influx of granulocytes at time of parturition, in line with the observed increase in the numbers of circulating neutrophils during pregnancy (48). ILC that play an important role in early pregnancy by facilitating spiral artery remodeling and trophoblast invasion, may in small proportions preserve their function (e.g., play a role in the clearance of infections) in term pregnancy where they display a less activated phenotype with the expression of inhibitory and tissue-residency receptors.

Most studies on decidual Treg have thus far focused on CD4+FOXP3+ T cells (16, 49, 50). Our present mass and flow cytometry data confirmed the presence of other, recently described, FOXP3$^{low/-}$ decidual Treg subtypes (51). Furthermore, we observed additional heterogeneity, with co-expression of inhibitory and stimulatory receptors and clusters lacking expression of FOXP3 and/or HELIOS, revealing a mixed population of Treg and Treg-like cells. It supports the hypothesis that both natural (nTreg) and induced Treg (iTreg) play a role, where bright expression of CD25 is not a prerequisite for Treg function. A decrease in FOXP3 and HELIOS expression toward term suggests a decline of nTreg and increase of iTreg throughout gestation (51). These Treg populations are induced, among others, by EVT and decidual Mφ (51), and may therefore have distinct cellular targets, which likely include the formerly unexplored heterogeneous group of memory CD4+ T cells. Evidence exists that 1st trimester decidual CD4+ T cells have transcriptional profiles compatible with antigen-induced activation and proliferation (52). Moreover, decidual CD4+ T cells isolated from term decidua showed fetal antigen-specific responses that were enhanced upon depletion of CD25+CD127- Treg (12). Presence of paternal antigen-specific Treg in the decidua has been suggested (29), and clonal expansion of both decidual Treg (53) and memory CD4+ T cells by locally presented antigens is suggested by preliminary data from our laboratory showing a restricted CDR3 length distribution of the TCRVβ repertoire in term decidual CD4+ T cells compared to peripheral CD4+ T cells (data not shown). The observed increase in activated CD4+ T cells may be counteracted by an increase in Treg in term parietalis to secure success of pregnancy. Evidently, functional assays are necessary to further explore the co-existence of effector memory CD4+ T cells with nTreg and iTreg, especially in the context of complicated pregnancies (54). Treg may also be essential in the regulation of distinct CD4+ and CD8+ NKT-like clusters in early pregnancy, as suggested by the increased percentages of NKT cells observed in women with unexplained recurrent spontaneous abortions (55).

Recent research on fetal-specificity (12), virus-specificity (56), and cross-reactivity of decidual CD8+ T cells with HLA-C (57), complemented by the herein described co-expression of inhibitory and stimulatory receptors, emphasizes the dual role of CD8+ T cells in both tolerance and immunity. Co-expression of CD45RO/RA in several clusters hints at local proliferative potential, and interactions with APC and Treg may be essential to control CD8+ T cells at the maternal-fetal interface. Furthermore, recently addressed contributions of TCRγδ T cells to transplantation outcomes and their role in HIV controllers (58, 59) advocate for an unexplored functional role of TCRγδ T cell subsets in early and term pregnancy, which requires further exploration. B cell clusters expressing CD39, a marker involved in the activation of B cells to suppress T cells (60), might resemble regulatory B cells. Alterations in B cell function in early pregnancy has been suggested to play a role in recurrent miscarriages, where a higher incidence of anti-HLA-C antibodies was observed in women with recurrent miscarriage (61).

Diffusion mapping revealed putative differentiation trajectories of effector, memory, and regulatory T cells throughout gestation, emphasizing the dynamic state and conceivable plasticity of decidual T cells in response to environmental cues. It should be kept in mind, however, that the cell phenotype trajectories may partly be influenced by recruitment of immune cells into the tissue as gestation progresses. In both the CD4+ and CD8+ compartment an increase in activated effector T cell phenotypes toward the end of pregnancy suggests an inflammatory state required for parturition. Subsequently, combining all identified immune cell clusters in a correlation network analysis demonstrated that the local immune landscape as a whole, and not isolated cell subsets, develops as an integrated system throughout gestation. Co-expression of inhibitory and stimulatory receptors in this system is prominent and needs to be finely balanced to ensure a successful pregnancy. The prominent connection between myeloid cells and T cells (network 1) at any time point during pregnancy reflects their bi-directional interactions both in a contact-dependent manner and through cytokine excretion. The connection between NKT-like cells and NK cells specifically in the first trimester (network 2) needs further exploration. Differences in immune cell networks and their prospective functions observed between term basalis (network 3) and parietalis (network 4) suggest possible distinct antigen availability and presentation at these two placental locations. More regulatory phenotypes were observed in the parietalis with increased percentages of Treg, M2 Mφ, and TRM CD8+ T cells. This observation may be in line with findings of a single cell analysis of separate placental compartments (62), showing that the basal plate (including the basalis) contains more activated T cells and less resting T cells compared to the chorioamniotic membranes (including the parietalis). The abundant density of lymphatic vessels in the region adjacent to the chorionic membrane, which is attached to the parietalis, suggests that antigen presentation and activation need to be carefully controlled at this site (63). Term basalis consistently showed cell clusters with higher expression of CCR6, a receptor involved in chemotaxis. The influx of immune cells might therefore be more common in term basalis.

This study has its limitations. First, in human pregnancy studies the unavailability of uncomplicated decidual samples between 24 and 37 weeks of gestation results in a gap in

our knowledge and understanding of the complete second trimester. Second, mass cytometry identifies phenotypic diversity based on preselected markers and provides little insight into the functionality of identified cell clusters. Here, we investigated the T cells in depth, but additional myeloid and B cell-specific markers are necessary to explore the complexity within these lineages. Although the rationale for defining a cluster is the presence of at least 100 cells with the same phenotype, further research needs to be performed to confirm if the identified subclusters represent true, functionally distinct, subpopulations. It is plausible that some of the phenotypically distinct cell clusters are differentiation stages between cell populations, as suggested by our diffusion mapping data. Therefore, the results from the current study should be considered as a basis for subsequent investigations. Future studies constituting a validation cohort with additional healthy decidual samples and including samples from complicated pregnancies will provide comprehensive insight into generalizable differences between healthy and complicated pregnancy. Although decidual and peripheral blood immune cells clustered completely separate in t-SNE analyses, trafficking of cells between these two entities almost certainly occurs (64, 65). In pregnancy complications both systems should be studied in parallel as the occurrence of certain cell subsets in the blood, possibly precursors, may predict what takes place locally in the decidua and thereby serve as biomarkers to predict complications.

In the field of reproductive immunology, a shift toward systems biology with a focus on interactions between cell types and away from studying isolated cell populations is required. This ecosystem where not only maternal immune cells but also EVT, decidual stromal cells, endothelial cells, and micro-organisms are coordinated with each other needs to be explored in more depth, and in relation to pregnancy complications presenting a more heterogeneous microenvironment than expected. In this context, single-cell RNA sequencing has revealed potential cell-cell interactions at the maternal-fetal interface (37). Future studies will benefit from combining mass cytometry data and RNA sequencing to cross-validate transcriptional activity and protein levels of singular cells, and from incorporating imaging CyTOF to define the cellular anatomical locations. Furthermore, the generation of trophoblast organoids as a model for maternal-fetal interactions (66), development of a placenta-on-a-chip (67), and interconnectivity analysis of multiple biological systems such as metabolomics and transcriptomics (37, 68) will further enhance our understanding of the placenta and the cellular interactions within this ecosystem.

Taken together, mass cytometry enabled us to visualize the complex and dynamic network of decidual immune cell populations at the maternal-fetal interface, where during uncomplicated pregnancy coordinated interaction is vital for a successful outcome. The immune atlas as presented here may serve as a foundation for further identification and functional analyses of immune subsets in healthy versus complicated pregnancies.

