IMMUNE INTERACTIONS DURING THE REPRODUCTIVE CYCLE

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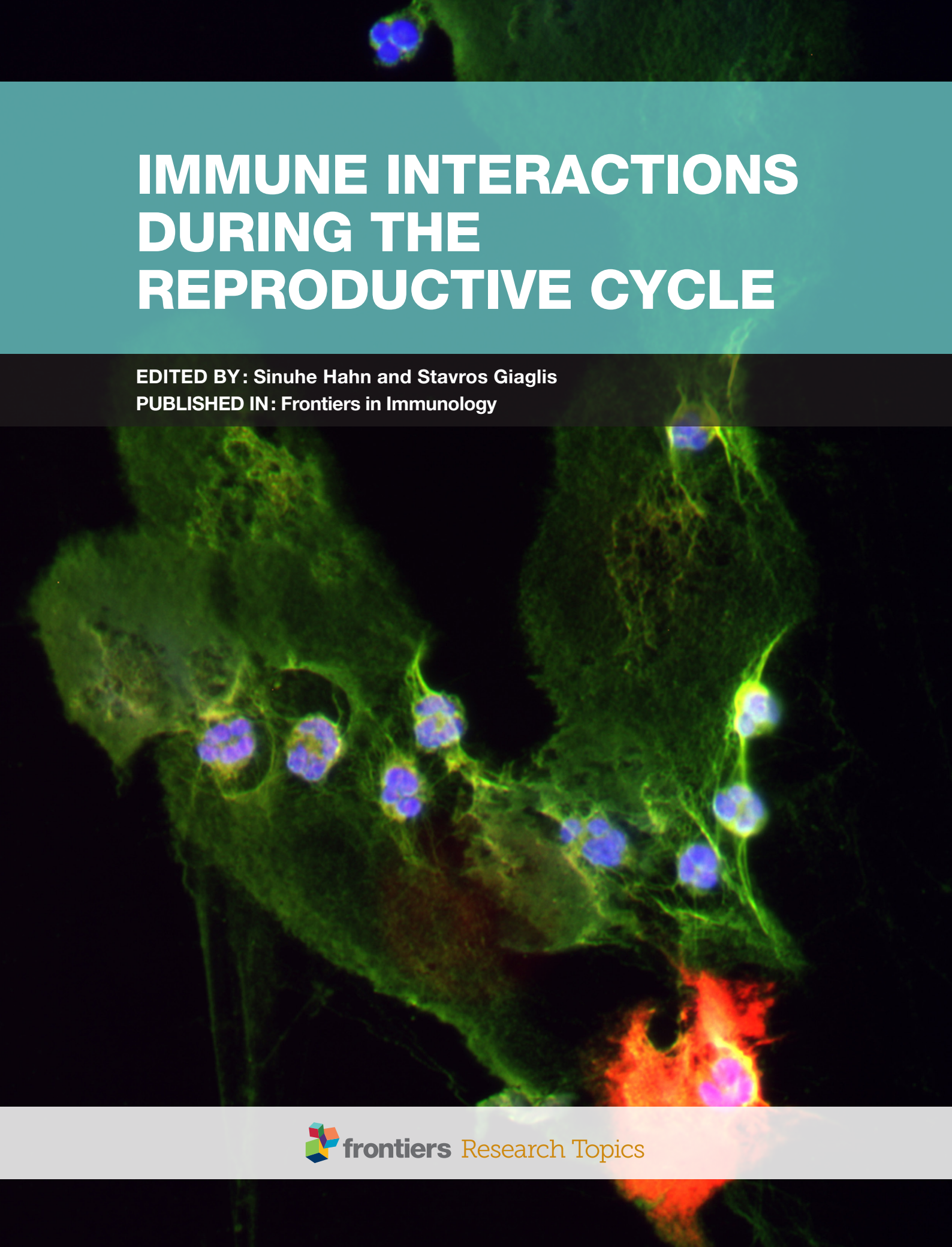