DATA AVAILABILITY STATEMENT

Mass cytometry data are available via Flow Repository (https://flowrepository.org/id/FR-FCM-Z3YF).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Medical Ethics Committee of the Leiden University Medical Center. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AZ, VU, FC, and SH designed the research and with help of FK wrote the manuscript. AZ performed the experiments with help of SL, CK, and HK. AZ performed the data analyses with help of VU, GB, SL, and TH. Conceptual input was provided by VU, GB, ME, and FK. Clinical samples were provided by M-LH and SC. All authors contributed to finalizing the manuscript. All authors contributed to the article 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.2020.571300/full#supplementary-material

FIGURE S1 | Identification of major immune cell lineages at the maternal-fetal interface using the T cell panel. **(A)** Gating strategy to select single, live CD45+ cells for downstream analysis. **(B)** Comparison of the absolute numbers and percentages of CD45+ cells measured by the general and the T cell panel. **(C)** Correlation plots of CD45+ cells measured by the general and T cell panel within the three trimesters, maternal peripheral blood mononuclear cells (mPBMC) and non-pregnant control samples (NP PBMC). **(D)** First-level HSNE visualization of

the major immune cell lineages derived from decidua and peripheral blood. Colors top left indicate tissue type (1st trimester $n = 11$; 2nd trimester $n = 5$; term basalis and parietalis $n = 8$; mPBMC $n = 8$; NP PBMC $n = 4$); colors bottom left indicate major immune cell types (CD8M, CD8 memory T cells; CD8N, CD8 naïve T cells; CD4M, CD4 memory T cells; CD4N, CD4 naïve T cells); colors for plots on the right indicate the arcSinh5-transformed expression values of the specified markers where every dot represents a landmark. Memory and naïve clusters were distinguished based on CD45RO and CD45RA expression. **(E)** t-SNE visualization of the separation between decidual and peripheral blood samples (as percentage of CD45+ cells); every dot represents a single sample. **(F)** Major immune cell lineages (as percentage of CD45+ cells) throughout gestation and within mPBMC and NP PBMC. Boxplots depict the 10–90 percentile and the Kruskal-Wallis with Dunn's test for multiple comparisons was applied. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

FIGURE S2 | t-SNE visualization of PBMC reference samples and partitioning of the myeloid cell compartment into subpopulations. Cell frequencies (as percentage of CD45+ cells) are plotted where every dot represents a single sample within the general panel **(A)** and within the T cell panel **(B)**. The gray arrow indicates the PBMC reference control samples clustering together. **(C)** HSNE overview (first) level embedding of all decidual samples with identification of the major immune cell lineages based on lineage marker expression. **(D)** Second-level HSNE embedding of the myeloid cells subdivided into six major subpopulations. **(E)** Second-level HSNE arcSinh5-transformed expression values of the specified markers where every dot represents a landmark.

FIGURE S3 | Analysis of staining fluctuations between batches for the general CyTOF antibody panel. Nine replicate control samples from the same blood donor stained with the general CyTOF panel measured throughout the 7-month study period. (A) A t-SNE embedding showing the collective CD45+ cells (14.5×10^4 cells) from nine replicate control samples and 20 experimental decidual samples. Colored dots represent single cells from replicate samples and gray represents experimental samples. (B) Same t-SNE embedding as in panel A, colored for each replicate sample. (C) A t-SNE plot showing 25 cluster partitions in different colors. (D) Composition of the cell clusters in the individual samples ($n = 29$) represented in horizontal bars where the size of the colored segments represents the proportion of cells as a percentage of total CD45. (E) Heat map showing the median ArcSinh5-transformed marker expression values of the clusters identified in C and hierarchical clustering thereof. (F) Graph depicting the standard deviation in cell cluster frequencies between the technical replicate control samples (black circles) and the experimental decidual samples (red triangles). Noticeable is differential abundance of cluster 21 and 22 within CD4+ T cells, due to minor fluctuations in the expression of CD127, CD27, and CCR7.

FIGURE S4 | Analysis of staining fluctuations between batches for the T cell CyTOF antibody panel. Ten replicate control samples from the same blood donor stained with the T cell CyTOF panel measured throughout the 7-month study period. **(A)** A t-SNE embedding showing the collective CD45+ cells (11.5×10^4 cells) from 10 replicate control samples and 13 experimental decidual samples. Colored dots represent single cells from replicate samples and gray represents experimental samples. **(B)** Same t-SNE embedding as in panel A, colored for each replicate sample. **(C)** A t-SNE plot showing 20 cluster partitions in different colors. **(D)** Composition of the cell clusters in the individual samples ($n = 23$) represented in horizontal bars where the size of the colored segments represents the proportion of cells as a percentage of total CD45. **(E)** Heat map showing the median ArcSinh5-transformed marker expression values of the clusters identified in C and hierarchical clustering thereof. **(F)** Graph depicting the standard deviation in cell cluster frequencies between the technical replicate control samples (black circles) and the experimental decidual samples (red triangles). Noticeable is differential abundance of cluster 18 and 19 within CD4+ T cells, due to minor fluctuations in the expression of CD127, CD38, and CCR7.

FIGURE S5 | Characterization of the innate lymphoid compartment. **(A)** Heatmap showing the marker expression values for the 14 identified NK and ILC clusters. Cluster IDs and cluster frequencies are displayed at the bottom of the heatmap. **(B)** Violin plots depicting expression values of indicated markers (arcSinh5-transformed) in the four tissue groups. **(C)** Heatmap showing the marker expression values for ILC clusters (CD3-CD7+) within the T cell panel. Only clusters expressing the co-inhibitory receptors CD39, TIM-3, TIGIT are depicted here. **(D)** Boxplots of sample frequencies, divided per trimester, of the clusters plotted as a fraction of total ILC. The Kruskal-Wallis with Dunn's test for multiple comparisons was performed. **(E)** Cluster frequencies (as percentage of total ILC) of the depicted clusters. **(F)** PCA of the sample frequencies (as percentage of total ILC) where the gestational age groups are depicted along the first two components. The centroid of each group is indicated in gray. $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.005$.

FIGURE S6 | Characterization of TCRγδ T cells. **(A)** Heatmap showing the marker expression values for the seven identified TCRγδ cell clusters within the general panel (36 samples; 114,875 cells). Cluster IDs and cluster frequencies are displayed at the bottom of the heatmap. **(B)** First-level HSNE embedding of the expression values of the indicated markers. **(C)** Boxplots of sample frequencies, divided per trimester, of the clusters plotted as a fraction of total TCRγδ cells. The Kruskal-Wallis with Dunn's test for multiple comparisons was performed. **(D)** PCA of the sample frequencies (as percentage of total TCRγδ cells) where the gestational age groups are depicted along the first two components. The centroid of each group is indicated in gray. $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.005$.

FIGURE S7 | In-depth characterization of the CD4+ Treg-like compartment where CD25+CD127- and CD25+CD127+ CD4+ T cells increase throughout gestation. **(A)** CD4+ NKT-like cell cluster identified within the general panel. **(B)** In-depth analysis of the regulatory-like CD4+ T cell (Treg-like) compartment, where the heatmap shows the marker expression values for the additional identified CD4+ Treg-like cell clusters within the T cell panel. Cluster IDs and cluster frequencies are displayed at the bottom of the heatmap. **(C)** Visualization of the five CD4+ Treg-like clusters, shown in **Figure 3A**, in a diffusion map along two components. Each color in the left panel represents a cluster of cells. In the right top panel, cells within the 1st trimester, 2nd trimester, and term decidua basalis and parietalis are portrayed. The bottom panel shows expression values of the specified markers in the diffusion map. **(D)** t-SNE embedding of the arcSinh-transformed expression values of CD127 and CD25 as observed in the three trimesters, measured by flow cytometry and gated within CD3+CD4+ T cells. CD4+CD25+CD127- clusters are circled in orange; CD4+CD25+CD127+ clusters are circled in blue.

FIGURE S8 | Characterization of the CD8+ T cell compartment, including CD8+ NKT-like cells. **(A)** Heatmap showing the marker expression values of CD8+ NKT-like cell clusters identified within the general panel. **(B)** Fourth-level HSNE arcSinh5-transformed expression values of the specified markers, measured by flow cytometry and gated within CD3+CD8+ T cells. Colors in the left plot indicate tissue type (1st $n = 3$; 2nd $n = 4$; term basalis and parietalis $n = 4$). **(C)** Pie charts depicting the contribution of major subpopulations to the CD4+ and CD8+ T cell compartments.

FIGURE S9 | Characterization of B cells. **(A)** Heatmap showing the marker expression values for the nine identified B cell clusters within the general panel (36 samples; 72,414 cells). **(B)** Percentage of CD20+ B cells in each trimester. **(C)** ArcSinh5-transformed expression values of the specified markers. $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.005$.

TABLE S1 | General CyTOF antibody panel[1].

TABLE S2 | T cell-specific CyTOF antibody panel[1].

TABLE S3 | Flow cytometry antibody panel[1].