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IMMUNE INTERACTIONS DURING THE REPRODUCTIVE CYCLE

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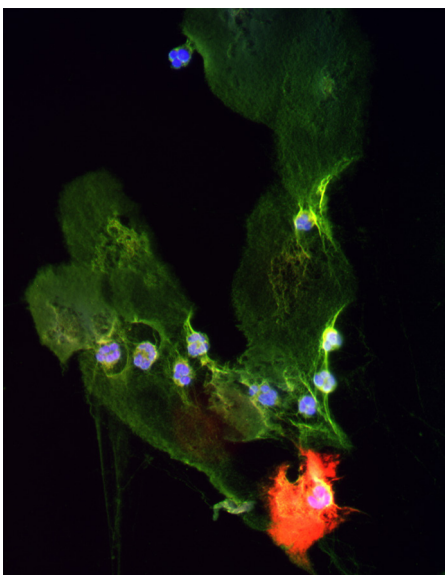

Human peripheral blood neutrophils isolated from a pregnant donor's whole blood were co-incubated with PMA. Three hours later, cells were fixed and stained with antibodies to the granular protein MPO (colored green) and the nuclear citrullinated histone H3. PMN nuclei were visualized using DAPI (colored blue).

Mammalian pregnancy represents a unique immunological riddle in that the mother does not reject her allogeneic fetus. In part this is largely due to a general sequestration or diminution of T cell activity, and an increased involvement of the innate immune system. The field of immunology is concerned primarily with how innate and adaptive mechanisms collaborate to protect vertebrates from infection. Although many cellular and molecular actors have evidently important roles, antibodies and lymphocytes are considered to be the principal players. Yet despite their importance, it would be definitely simplistic to conclude that they are solely essential for immunity overall. A major distinction between adaptive and innate immunity is the spontaneity of the innate immune response, which utilizes an already pre-existing but limited repertoire of responding modules. The slower onset of adaptive immunity compensates by its ability to recognize a much broader repertory of foreign substances, and also by its power to constantly improve during a response, whereas innate immunity remains relatively unaffected.

The interactions between the reproductive system and the immune system are of particular interest, since the reproductive system is unique in that its primary role is to assure the continuity of the species, while the immune system provides internal protection and thus facilitates continued health and survival. The modus operandi of these two morphologically diffuse systems involves widely distributed

chemical signals in response to environmental input, and both systems must interact for the normal functioning of each. Furthermore, dysregulation of normal physiological interactions between the reproductive and immune systems can lead to severe pregnancy-related disorders or complications. On the other hand, by ameliorating auto-inflammatory conditions such as MS and RA, pregnancy may provide a unique insight into novel immune modulatory strategies.

The scientific focus on reproductive–immune research has historically provided substantial insight into the interface between these two physiological systems. A translational research approach would involve a tight interaction between diverse scientific and clinical disciplines including immunology, obstetrics, haematology, haemostasis and endocrinology. With so much recent progress in the field, we believe that it is valuable and well-timed to review the broad variety of the relevant physiologic and pathologic aspects – from menstruation to fertilization and implantation, and from placentation and pregnancy *per se* to the post partum condition – in which the immune system takes part.

We are looking forward to a wide and vivid discussion of these and related issues, and we sincerely expect that our readers profoundly benefit from new exciting insights and fruitful collaborations.

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EDITORIAL
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Reproductive immunology research: a tight interaction between diverse scientific and clinical disciplines including immunology, obstetrics, hematology, and endocrinology

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The unique immunological riddle of human gestation involves a broad variety of cells and molecules playing evidently important roles (1). The reproductive and immune systems are morphologically diffused, and their *modus operandi* involves widely distributed signals in response to the given environmental input, while both systems must interact to obtain their normal functionality (2). Furthermore, dysregulation of physiological interactions between the two systems can lead to severe pregnancy-related disorders or complications, such as fetal loss, preterm labor, preeclampsia (PE), and poor fetal development (3). On the other hand, by ameliorating autoimmune conditions such as multiple sclerosis and rheumatoid arthritis, while other conditions such as systemic lupus are deteriorated, pregnancy as a condition may provide a unique insight into novel immunomodulatory strategies (4, 5).

The scientific focus on reproductive–immune research has historically provided substantial understanding of the interface between these two physiological systems. With such recent progress in the field, we felt that it is valuable and well-timed to review the broad variety of the relevant physiologic and pathologic aspects – from menstruation to fertilization and implantation, and from placentation and pregnancy *per se* to the post partum condition – in which the immune system takes part.

As editors, we are delighted by the keen response of 15 groups of scientists from 4 different continents, which kindly contributed their unique expertise to our effort to recap, value, and extend our insights concerning the field of reproductive immunology. We sincerely hope that the present eBook will succeed to share with the reader the broad and vivid discussion between the authors and editors.

Schumacher et al. (6) explore the interplay between the endocrine and immune systems during gestation, with focus on progesterone, estradiol, and human chorionic gonadotropin. Pregnancy hormones are critically for the successful establishment, maintenance, and completion of pregnancy. They suppress detrimental maternal alloresponses while promoting tolerance pathways through the antigen-presenting capacity of DCs, monocytes, and macrophages as well as the blockage of NK, T, and B cells. These findings highlight the importance of endocrine factors for tolerance induction during pregnancy.

Hsu and Nanan (7) discuss the recent advances in the complex crosstalk between the innate and adaptive immune system during human pregnancy and PE. They present many lines of evidence supporting an immunological origin to PE, implicating decidual NK cells and APC DCs and macrophages as major players in the regulation of vascular remodeling and trophoblast invasion. On the other hand, within the adaptive immune system, $Foxp3^+$ Tregs and $CD4^+HLA\text{-}G^+$ suppressor T-cells seem to be essential for guaranteeing immune tolerance.

Getting deeper into the mechanisms of placental pathology, Faas et al. (8) focus on the role of monocytes and macrophages in pregnancy and PE. Given the generalized activation of the acute inflammatory response, monocytes may play a central role in this reaction, since they are short-lived cells that mature in the circulation and transmigrate into affected tissues upon an inflammatory stimulus, developing into macrophages. Macrophages in turn are abundant in the endometrium and play a crucial part in implantation and placentation. In PE, these macrophages appear to be activated and in larger numbers.

In the same context, Ruocco et al. (9) provide insight concerning the role of Tregs in pregnancy reassessing the original concept of "suppressor T-cells" in pregnancy, putting it in a historical perspective, and highlighting the main data revising the concept of Tregs in gestation. Moreover, they focus to the most important questions in the field, such as Treg antigen specificity, Treg subsets, the functional crosstalk of Tregs with NK and DCs.

Joerger-Messerli et al. in Basel (10) observed that the inflammatory reaction in monocytes is initiated by the interaction of syncytiotrophoblast microparticles (STBM) with TLRs, which in turn signal through NF-κB to mediate the transcription of pro-inflammatory mediators. Since pregnancy is accompanied by a mild systemic inflammatory response, they show that *in vitro* generated STBM from normal placentas stimulate monocytes. Furthermore, STBM derived from PE placentas up-regulated CD54 expression, and stimulated IL-6 and IL-8 secretion in a dose-dependent manner, which was impaired in the presence of TLR signaling inhibitors or when blocking NF-κB activation.

Fettke et al. (11) examine B cell involvement in the immune response against paternal antigens and tolerance mechanisms. Such pleiotropic cells play a considerable role by secreting immunomodulatory IL-10, while they can harm pregnancy due to their capacity to produce autoantibodies. New evidence in mouse models suggests that IL-10 producing B cells (B10), contribute

in maintaining tolerance, fighting danger signals at the fetal–maternal interface. In human pregnancies, B10 cells increase with onset but not in case of spontaneous abortions, suppressing TNF-α production by T-cells.