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REVIEW
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Molecules and Prostaglandins Related to Embryo Tolerance

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It is generally understood that the entry of semen into the female reproductive tract provokes molecular and cellular changes facilitating conception and pregnancy. We show a broader picture of the participation of prostaglandins in the fertilization, implantation and maintenance of the embryo. A large number of cells and molecules are related to signaling networks, which regulate tolerance to implantation and maintenance of the embryo and fetus. In this work, many of those cells and molecules are analyzed. We focus on platelets, polymorphonuclear leukocytes, and group 2 innate lymphoid cells involved in embryo tolerance in order to have a wider view of how prostaglandins participate. The combination of platelets and neutrophil extracellular traps (Nets), uterine innate lymphoid cells (uILC), Treg cells, NK cells, and sex hormones have an important function in immunological tolerance. In both animals and humans, the functions of these cells can be regulated by prostaglandins and soluble factors in seminal plasma to achieve an immunological balance, which maintains fetal-maternal tolerance. Prostaglandins, such as PGI2 and PGE2, play an important role in the suppression of the previously mentioned cells. PGI2 inhibits platelet aggregation, in addition to IL-5 and IL-13 expression in ILC2, and PGE2 inhibits some neutrophil functions, such as chemotaxis and migration processes, leukotriene B4 (LTB4) biosynthesis, ROS production, and the formation of extracellular traps, which could help prevent trophoblast injury and fetal loss. The implications are related to fertility in female when seminal fluid is deposited in the vagina or uterus.

Keywords: prostaglandins, PGE2, platelets, polymorphonuclear leukocyte, group 2 innate lymphoid cells, embryo tolerance

INTRODUCTION

Prostaglandins (PGs) belong to a subclass of eicosanoids known as prostanoids, these are comprised of C20 atoms, including a cyclopentane ring. PGs are hormone-like chemical messengers which act as autacoids (1) through prostanoid receptors (G protein-coupled receptors) and their variants or isoforms such as $E_{1\text{-}4}$, $DP_{1\text{-}2}$, FP, TP, and IP (1, 2). The main precursor of eicosanoids is arachidonic acid (AA), this is released by the action of phospholipases A2 (PLA2) and C (PLC) (3), AA is then converted into different metabolites through the COX, LOX, and CYP450 pathways (4). The importance of prostaglandins becomes evident when ovulation and fertilization are affected, e.g., as cyclooxygenase (COX) is inhibited by aspirin or indomethacin (5).

PGs have a significant role in maternal immune tolerance and the conception process. We consider prostaglandins in seminal fluid as key in modulating responses in different types of cells participating in fetal-maternal tolerance.

The balance of the immune response in maintaining fetal-maternal tolerance is due to a complex network of soluble molecules and cells, such as macrophages, and dendric, decidual, and NK cells. In **Table 1**, cells and biological processes are summarized. Moreover, many molecules are released by these cells and have a fundamental role in the tolerance process. **Table 2** summarizes the most important of these.

The molecules are released through macro-, micro-, and nanovesicles, including exosomes from placenta cells, syncytiotrophoblasts, denudated syncytiotrophoblasts, and extravillous trophoblasts. All are part of the complex intercommunication between the foetus and the mother. These vesicles transport immunomodulatory proteins such as Fas ligand, TRAIL, CD274, CD276, HLA-G5, Syncytin-1, hCG, glycodelin, galectin-1 (107), which may maintain fetal-maternal tolerance, and may even be related to recurrent early miscarriage (108).

The accumulated evidence indicates that when sexual intercourse occurs and seminal fluid is deposited in the female reproductive tract, the prostaglandins in the seminal fluid, i.e., PGE2, PGE1, PGE3, and PGF2 (109), initiate a signaling cascade toward the woman's innate immune cells. The cells mentioned in **Table 1**, such as platelets, polymorphonuclear leukocytes, and Group 2 innate lymphoid cells participate in the physiological mechanisms in embryo tolerance and implantation, allowing successful fertilization.

PREIMPLANTATION, IMPLANTATION, AND DECIDUALIZATION

Implantation begins by apposition and adhesion of the embryo to the luminal epithelium of the endometrium. Following its invasion toward the stromal bed, the union of the embryo to the luminal epithelium transforms the underlying stromal fibroblasts into secretory cells of the epithelioid type, or decidualization (110). Through different molecules such as IL-1β, steroid hormones, insulin-like growth-factor-binding protein-1 (IGFBP-1) and prostaglandin-endoperoxide synthase-2 (PTGS-2), the decidualized cells regulate this stage with the invasion of embryos, and the formation of the placenta (110).

Prostaglandins participate in each stage of the interaction of the embryo with the endometrium, for example in preimplantation, implantation (apposition, adhesion/attachment, invasion/penetration) and decidualization; as well as affecting many other cells and molecules. PGs have a complex role in each of these stages, e.g., the essential role of prostaglandin E2 (PGE2) in the oocyte is to enhance the cumulus expansion in ovulation for sperm penetration, to regulate extracellular matrices disassembly (111), and also, importantly, to participate during transport and embryo implantation (112).

PROSTAGLANDIN SIGNALING BY SEMINAL FLUID AND FERTILIZATION

Preceding evidence shows that sperm induces immunosuppression against hapten-modified self and alloantigens, including cytotoxic T-cell in mice responses (113). Also, seminal plasma contains high concentrations of prostaglandins, key molecules in the regulation of sexual intercourse signaling (114). The female immune response tolerates seminal plasma and supplies cytokines and prostaglandins, which are synthesized in the male accessory glands. In addition, it causes molecular and cellular changes in the endometrium. This facilitates the development and implantation of the embryo when prostaglandins, cytokines and hormones bind to receptors in target cells in the cervix and uterus (115).

The prostaglandins present in seminal fluid have a role in immune modulation. They regulate the pathways that may exacerbate inflammation in the female reproductive tract during physiological processes such as ovulation, implantation, and parturition (116), e.g., ejaculation or the spermatozoa induce an inflammatory response in the endometrium in the preimplantation period after mating, in which IL-1 (alpha and beta), and TNF-alpha participate (117).

Seminal plasma derived from the male accessory sex glands performs a fundamental function in fertilization in animals. The components of seminal plasma participate in the transport and survival of viable sperm and the elimination of non-viable sperm from the uterus (118). In the quail species, the cloacal gland produces prostaglandin F2α (PGF2α), which contributes to successful fertilization and acts as a natural mechanism for the protection of sperm from rejection or death by the female reproductive tract (119). Seminal fluid factors exert significant effects on the female reproductive tract, as shown by Shahnazi et al. (120). Also, in the uterine tissues of mice that were paired with mice without seminal vesicles, implantation rates, enzyme cytosolic PGE synthase (cPGES), microsomal PGE synthase (mPGES) and receptors EP2 and EP4 involved in the signaling pathway of PGE2, were all significantly low (120). In addition, 19-hydroxy PGE and 19-hydroxy PGF are regulators of sperm motility, and its effects may be mediated by the content of ATP in sperm (121). Prostaglandins such as PGE-1 are potent stimulators of adenylate cyclase in various

TABLE 1 | Cells related to maternal-fetal tolerance and implantation.