A review on the unique neonatal NK cell population and its role in gestational autoimmunity is provided by Rival et al. (12) from Kenneth Tung's group. Maternal autoantibodies can trigger autoimmune ovarian disease (AOD) in the progeny of women with SLE or Sjogren's syndrome. The pathogenic effect of autoantibody exposure is investigated in mice, in which immune complexes are formed in adult and neonatal ovaries, but a specific Ab-species triggers severe AOD only in young mice. Propensity to AOD is due to the uniquely hyper-responsive neonatal NK cells. Resistance to AOD in older mice results from specific NK cells that regulate effector NK cells and Tregs. Activated by ovarian immune complexes, NKs migrate to lymphoid organs where priming occurs. These insights uncover new properties of the neonatal innate and adaptive responses, lethality of premature infant infection, and novel neonatal antiviral vaccine design.

Woidacki et al. (13) summarize the existing knowledge concerning the course of pregnancy in women affected by mast cell (MC) mediated or associated disorders. While physiological numbers of MCs influence positively the outcome of pregnancy, uncontrolled augmentations in quantity and activation can lead to dangerous complications. Women with the desire of getting pregnant and diagnosed with MC mediated disorders – urticaria, mastocytosis, or MC-related chronic inflammatory diseases – may benefit from specialized medical support to ensure a positive pregnancy outcome.

Than et al. (14) provide evidence concerning the protective role of placental protein 13 (PP13), an immunoregulatory galectin. Three of the five human galectins are expressed in the placenta, and galectin-13 (PP13) is predominantly expressed by the syncytiotrophoblast and released from the placenta into the circulation. Its ability to induce apoptosis of T-cells *in vitro* and to kill T-cells and macrophages in the maternal decidua, suggests important immune functions. Indeed, *LGALS13* mutations and decreased placental expression of PP13 and its low concentrations during first trimester are associated with elevated risk of PE. PP13 turned to be a good early biomarker to assess maternal risk for the subsequent development of pregnancy complications, which might enable its potential in directing patient management.

Sedlmayr et al. (15) highlight the role of tryptophan catabolism in the placenta, focusing mainly on the role of indoleamine 2,3-dioxygenase-1 (IDO1), one of three enzymes involved in the tryptophan degradation pathway. IDO1 has been implicated in regulation of feto-maternal tolerance in the mouse. Depletion of tryptophan mediates immunoregulation and antimicrobial functions. In addition to the decidual glandular epithelium, IDO1 is localized in the vascular endothelium of the chorion and the endothelium of the decidual spiral arteries. Possible consequences of tryptophan catabolism in the endothelium are relaxation of the placental vasotonus, contributing to placental perfusion and growth of both placenta and fetus.

Chatterjee et al. (16) from the Mitchell lab examine how immune cells that produce IL-4 and IL-10 are regulated throughout pregnancy and the effects of reduced IL-4 and IL-10 signaling on fetal and maternal physiology. The resolution of inflammation plays an important role throughout pregnancy and is largely mediated by immune cells producing IL-4 and IL-10. The temporal and spatial aspects of reducing inflammation during pregnancy are thoroughly discussed.

Perez-Sepulveda et al. (17) discuss the involvement of the innate immune system in the establishment of an environment that favors pregnancy and possible alterations related to the development of PE. Since normal pregnancy is considered as a Th2 immunological state, PE has been classically described as a Th1/Th2 imbalance; recent studies have expanded the Th1/Th2 into a Th1/Th2/Th17 and regulatory T-cells paradigm and where DCs could have a crucial role.

An insight into the impact of bacterial infections, such as *Helicobacter pylori* (HP), in PE is delivered by Tersigni et al. (18). Since the primary trigger of PE is unknown, a hypothesis concerning the disease onset is triggering by infectious agents. Consistently, higher seroprevalence of HP infection is evident in women with PE. As trophoblast invasion is a crucial step in implantation and placental development, the proposed infection-induced autoimmune mechanism, interfering negatively with the fetal side of the developing placenta, may explain the higher seropositivity for HP infection PE cases.