	Cells	Biological process	Molecules related	Prostaglandins related	Authors
Dendritic cells	ILT4$^+$ Dendritic cells (DCs)	Induction of Foxp3$^+$ Treg cells. DCs suppress T-cell activity, induce T helper cell anergy and inhibit the differentiation of cytotoxic T cells.	IL-10		Liu et al. (6)
	Tolerogenic dendritic cells (tol-DCs)	Present the antigen to Th0 cells, which become activated, proliferate and differentiate into peripherally derived Tregs (pTregs).		PGE2–EP4 receptor signaling inhibits IL-12 and promotes IL-23 production. PGE2 regulates IL-10 production.	Flórez-Grau et al. (7) Robertson et al. (8)
Macrophages	M1 macrophages	Skew T cell responses to a TH1 mediated immune response.	IL-12, IL-23, ROS	PGE2 is essential to corpus luteum formation by stimulating macrophages to induce angiogenesis through EP2/EP4.	Brown et al. (9) Liu et al. (10)
	M2 macrophages	Promote TH2 or antibody mediated immune responses.	IL-10, TGF-β	PGD2, PGF2α, and PGE2 contribute to differentiation toward M2-like macrophages	Brown et al. (9) Magatti et al. (11)
NK cells	Uterine NK cells (uNK)	Respond to fetal MHC class I molecules. Stimulate fetal growth. Regulate decidual blood vessel remodeling.	IFN-γ, growth-promoting factors		Sojka et al. (12) Fu et al. (13)
	Endometrial NK cells (eNK)	Inactive cells (before IL-15 activation) that are present in the endometrium before conception and pregnancy.	IP-10 or IFN-γ		Yang et al. (14) Manaster et al. (15)
	Decidual NK cells (dNK) (CD56^{bright}CD16$^-$)	Widen maternal blood vessels and promote fetal growth. Interact with resident myeloid cells and participate in the induction of regulatory T cells	IL-24, Angiopoietin 1 and 2 (Ang 1, Ang 2), vascular endothelial growth factor C (VEGF-C), TGF-β1, SDF-1, pleiotrophin, osteoglycin, IL-8, protein-10.	Suppression of their activity has been observed in humans and mice by PGE2.	Yang et al. (14) Yu et al. (16)
Decidual cells	Decidual stromal cells (DSCs)	Differentiation and development of dNK during decidualization. Induce the downregulation of activating NK receptors and inhibit NK cell proliferation, cytotoxicity, and IFN-γ production.	IL-24, TGF-β	The DSC-induced inhibition is primarily mediate by PGE2.	Yang et al. (14) Sojka et al. (12) Vacca et al. (17) Croxatto et al. (18)
	Decidual ILC3 (NCR$^+$NCR$^-$)	Establish physical and functional interactions with neutrophils and produce factors for pregnancy induction/maintenance and promotion of the early inflammatory phase.	IL-8, IL-22, GM-CSF, TNF, IL-17		Vacca et al. (17)
	Decidual Tregs	Express CD25, CTLA4, and PD-L1, which are hallmark mediators of Treg suppression. Downregulate DC costimulatory molecules CD80 and CD86 needed for T effector (Teff) activation.	IL-10, TGF-β		Robertson et al. (8)
	Decidual T cells	Proliferate in response to fetal tissue. Elevated expression of proteins associated with the response to interferon signaling.	IL-4, IL-10, IFN-γ, leukaemia inhibitory factor and colony-stimulating factor 1 (M-CSF).		Ernerudh et al. (19) Powell et al. (20)
	Decidual myeloid cells (dCD14$^+$)	Induce Treg, dNK and dCD14$^+$ cells resulting in the production of IFN-γ.	TGF-β, indoleamine 2,3-dioxygenase (IDO).		Vacca et al. (17)
	Decidual CD4$^+$EM cells	Increase expression of the immune inhibitory checkpoint receptors PD-1, Tim-3, cytotoxic T lymphocyte antigen 4 (CTLA-4), and lymphocyte activation gene 3 (LAG-3).	IFN-γ, IL-4		Kieffer et al. (21)
	Decidual CD8$^+$EM cells (CD45RA$^-$CCR7$^-$)	The interaction with trophoblasts induces the upregulation of Tim-3 and PD-1. Trophoblasts may induce tolerance in CD8$^+$ EM cells in the decidua. Reduced expression of perforin and granzyme B.	IFN-γ, IL-4	PGE2 is an important modulator of CD8 membrane expression in human lymphocytes.	Kieffer et al. (21) Tilburgs et al. (22) Ouellette et al. (23)
T Cells	Tregs (CD4$^+$CD25$^+$FOXP3$^+$)	Inhibit the activation and function of Th1 and Th17 cells and control inflammation. Control IL-15 release from DCs and suppress uNK cytolytic activity.	TGF-β, IL-10, Heme oxygenases-1(HO-1)	PGE2 promotes the development of regulatory T cells.	Robertson et al. (8) Erkers et al. (24)

(Continued)

TABLE 1 | Continued

	Cells	Biological process	Molecules related	Prostaglandins related	Authors
	T helper 3 cells (Th3 cells)	Induce the mucosal environment that is intrinsically rich in TGF-b, IL-10, and IL-4. Vasoactive intestinal peptide (VIP) modulates to toward a tolerogenic profile.	VIP, TGF-β		Ernerudh et al. (19) Grasso et al. (25)
	Th17 cells	Express higher levels of T cell immunoglobulin- and mucin-domain-containing molecule-3 (Tim-3) and programme death-1 (PD-1) inducing inflammation. Regulating trophoblast function.	IL-10		Ahmadi et al. (26) Wang et al. (27)
Endometrial and umbilical cells	Endometrial stromal cells (ESCs)	In humans, the so-called "decidualization window" transforms endometrial stromal cells into larger round decidual cells. This phenomenon is largely dependent on hemodynamic forces, progesterone, and prostacyclin.	IL-15	PGE2 can stimulate IL-15 expression and release by ESCs	Dunn et al. (28) De Clercq et al. (29) Gnecco et al. (30)
	Human Umbilical Vein Endothelial Cells (HUVECs)	Possess immune-regulating properties and are one of the first fetal cells to make contact with foreign maternal immune cells. Also, increase the Treg cell population.	TGF-β		Oettel et al. (31)

cellular systems (122). An increase in adenylate cyclase activity and subsequent entry into cAMP levels may also be involved. PGs stimulate the fertilization capacity of human sperm by facilitating the transport of calcium through their plasma membrane (123).

The amplification of effects by microparticles from epididymal fluid (epididymosomes) and prostasomes could lead to the activation of many genes and the expression of related molecules, as reported in humans and mice, some species of cows, pigs and sheep (123, 124). More specifically, signaling may affect the enzymes of the cyclooxygenase pathway and other molecules related to the metabolism of arachidonic acid, e.g., Cytochrome P450 in blastocyst implantation (125), and prostaglandin D2 in the maintenance of pregnancy through Th1/Th2 and T-cytotoxic (Tc) 2 cells balance (126, 127).

The change induced by seminal plasma in a porcine uterus makes conception and pregnancy possible (128), it also reduces embryonic mortality in pigs and other livestock (129). In addition, seminal plasma possesses potent immunosuppressive activity caused by immune-deviating soluble factors, inducing tolerance, with molecules, such as Transforming growth factor-β (TGFB) and prostaglandin E (PGE).

EFFECTS OF PROSTAGLANDINS AND RELATED MOLECULES ON INNATE IMMUNITY AND FEMALE REPRODUCTIVE TRACT CELLS

Cells of the innate immune response are modulated by prostaglandins (130), among them, are the following:

1. M1 macrophages (Mø1) which produce proinflammatory cytokines (TNFα, IL-6, IL-12, IL-23, and IL-1β), M2 macrophages(Mø2) which produce IL-10 and TGFβ (transforming growth factor β) and have anti-inflammatory and immune down-regulating properties. Both are regulated by prostaglandins in pregnancy (9) (**Table 1**).
2. Dendritic cells (DCs) have several subclasses, e.g., $CD103^+$, myeloid, plasmacytoid, the latter are related to the production of high IFNα levels. In infertile patients with endometriosis, $CD4^+$, $CD25^+$, and $CD103^+$ dendritic cells are increased in peritoneal fluid (131), dendritic cells $CD103^+$ have a relevant role in implantation (132); in addition, $CD103^+$ dendritic cells are regulated by prostaglandin D2 in different disorders (133).
3. Endothelial cells have innate and immune tolerogenic function (134). In patients with preeclampsia (PE), in the presence of vascular endothelial growth factor (VEGF), these cells increase levels of prostacyclin (135). In the pathogenesis of PE, VEGF (VEGF-A) participates in the proliferation, migration and angiogenesis of endothelial cells, and works through the receptors VEGFR-1 (or Flt-1) and VEGFR-2. In PE this increases the release of FMS-like tyrosine kinase-1 (sFlt-1) and blocks free VEGF to protect the fetus from toxicity (136).
4. Neutrophils (PMN) are regulated by cytokines and prostaglandins (137). The aspirin (ASA) is used for prevention of preeclampsia in high-risk patients (138, 139). ASA triggers

TABLE 2 | Principal soluble molecules acting in implantation (apposition/adhesion/invasion) to maintain fetal-maternal tolerance.

Effects	Soluble molecule	Biological process	Steroid hormones and related molecules	Author
Attachment and implantation	Oestrogen	Regulation of oestrogen receptors β/IL-24 (ERβ/IL-24) signal pathways. Induces the recruitment of macrophages and DCs.	Promotes the conversion of peripheral Tregs in secondary lymphoid organs. Prolongs the survivals of H-Y skin grafts by the expansion of Tregs, suppression of CD3(+) CD8(+) effector T-cells and immune shifts toward Th2 cytokines.	Padmanabhan et al. (32) Vrtačnik et al. (33) Lin et al. (34)
	17β-oestradiol (E2)	Promotes uterine blood flow, myometrial growth stimulates breast growth and later promotes cervical softening and expression of myometrial receptors. Expansion and activation of monocytic-myeloid-derived suppressor cells (M-MDSCs) through signal transducer and activator of transcription (STAT)-3.	E2-treated MDSCs have a stronger capability in suppressing T cell responses. 17β-oestradiol, FSH, oxytocin, and arachidonic acid (AA) induce receptors and enzymes through the synthetic pathway for PGE2.	Rahimipour et al. (35) Pan et al. (36) Falchi and Scaramuzzi, (37)
	Progesterone (P4)	Stimulates the activity of some specific enzyme matrix metalloproteinases and adhesion molecules. Inhibits antibody production and suppresses T-cell activation and cytotoxicity and modifies the activity of natural killer cells; influences B cells and induces secretion of protective asymmetric antibodies.	Progesterone-induced blocking factor (PIBF) mediates the immunomodulatory effects of progesterone. Consumption of IL-4 increases and the number of cells undergoing apoptosis. Increases secretion of IL-10, IL-27, causes increased secretion of IL-13 and decreased secretion of IL-23 by the monocyte-derived dendritic cells. Upregulates macrophage-colony-stimulating factor (M-CSF) and downregulates granulocyte-macrophage colony-stimulating factor (GM-CSF). Progesterone and prostaglandin E have synergistic inhibition effects on T-cell mitogenesis.	Rahimipour et al. (35) Kyurkchiev et al. (38) Svensson et al. (39) Fujisaki et al. (40)
	Chorionic gonadotropin (CG)	hCG is comprised of 4 molecules, one produced by villous syncytiotrophoblastic cells, another hyperglycosylated hCG produced by cytotrophoblast cells, the free beta subunit, and hCG produced by anterior pituitary gonadotropic cells. Stimulates P4 production by the corpus luteum, facilitating trophoblast invasion, and promoting angiogenesis.	It is a pleiotropic molecule that mediates implantation. Upregulation of indoleamine 2,3-dioxygenase activity of dendritic cells. hCG may have a biological role in the regulation of PG (PGE and 6-keto-PGF1) synthesis in trophoblasts. In particular, the hyperglycosylated form stimulates implantation through the invasion of cytotrophoblast cells.	Cole, 2020. (41). Szmidt et al. (42) Bansal et al. (43) Schumacher et al. (44) North et al. (45)
	Neuropeptide kisspeptin (KP)	Kisspeptins participate in reproduction. Regulates trophoblast cell invasion alongside tumor necrosis factor α.	KP is a regulator of Gonadotropin (GnRH) secretion and stimulates LH secretion and LH pulse frequency. KP-10 moderates trophoblast invasion and regulating implantation.	Mumtaz et al. (46) Francis et al. (47) Skorupskaite et al. (48) Pinilla et al. (49)
	Platelet-Activating Factor (PAF)	Platelet-activating factor is an acetylated Glycerophospholipid, releasing histamine from platelets, which increase vascular permeability.	PAF is related to processes of ovulation, implantation and parturition, and is regulated by ovarian steroid hormones. PAF is associated with sperm motility, acrosome reaction, and fertilization.	Harper, 1989. (50) Tieman, 2008. (51) Roudebush, 2001. (52)
Cytokine mediators of implantation and decidualization	IL-6	IL-6 is a cytokine with functions in immunity, metabolism and tissue regeneration. It is produced in the endometrial epithelium and stromal cells during implantation.	Variation in the expression of pro-inflammatory cytokines such as IL-6, CSF-1, macrophage colony-stimulating factor (CSF-1), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin 1-alpha, interleukin 1-beta, and tumor necrosis factor-alpha (TNF alpha) has been reported in the uterus immediately after mating in mice. Changes in the bioavailability of IL-6 are important for pregnancy. The increase of IL6 is related to unexplained infertility, recurrent miscarriage, preeclampsia and preterm delivery and inhibition of the generation of CD4 + regulatory T cells in pregnancy tolerance. Local IL-6 insufficiency could also contribute to recurrent spontaneous abortion. IL6 activate cathepsin S (CTSS) in dendritic cells, in decidualized endometrial stromal cells, this process is regulated by cystatins CST7 and CST3.	De et al. (53) Ochoa-Bernal et al. (54) Cork et al. (55) Prins et al. (56) Baston-Buest et al. (57)

(Continued)

TABLE 2 | Continued

Effects	Soluble molecule	Biological process	Steroid hormones and related molecules	Author
	Leukaemia inhibitory factor (LIF)	It is a member of the interleukin-6 family of cytokines. Upregulation of poFUT1, promotes trophoblast cell migration, invasion and differentiation at the fetal-maternal interface through activating the Janus kinase/signal transducers and fetal transcription (JAK/STAT) and a *mitogen-activated protein kinase* (MAPK) signaling pathway.	Urokinase-type plasminogen activator receptor (uPAR) is upregulated by LIF, also it is mediated by phosphoinositide-3-kinase–protein kinase B/Akt (PI3K/AKT) signaling pathway. LIF participates in placentation by up-regulating PGE2 production and PGE2 receptor expression.	Szmidt et al. (42) Liu et al. (58) Zheng et al. (59) Horita et al. (60)
	IL-1	Acts on blastocysts, syncytiotrophoblasts and endometrial glands.	Stimulates endometrial secretion of endometrial leukaemia inhibitory factor (LIF), prostaglandin E2, and integrin β3 subunit expression.	Viganò et al. (61) Hambartsoumian, 1998. (62) Fouladi-Nashta et al. (63)
	IL-11	IL-11 regulates endometrial epithelial cell increasing adhesion to fibronectin and collagen IV, similar to IL-6.	IL-11 decreases TNFα in a dose-dependent way in epithelial and stromal cells, in endometria, through gp130. IL-11 production is maximal during decidualization, its production depends on steroid hormones, relaxin and PGE2.	Cork et al. (55) Marwood et al. (64) von Rango et al. (65)
	IL-15	Promotes the differentiation of the local eNK cells toward dNK cells.	Decidual NK cells secrete cytokines and angiogenic factors to placental vascular remodeling and differentiation. IFN-γ, IP-10, vascular endothelial growth factor (VEGF), Placenta growth factor (PlGF). Suppression of IL-15-activated NK cell is mediated by PGE (2).	Manaster et al. (15) Kopcow and Karumanchi, 2007. (66) Joshi et al. (67)
	IL-24	Regulates the function of eNK and pNK through the Janus kinase (JAK)/STAT3 pathway.	Contributes in differentiation to $CD56^{bright}CD16^{-}$dNK with low cytotoxic activity, high immunomodulation and angiogenic activity by inhibiting CD16, Granzyme B and perforin, IFN-γ, upregulating KIR2DL1, KIR3DL, TGF-β, IL-10, and IL-8.	Yang et al. (14)
	Cytokine-like protein 1 (Cytl1)	Regulation of embryo implantation. It is an ovarian hormone-dependent protein expressed in the endometrium that stimulates the secretion of LIF and heparin-binding epidermal growth factor (HB-EGF). Induces endometrial cell proliferation.	Releases LIF, HB-EGF, and IL-1, in decidualization.	Ai et al. (68) Wang et al. (69) Moghani-Ghoroghi et al. (70)
Implantation and decidualization	Cellular Adhesion Molecules (CAMs)	Adhesion molecules include integrins, cadherins, selectins, and the immunoglobulin superfamily.	Numerous integrins interact with the trophoblast, especially the αVβ3, with its ligand osteopontin. HOXA 10 and IL-1 regulated β3 subunit expression in the receptive endometrium. The absence of L-selectin and its Meca-79 ligand is associated with recurrent implantation failure (RIF), also, a significant reduction of HOXA-10 and E-cadherin in recurrent implantation failure (RIF) and recurrent miscarriage (RM). ICAM-1, VCAM-1, NCAM, CD44, and CD49d provide interaction between the embryo and maternal cells.	Achache and Revel, 2006. (71) Foulk et al. (72) Yang et al. (73) Lu et al. (74)
	Melatonin	Melatonin is an indoleamine acting as an antioxidant, free radical scavenger, and it promotes embryo development in different species	A positive feedback loop among p53, p38, and p21 inhibiting mucin 1 and activating LIF is realized by melatonin signaling, which improves adhesion proteins, present at the membrane level on endometrial cells and the blastocyst, in the pre-implantation stage. Melatonin is associated with the inhibition of prostaglandin synthesis.	Carlomagno et al. (75) Voiculescu et al. (76) Gimeno et al. (77)
	Calcitonin (CT)	It is a peptide hormone which regulates calcium homeostasis	Promotes endometrial receptivity and embryo implantation.	Xiong et al. (78) Xiong et al. (79)
	Platelet-derived growth factor (PDGF-BB)	Decidualized endometrial stromal cells migrate upon exposure to PDGF-BB.	Involvement of ERK1/2 and PI3K/Akt signaling in endometrial stromal cell chemotaxis. Both epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) participate in implantation in the first days of gestation.	Schwenke et al. (80) Jaber and Kan, 1998. (81)