Nilsson et al. (19) discuss the role of the human HLA-Ib protein (HLA-G) in the regulation of the immunological crosstalk during conception and pregnancy: from genetics to physiological effects, from pregnancy and pregnancy complications to a short discussion on future possible means of preventative measures and therapies. As HLA-G expression is limited to gestation, it is proposed as a key player in the maintenance of immunological tolerance. HLA-G might be involved in immune processes even before conception, since HLA-G is detected in non-pregnant women genital tract and blood, in men's seminal fluid, and in the pre-implanted embryo. Therefore, a combined contribution from the mother, the father, and the embryo/fetus is important.

The series of articles included in the present collection is completed with a commentary by Khoury et al. (20) on the promising potential of menstrual stem cells for antenatal diagnosis and cell therapy. Menstrual-derived stem cells (MenSCs) are a new source of MSC isolated from the menstrual fluid. Currently, there is a growing interest in their clinical potential due to their multipotency, high proliferative capacity, and facile way to obtain non-invasively. This review details their distinctive biological properties regarding immunophenotype and function, proliferation/differentiation potential, and paracrine effects. Their possible role in antenatal diagnosis is also discussed.

ACKNOWLEDGMENTS

We cordially thank all the participants and the numerous committed reviewers for their major support, substantial effort, and great enthusiasm. We hope that this special group of contributors and others drawn to this field will remain highly productive in the future and we are sincerely looking forward for our readers to profoundly benefit from new exciting insights and fruitful collaborations.

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Endocrine factors modulating immune responses in pregnancy

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How the semi-allogeneic fetus is tolerated by the maternal immune system remains a fascinating phenomenon. Despite extensive research activity in this field, the mechanisms underlying fetal tolerance are still not well understood. However, there are growing evidences that immune–immune interactions as well as immune–endocrine interactions build up a complex network of immune regulation that ensures fetal survival within the maternal uterus. In the present review, we aim to summarize emerging research data from our and other laboratories on immune modulating properties of pregnancy hormones with a special focus on progesterone, estradiol, and human chorionic gonadotropin. These pregnancy hormones are critically involved in the successful establishment, maintenance, and termination of pregnancy. They suppress detrimental maternal alloresponses while promoting tolerance pathways. This includes the reduction of the antigen-presenting capacity of dendritic cells (DCs), monocytes, and macrophages as well as the blockage of natural killer cells, T and B cells. Pregnancy hormones also support the proliferation of pregnancy supporting uterine killer cells, retain tolerogenic DCs, and efficiently induce regulatory T (Treg) cells. Furthermore, they are involved in the recruitment of mast cells and Treg cells into the fetal–maternal interface contributing to a local accumulation of pregnancy-protective cells. These findings highlight the importance of endocrine factors for the tolerance induction during pregnancy and encourage further research in the field.

Keywords: progesterone, estradiol, human chorionic gonadotropin, luteinizing hormone, alpha-fetoprotein, immune regulation, pregnancy

INTRODUCTION

It is said that mammalian pregnancy defies the immunological rules because a semi-allogeneic conceptus is tolerated rather than rejected. It is therefore, a fascinating phenomenon and target of many immunological studies. Pregnancy is however a natural phenomenon that ensures the survival of species and exists since millions of years. By contrast transplantation, where empirical observations led to the definition of the immunological rules, is an artificial process that was first described in 1905. Thus, understanding how natural tolerance operates may help creating novel strategies to ensure tolerance in other models. Especially because of the fact that initial allorecognition of foreign fetal antigens by the maternal immune system is advantageous for a successful pregnancy, the mechanisms behind gestational tolerance are of interest for other disciplines. Local suppression of alloreactive immune responses to paternal antigens is a prerequisite for fetal acceptance. Steroid hormones like progesterone (P4) and estradiol (E2) as well as gonadotropins such as the human chorionic gonadotropin (hCG) are fundamentally involved in the regulation of the menstrual cycle and in the establishment and maintenance of pregnancy (1, 2). Through binding their specific receptors expressed by immune cells and/or by acting via mediators these hormones support fetal tolerance by inhibiting destructive immune responses and inducing tolerance pathways. This review highlights the effects of pregnancy-associated hormones on different immune cell types with a special focus on P4, E2, and hCG.

PROGESTERONE

P4 is a member of the steroid hormone family and has been described as the "pregnancy hormone" due to its indispensable role for pregnancy maintenance (3). During the menstrual cycle, P4 levels are relatively low during the preovulatory phase, rise after ovulation, and are elevated during the luteal phase (4). If pregnancy occurs, hCG initially maintains P4 levels by inducing its production by the *corpus luteum*. After the luteal–placental shift, the placenta takes over P4 production (5). P4 prepares the uterus for implantation as it induces differentiation of stromal cells into decidual cells (decidualization) and decreases the contractility of uterine smooth muscle cells (6, 7). Additionally, P4 withdrawal is associated with the initiation of labor (8). P4 has been shown to affect immunity, mainly at pregnancy concentrations. These effects are primarily mediated via the intracellular P4 receptors (PR), PR-A and PR-B, which act as transcription factors, although non-genomic effects of PR activation have been reported (9). In addition, P4 mediates its immune regulatory function via mediators such as the progesterone-induced blocking factor (PIBF) and glycodelin A (10, 11).

ESTRADIOL

Like progesterone, estrogens belong to the steroid hormones. Three major naturally occurring estrogens have been described in women, namely estrone (E1), estradiol (E2), and estriol (E3). Within those, E2 is the predominant estrogen produced during the reproductive years. High levels of E2 are produced by the ovary, while smaller amounts are also produced by the adrenal cortex and from E2 precursors in fatty tissues (12). In the normal menstrual cycle, E2 levels rise with follicular development, drop briefly at ovulation, and rise again during the luteal phase for a second peak. At the end of the luteal phase, E2 levels drop to their menstrual levels unless there is a pregnancy (13). During pregnancy, E2 levels increase continuously until term due to the production by the growing placenta (14). Several important functions have been described for E2. During the menstrual cycle, E2 triggers the luteinizing hormone (LH) surge resulting in ovulation. After ovulation, in the luteal phase, E2, in conjunction with P4 prepares the endometrium for implantation. Upon pregnancy, E2 is shown to promote uterine blood flow, myometrial growth, stimulate breast growth and at term, promote cervical softening and expression of myometrial receptors. Besides, E2 was suggested to affect different immune cell populations in their number and function and thereby contributes to fetal tolerance. These effects are mediated via binding of E2 to its intracellular receptors, estrogen receptor alpha (ERα) and beta (ERβ), which in turn modulate the expression of many genes (15). Both receptors are expressed in various lymphoid tissue cells as well as in lymphocytes, macrophages, and dendritic cells (DCs) (16, 17).

HUMAN CHORIONIC GONADOTROPIN

Human chorionic gonadotropin is a primate-specific heterodimeric placental glycoprotein. Four different hCG variants, namely total hCG, hyperglycosylated hCG (hCG-H), free β-subunit, and pituitary hCG, have been reported, each produced by different cells with separate biological functions (18). In humans, after pregnancy onset, total hCG increases rapidly during the first trimester, peaks between the 9th and 12th week of pregnancy and then declines, until the woman gives birth, although remaining higher than in a non-pregnant woman (19). hCG is produced by differentiated syncytiotrophoblasts and its main function is to stimulate P4 production by the *corpus luteum* (20). Moreover, hCG supports pregnancy by facilitating trophoblast invasion (21–23), promoting angiogenesis, and ensuring nourishment of the fetus (24–26). In rodents, similar functions are mediated by the highly homologous LH. During the last years, there is growing evidence that hCG and LH are involved in immune tolerance mechanisms leading to fetal survival. Both gonadotropins were shown to affect immune cells by binding to the LH/CG receptor expressed by several immune cell types. Moreover, hCG also acts through the mannose receptor.