(Continued)

TABLE 2 | Continued

Effects	Soluble molecule	Biological process	Steroid hormones and related molecules	Author
	Platelet-derived growth factor (PDGF-AA)	Secreted by the trophoblast cell line AC-1M88 and by first trimester villous explants. Trigger endometrial stromal cell chemotaxis.	Participates in attracting decidualized endometrial stromal cells to the implantation site. Modulates early post-implantation.	Schwenke et al. (80) Haimovici and Anderson, 1993. (82)
	Tissue inhibitor of MMP (TIMP)	Endogenous inhibitor of MMP activity in tissues.	Inhibits trophoblast invasion. Decidual cell production. TIMP-2 attenuates the proteolysis of IGFBP-1 by MMP-3.	Liu et al. (58) Coppock et al. (83)
	Heparin-binding epidermal growth factor (HB-EGF)	HB-EGF has a function in implantation, decidualization and placenta development. Promotes differentiation of trophoblast cells to the invasive phenotype. Stimulates the migration of decidualized endometrial stromal cells.	Endometrial stromal cells with HB-EGF increase the level of the tetraspanin CD82, a metastasis suppressor found in decidual cells at the implantation site. A decreased level of HB-EGF is related to pregnancy complications.	Schwenke et al. (80) González et al. (84) Ozbilgin et al. (85)
	Lipoxins	These are derived from arachidonic acid, an ω-6 fatty acid. They exert their anti-inflammatory effects through binding to high-affinity G protein-coupled lipoxin receptors.	Lipoxins, calcitonin, leukaemia inhibitory factor, and homeobox A10 are essential in implantation. Lipoxin A4 is regulated by human chorionic gonadotrophin (hCG) during early pregnancy and it has anti-inflammatory activity in human endometrium and decidua tissue.	Xiong et al. (79) Macdonald et al. (86)
	Complement components and their receptors (C1q, gC1q, α4β1 integrin)	It is produced at the fetal-maternal interface by macrophages, decidual endothelial cells and invading trophoblasts.	Synthesis of C1q by decidual endothelial cells is crucial for the replacement by endovascular trophoblasts. Surfactant proteins SP-A and SP-D play a role in implantation, trophoblast invasion and placental development.	Agostinis et al. (87) Madhukran et al. (88)
	Protein O-fructosyltransferase 1 (poFUT1)	Favors trophoblast cell migration and invasion at the fetal-maternal interface.	Increases Tissue inhibitors of metalloproteinases 1 and 2 (TIMP-1, TIMP-2) expression further inhibited MMP-2 activity. Activates MAPK and PI3K/Akt signaling pathways.	Liu et al. (58) Liu et al. (89)
	Matrix metalloproteinase (MMP-2) -2	Implicated in the remodeling of the extracellular matrix (ECM) during the trophoblast invasion process.	Synthesis and degradation of the extracellular matrix under physiological and pathological conditions. It is capable of degrading collagen. During the implantation process, matrix metalloproteinase (MMP)/insulin-like growth factor binding protein-1 (IGFBP-1) activity is stimulated by leukaemia inhibitory factor (LIF) and colony-stimulating factor (CSF).	Liu et al. (58) Ortega et al. (90) Herrler et al. (91)
	Gonadotropin-releasing hormone type II (GnRH-II) agonist	Promotes cell motility of human decidual endometrial stromal cells through the GnRH-IR by phosphorylation of ERK1/2 and JNK in decidual endometrial stromal cells.	Increased expression and proteolytic activity of matrix metalloproteinase-2 and -9 (MMP-2, MMP-9) is due to GnRH-II	Wu et al. (92)
Immune tolerance	Human leukocyte antigen G (HLA-G)	Promotes proliferation and cytokine production by uNK cells.	Secretion of growth-promoting factors essential for fetal development by uNK cells. Levels of sHLA-G ≥ 2 U/ml in embryos which were selected for transfer after IVF based on culture media gave a 65% pregnancy rate compared with low levels of sHLA-G. The HLA-G -725 promoter polymorphism has a high risk for recurrent miscarriage.	Sojka et al. (12) Roussev and Coulam, (93)
	Soluble MHC class I (sMHC-I)	sMHC-I induces apoptosis by stimulating expression of CD95-L and regulates the Fas/FasL system.	sHLAs downregulates T-cell responses.	Bakela and Athanassakis, (94) Zavazava and Krönke, 1996. (95)
	Soluble MHC class II (sMHC-II)	It has important immunoregulatory properties, stimulates proliferation of CD25– CD4+, CD25+ CD8+ and CD25+ CD4 + cell, as well as inhibits CD25– CD8+ cells.	sMHC-II decreases IL-2, increases IL-10, and inhibits phosphorylation of ZAP-70, particularly LAT proteins in the pathways of TCR signaling in CD4+ cells.	Bakela and Athanassakis, 2018. (95) Athanassakis and Vassiliadis, 2003. (96)

(Continued)

TABLE 2 | Continued

Effects	Soluble molecule	Biological process	Steroid hormones and related molecules	Author
	Pregnancy specific beta-1-glycoprotein 9 (PSG9)	Binds to the 250-residue latency-associated peptide (LAP) and activates the latent form of TGF-β1.	Induces the secretion of TGF-β1 from macrophages. Induces the differentiation of FoxP3+ regulatory T-cells from naive T-cells. PSG participates in immune tolerance in pregnancy by suppressing the CD16/56 expression by NK-cells and enhancing the CD16/56 expression by NKT-cells.	Jones et al. (97) Zamorina and Raev, 2015. (98)
	Alpha-fetoprotein (AFP)	It is released by trophoblasts during pregnancy. Acts as a fetal transport protein. Influences fetal-maternal immunologic relationships during the first trimester and helps to protect the foetus against attacks by the maternal immune system.	Suppresses the production of TNFα and IL-1β. Controls the production of HLA-G and the Ia antigen, it stimulates the growth of trophoblasts containing paternal H2 antigens. Inhibits macrophage expression of Ia antigens. AFP is capable of driving B cells into apoptosis to avoid maternal B cells in order to reach the foetus.	Schumacher et al. (44) Lafuste et al. (99) Lu et al. (100) Fettke et al. (101)
	Indoleamine-2,3-dioxygenase (IDO)	IDO is involved in tolerance	IDO activity promotes tolerance due to the conversion of mature dendritic cells (DCs) into tolerogenic antigen-presenting cells (APCs) that suppress effector T cells (Teff) and promote regulatory T cells (Tregs). Factors which are expressed by Human amniotic membrane-derived mesenchymal stem cells (hAM-MSCs) including hepatocyte growth factor (HGF), TGF-β, prostaglandin E2 (PGE2), and indoleamine 2,3 dioxygenase (IDO) have immunomodulatory effects.	Mellor et al. (102) Mellor et al. (103) Kang et al. (104)
	Preimplantation Factor (PIF)	It is a fifteen amino acid linear peptide secreted by embryos two-cell, four-cell and six-cell stages in mice, in humans and bovines, respectively	PIF promotes immunological tolerance due to increasing the expression of HLA-G, -C, -E, and -F slightly. It also potentiates the effect of the endogenous steroid and promotes the secretion of Th1/Th2 cytokines.	Hakam et al. (105) Zare et al. (106)

transcellular biosynthesis of eicosanoids by acetylation of PGHS-2. Eicosanoids correspond to 15R-epimers of lipoxins (ATL) and are potent inhibitors of leukotriene B4-mediated neutrophils (140). Considering that preeclampsia is associated with increased proinflammatory, antiangiogenic and PMN-endothelial cell adhesion, Gil-Villa et al. (141) shows that PMN adhesion in patients with preeclampsia is reduced by Aspirin-triggered lipoxin (ATL) when aspirin is used.