ALPHA-FETOPROTEIN

Alpha-fetoprotein (AFP) is a glycoprotein that is produced by the yolk sac and fetal liver during pregnancy (27). It is the most abundant plasma protein found in the human fetus, acting as a fetal transport protein. AFP levels increase in the 4-week-old fetus, peak between the 12th and 16th week and remain low after birth. Although several studies provide evidence for an immune regulatory potential of AFP (28–32), it is still not explored whether AFP contributes to pregnancy success by modulating immune responses.

HORMONAL INFLUENCE ON IMMUNE CELLS DURING PREGNANCY

EFFECT OF PREGNANCY HORMONES ON MACROPHAGES

Monocytes and macrophages are major representatives of the innate immune system in the cycling and pregnant mammalian uterus. Several studies provide evidence that monocyte recruitment, differentiation into macrophages, and function in the reproductive tract is modulated by pregnancy-associated hormones (33). Hormonal influence may be achieved by directly binding to the appropriate hormone receptors expressed on human and murine macrophages (16, 34, 35) or indirectly by modulating the levels of cytokines and growth factors that target the resident macrophages and influence their secretory profile. Hunt and colleagues reported that P4 reduced macrophage migration into the murine uterus (36), while Kitzmiller and colleagues showed that E2, P4, and hCG did not affect macrophage migration in guinea pigs (37). Differentiation of monocytes into macrophages was hindered by glycodelin A, a P4 mediator, by induction of apoptosis in human monocytes. However, after differentiation glycodelin A was not able to alter phagocytic capacity of macrophages (11). Macrophages are important regulators of trophoblast activity that promote tissue remodeling and angiogenesis (38). In this regard, E2, hCG, and LH have been demonstrated to enhance the production of the vascular endothelial growth factor (VEGF) in human macrophages (39, 40), supporting vessel formation in the placenta. In addition, P4 impairs the ability of human and murine macrophages to produce potent effector molecules such as nitric oxide and IL-1 proven to be detrimental for successful pregnancy outcome (36, 41, 42). Moreover, P4 suppresses toll-like receptor-triggered activation of murine macrophages by regulating miR-155 expression (43). Menzies and colleagues recently suggested an involvement of P4 in the regulation of genes associated with alternative macrophage activation (44). By contrast, hCG treatment of human and murine IFN-γ-primed macrophages resulted in increased production of nitric oxide, reactive oxygen species, IL-6 and IL-12p40, and enhanced phagocytosis of apoptotic cells (45, 46). However, hCG treatment of murine IFN-γ-primed macrophages did not affect the induction of allogeneic T cell proliferation (45). Interestingly, macrophages regulate excess of hCG known to be teratogenic to fetal tissues. Here, human macrophages are proposed to incorporate and degrade hCG in a time-dependent manner that protect fetal gonadogenesis from excess hCG (47, 48). More precisely, Katabuchi and colleagues recently demonstrated that hCG induces transient vacuole formation in human monocytes, morphologically mimicking Hofbauer cells. The authors suggest that Hofbauer cells and especially their vacuoles are involved in the protection of fetal tissue from high amounts of maternal hCG (49). Besides an effect of steroid hormones and gonadotropins on monocytes and macrophages, AFP is suggested to have an influence on both innate immune cell