5. Natural killer and innate lymphoid cells (ILC). According to the cytokine profile and transcription factor, ILCs are divided into two groups, cytotoxic and "helper"-ILC (17). The cytotoxic ILC group is represented by Natural Killer (NK). The "helper"-ILC in humans has three subclasses, ILC1 with two subsets, producing IFNγ; ILC2 produces IL-5, IL-13, and IL-4; and ILC3 releases IL-17 and IL-22. The NK cells in a decidua (dNK) microenvironment are around 50% to 70% of the total of lymphoid cells in decidual tissue. They have $CD56^{bright}$ $CD16^{-}$ KIR^{+} $CD9^{+,}$ and activate the NK receptor phenotype, participate with cytokines, which mediate new vessel formation, aid in the renovation of existing tissues and placentation through the release of VEGF, stromal-derived factor-1 (SDF-1) and IFN-γ-inducing protein 10 (9). In stromal tissue, the decidual stromal cells (DSCs) participate in the induction of maternal tolerance, physically concur and have a regulatory mechanism in dNK, and $CD14^{+}$ myelomonocytic cells, and induce regulatory Treg. Also, DSCs inhibit dendritic cells through prostaglandin E2 (PGE2) and Indoleamine 2,3-dioxygenase (IDO), this inhibition favors the maintenance of the pregnancy (18).

In the normal eutopic endometrium, the Mø2 together with the Tregs predominate, providing an anti-inflammatory environment for the implantation of the embryo, while in endometriosis, they can cause infertility. The Mø1 provide a pro-inflammatory environment which affects embryo implantation, the dendritic cells (DC) do not increase in endometrial tissue, also the Treg is dysregulated. Therefore, DC does not eliminate the cellular debris which could migrate to the peritoneal cavity and grow in ectopic sites, developing as endometriosis. On the other hand, Treg and NK have abnormal behavior, the first favors a pro-inflammatory state and the second is less cytotoxic which impacts embryo implantation (142). COX2 and PGE2 are related to the pathogenesis of endometriosis. A high level of COX-2 due to various factors such as estrogens, hypoxia and environmental pollutants could suppress apoptosis and increase cell proliferation through PGE2 and its receptors EP2, and EP4 in endometriosis (143). In addition, experimental studies with intralesional injections of ASA, in rabbits with peritoneal endometriosis, eliminate endometriotic lesions (144).

PROSTAGLANDINS IN IMPLANTATION AND MAINTENANCE OF GESTATION

The generation of prostaglandins and expression of receptors in a mouse uterus has demonstrated their importance during implantation and decidualization (145). In mice, PGE2 levels increase from the 2-cell embryo stage to the blastocyst,

demonstrating the importance of PGE2 in early development (112). PGE2 also plays a significant role in peri-implantation in a mouse uterus through the expression of EP2 and EP4 receptors, which increase cAMP levels during the implantation and decidualization processes. EP4 induces the activation of VEGF (growth factor vascular endothelial), increasing vascular permeability of the endometrium (146), implantation and decidualization, together with PGF2 (132).

Inadequate production of prostaglandins in mice, and possibly in humans, may explain some cases of infertility (147). Low concentrations of PGE2, PGF and PGI2 cause failure in ovulation, fertilization, implantation, and decidualization (133). In mice, prostacyclin (PGI2) is the primary prostaglandin at the implantation site. It participates in implantation and decidualization through the peroxisome proliferator-activated receptor (PPAR-δ) and the RXRα signaling pathway in the uterus (148).

As an example, PGF2α is used in fertilization procedures, in addition to GnRH, to pre-synchronize ovulation before applying for a resynchronization program in cows in dairy herds with acceptable pregnancy outcomes (149).

PROSTAGLANDINS IN MATERNAL IMMUNE TOLERANCE

When intercourse occurs, endothelial cells release IL-8, IL-1, INF-α, and TNF-α to recruit immune cells (150). Neutrophils are mobilized in the oviduct in female mammals in response to the presence of sperm (151). This process may also induce a state of unresponsiveness by the presence of anti-inflammatory cytokines, such as IL-4, IL-10, IL-13, and TGF-β (152) **Figure 1**.

In order to prevent a compromised systemic maternal immune response, local immune regulation in the fetal-maternal interface is very important. This is achieved by several mechanisms. One of these is local immunoregulation at the fetal-maternal interface, e.g., Human amniotic membrane-derived mesenchymal stem cells (hAM-MSCs) release factors such as indoleamine 2,3 dioxygenase (IDO), TGF-β, prostaglandin E2 (PGE2), and others inducing immunomodulatory effects (153).

PGs release or regulate different kinds of cells, such as Tolerogenic dendritic cells (tol-DCs), Mø1 and Mø2 macrophages, Decidual NK cells (dNK) ($CD56^{bright}CD16^{-}$), Decidual stromal cells (DSCs), Endometrial stromal cells, Tregs ($CD4^{+}CD25^{+}FOXP3^{+}$), and Decidual $CD8^{+}$EM cells ($CD45RA^{-}CCR7^{-}$) (**Table 1**).

Prostaglandin E (PGE), specifically, induces T-helper type 3 (Th3) and T-regulatory 1 cells (Tr1), as shown by Lewis´ rat and mouse test (154, 155). PGE2 secretion by human deciduous cells in the first trimester of pregnancy blocks the activation of maternal leukocytes in the decidua and inhibits IL-2 production and its receptor (156).

Other cells assisting in the decidualization of endometrial stromal cells (ESCs) and pregnancy maintenance are decidual natural killer (dNK) cells (157) and $CD14^{+}$ cells for Treg induction and immunosuppression (158). Also, Treg and Breg may contribute to the regulation of type 1 and 2-like T helper anti-fetal immune mechanisms during human pregnancy (159) (**Table 1**).

PLATELETS

It is evident that platelets may be important in tolerance mechanisms. Platelet activity is inhibited post-coitus, and this inhibition depends on prostaglandins (160). Seminal fluid has factors that favor clot formation, similar to peripheral blood, such as Factor VIII: Ag, FVIII: C and Von Willebrand factor (vWF), in addition to other factors (161). vWF (162), fibronectin (163), and vitronectin (164) are proteins that favor platelet adhesion (165). This implies that inhibition of platelet aggregation by PGI2 could be a compensatory mechanism for pro-adhesive molecules.

Using a mouse model, Etulain et al. (166) found that platelets act through P-selectin glycoprotein ligand-1 (PSGL-1), and directly affect neutrophil extracellular traps (NETosis). Platelet P-selectin is crucial for neutrophil recruitment (167). Furthermore, NETs cause the recruitment and activation of platelets and induce procoagulant activity due to the expression of histones H3 and H4, toll-like receptor 2 (TLR2) and TLR4 platelets. NETs present a surface for the activation of coagulation factor XII (168) in order to promote thrombosis as a mechanism of rejection (169).

Platelets cause a decrease in the formation of extracellular traps when preincubated with PGI2, followed by stimulation with lipopolysaccharide (LPS), arachidonic acid, and a synthetic diacylated lipopeptide (Pam3SCK4). This highlights the physiological role of PGI2 in platelet modulation (170). Prostaglandins may also inhibit the function of neutrophils by increasing levels of cyclic adenosine monophosphate (cAMP) (171).

The interaction of PMN-platelets releases products of arachidonic acid serving as precursors of neutrophil eicosanoids (172). In polymorphonuclear neutrophils (PMN), PGE2 modulates their response through the expression of EP2 and EP4 receptors (173).

In addition, other mechanisms of maternal immune tolerance are mediated by placental trophoblast derived microvesicles (MVs) and maternal thrombocyte-derived MVs. These bind to circulating peripheral T lymphocytes through P-selectin (CD62P)–PSGL-1 (CD162) interaction induces STAT3 phosphorylation in T cells (174).

The above mentioned may explain why platelet aggregation is inhibited post-intercourse and has a possible reduction in the formation of NETs to protect the embryo. It is possible that the release of extracellular traps may contribute to trophoblast lesions.

Many other cells mentioned above participate through high complexity fetal-maternal interface interaction to induce a tolerance stage, which protects the embryo (175).

POLYMORPHONUCLEAR CELLS

In mammalian species, PMNs are implicated in endometrial remodeling as being receptive to oocyte implantation. Human neutrophils exposed to progesterone and estriol hormones

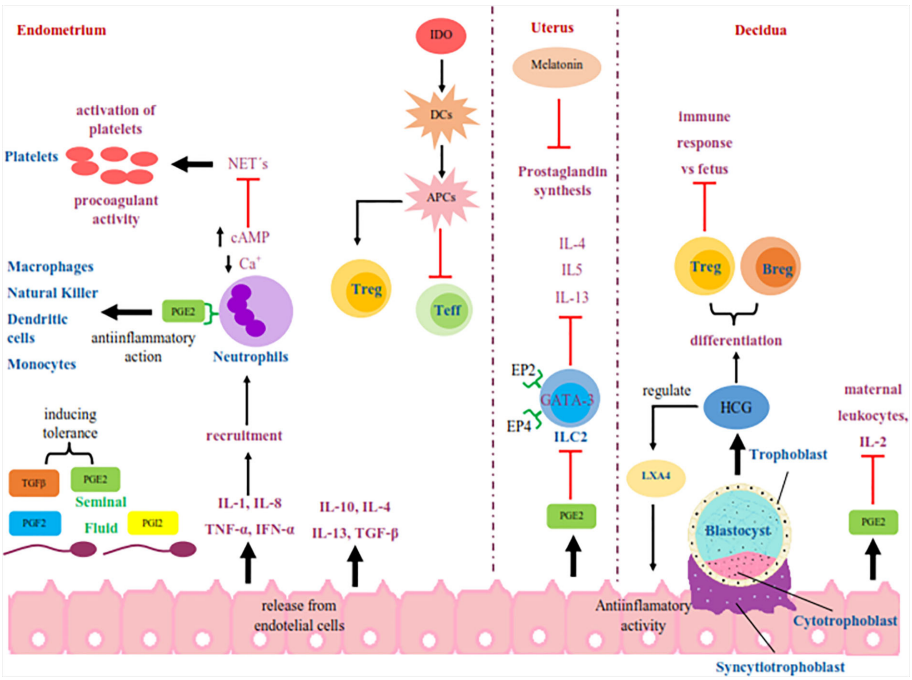

FIGURE 1 | Schematic representation of the signaling in the maternal immune response that begins with the deposition of seminal fluid in the female reproductive tract during intercourse. The seminal fluid start an immune signaling pathways mediated by PGE2 and PGI2 in the functions of endothelial cells, platelets, neutrophils, ILC2, lymphocytes, macrophages, natural killer, dendritic cells and monocytes during oocyte fertilization and early implantation. In addition, the molecules released by these cells like interleukins, HCG, IDO, and LXA4 have a fundamental role in this tolerance process. PGE2, prostaglandin E2; PG12, prostaglandin I2; PGF2, prostaglandin F2; TGFβ, transforming growth factor beta; IL-1, interleukin-1; IL-2, interleukin-2; IL-4, interleukin-4; IL-5, interleukin-5; IL-8, interleukin-8; IL-10, interleukin-10; IL-13, interleukin-13; TNF-α, tumor necrosis factor-alpha; INF-α, interferón alpha; Ca+, calcio; cAMP, cyclic adenosine monophosphate; NET´s, neutrophil extracellular traps; IDO, indoleamine-2,3-dioxygenase; DCs, mature dendritic cells; APCs, tolerogenic antigen presenting cells; Treg, regulatory T cells; Teff, effector T cells; GATA-3, GATA-3 transcription factor; EP2, prostaglandin E2 receptor 2; EP4, prostaglandin E2 receptor 4; ILC2, group 2 innate lymphoid cells; Breg, regulatory B cells; HCG, human chorionic gonadotropin; LXA4, Lipoxin A4.

promote the establishment of maternal tolerance through the induction of CD4+ T cells (176).

In humans, during coitus, sperm is deposited into the female reproductive tract (FRT). Neutrophils are then recruited for the elimination of excess sperm through phagocytosis (177).

However, bovine seminal plasma is shown to reduce the ability of PMNs to phagocytize bull sperm. Furthermore, equine seminal plasma is reported to contain factors that reduce the binding of neutrophils to sperm, avoiding the formation of NETs (178). In humans, when granulocytes are exposed to the seminal plasma, the respiratory burst is inhibited (179). These mechanisms allow more of the healthy motile sperm to reach the oviduct, which makes it clear that seminal plasma contains factors that modulate the response of PMN.

In addition, PGE2 can exert anti-inflammatory action on neutrophils and other innate immune cells such as macrophages, natural killer cells, dendritic cells, and monocytes (180, 181). Also, it inhibits the production of IFN-α in plasmacytoid dendritic cells and the production of IL-12 in myeloid dendritic cells.

Finally, polymorphonuclear leukocytes contribute to preterm labor by activating prostaglandin production from human fetal membranes (182).

GROUP 2 INNATE LYMPHOID CELLS

Specific ILC2s (Group 2 innate lymphoid cells) and uterine innate lymphoid cells (uILCs, uILC1, uILC2, and uILC3) (183) in the uterus are regulated by PGD2, PGE2, PGI2, and sex hormones, in particular, oestrogen (151, 184). Together, these may play a role in the balance between immunity and tolerance at the beginning of placenta formation and could be related to pregnancy loss, as shown in mice (185). Some studies show that ILC2 is the most abundant subset in the human fetal-maternal interface during premature and full-term pregnancies, in which its presence is regulated by sex hormones (e.g., oestrogen) (186). PGI2 decreases the proliferation of ILC2 and significantly inhibits the expression of IL-5 and IL-13 induced by IL-33 (187).

The production of PGE2 could also suppress the function of neutrophils and uILCs, a particular cell, similar to ILC2, through its EP2 and EP4 receptors in both healthy humans and mouse models (188, 189). PGE2 inhibits the expression of GATA-3, as well as the production of type 2 cytokines (IL-5 and IL-13) (144). These effects are mediated by the action of the EP2 and EP4 prostanoid receptors, which are specifically expressed in ILC2 (151, 190).

In addition, Group 1ILCs, uNK cells, and uILC3s significantly increase in abortion in mice. They also have a lower proportion of uILC2s (183).

DISCUSSION

Of the hundreds of molecules released with cells in the preimplantation, implantation, and decidualization processes; prostaglandins are integrated into each of these stages by seminal fluid, even until parturition. In particular, some of these molecules are found to be related to infertility and abortions, such as PGE2, PGF, and PGI2, which, in turn, are related to ovulation, fertilization, implantation, and decidualization (133). Increased levels of IL6 are also related to unexplained infertility, recurrent miscarriage, and pre-eclampsia among other disorders (9), e.g., in humans, cases of placental insufficiency, manifesting as intrauterine fetal growth restriction, are observed where the level of melatonin, a molecule with pleiotropic effects that regulates inflammatory processes (191), is decreased (192). Melatonin inhibits prostaglandin synthesis and is a potent inducer of uterine contractility (54, 193), in addition, there is evidence that in fish, melatonin is produced in the granulosa cells and is a critical factor for ovulation (194). Likewise, in women, it increases progesterone and regulates the corpus luteum (195). Also in a recent clinical trial, melatonin is shown to improve intrafollicular oxidative balance and gives a slight increase in the rate of human live births (196). Another example is Polish landrace gilts treated with pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) (PMSG/hCG-induced). Treatment with exogenous progesterone increases pregnancy success through the expression of genes responsible for vascular function and PGE2 synthesis (197). Therefore, the administration of inhibitors of prostaglandin synthesis, e.g., PGE2, must be carefully considered due to the multiple mechanisms of female fertility in which they participate (111).

Also, the mechanism of control over the rate of gene transcription or transcriptional regulation is altered in genes involved in chronic endometritis and the inflammatory response (IL-11, CCL4), growth factors (IGFBP1), and apoptotic proteins (BCL2, BAX, CASP8) in infertile patients (198).

Another mechanism of transcriptional regulation is that of Uterine Vascular Endothelial Growth Factor (UVEGF), in which PGE2 regulates vascular development through receptors EP2 and EP4.

CONCLUSIONS

To maintain fetal-maternal tolerance in the process of implantation (apposition/adhesion/invasion), a whole network of cells and molecules regulate different factors and responses according to the stage of pregnancy. Among the most highly studied cells and molecules are tolerogenic dendritic cells (tol-DCs), M1 and M2 macrophages, Decidual NK cells (dNK) ($CD56brightCD16^{-}$), Decidual stromal cells (DSCs), Endometrial stromal cells, Tregs ($CD4^{+}$ $CD25^{+}$ $FOXP3^{+}$) and Decidual $CD8^{+}$ EM cells ($CD45RA^{-}$ $CCR7^{-}$), progesterone, oestrogen, Leukaemia inhibitory factor (LIF), Indoleamine-2,3-dioxygenase (IDO), and melatonin. Within this complex network, prostaglandins, specifically, PGD2, PGF2α, and PGE2, are important modulators and regulators in maintaining maternal-fetal tolerance, as we deduced. Nevertheless, other cells such as platelets, uILCs, and polymorphonuclear leukocyte/Nets require more research.

AUTHOR CONTRIBUTIONS

Conceptualization: EP-C and GM. Writing—original draft preparation: GM, GV, LP-C, MH-H, EC-P. Manuscript revision: GM, LP-C, MH-H, EZ, EP-CM, MM, RM, CM-C, NM, CR, EC-P, and EP-C. All authors contributed to the article and approved the submitted version.

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