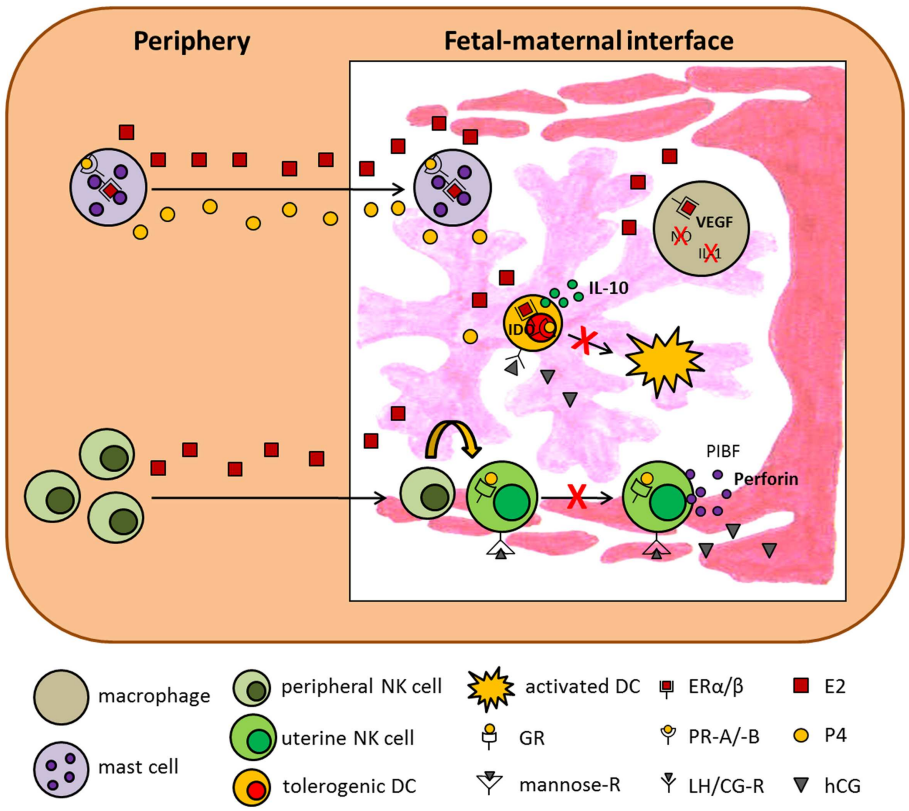

FIGURE 1 | Hypothetical scenario presenting the influence of pregnancy-associated hormones on innate immunity. The scenario suggests several mechanisms by which E2, P4, and hCG influence innate immune cells and thereby support pregnancy success. ER, estrogen receptor; GR, glucocorticoid receptor; IDO, indoleamine 2,3-dioxygenase; IL-1, interleukin-1; IL-10, interleukin-10; LH/CG-R, luteinizing hormone/chorionic gonadotropin receptor; NO, nitric oxide; PIBF, progesterone-induced blocking factor; PR, progesterone receptor; VEGF, vascular endothelial growth factor.

types. It has been demonstrated that AFP significantly suppresses the production of TNFα and IL-1β and induces a rapid down-regulation of surface MHC class II expression in a stimulated human monocyte cell line (29, 31). Moreover, Lu and colleagues showed that AFP inhibits the cell surface expression of Ia antigens on macrophages but does not affect macrophage viability (50). Hormonal effects on macrophages are summarized in **Figure 1**.

EFFECT OF PREGNANCY HORMONES ON NATURAL KILLER CELLS

NK cells and, in particular, uterine NK (uNK) cells are of special interest when analyzing mechanisms underlying normal pregnancy. This becomes obvious when taking into account that uNK cells are the predominant lymphocyte population in the late secretory phase of the menstrual cycle and in the early pregnant uterus representing circa 70% of all leukocytes in decidual tissue. uNK cells differ from peripheral NK cells in the expression of their receptor repertoire and in the expression of some genes induced by the hormonal environment. The main function of uNK cells is to regulate maternal uterine vasculature remodeling (51). Therefore, it has been demonstrated in the murine system that uNK cells produce proangiogenic factors such as VEGF and growth factors and provide local IFN-γ for initiation of spiral artery formation (52–54). Their origin or expansion remains a matter of discussion. They may migrate from the periphery, differentiating from NK cell progenitors under the control of different factors, including steroid hormones (55), and/or by recruitment of peripheral NK cells into the uterus (56–58) or expand *in situ* after pregnancy was established (59). Qu and colleagues demonstrated a P4-dependent osteopontin expression in human decidual stroma cells and human uNK cells and proposed a role for osteopontin in uNK cell accumulation in uterine tissue (60). Moreover, human uNK cell recruitment from the peripheral blood into the uterus seems to be favored by rising E2 and LH levels and restricted by increasing amounts of P4 (61). Interestingly, mature human and rodent uNK cells do not express steroid receptors (55, 62–64). Thus, it is suggested that, at least for P4, effects are mediated through the glucocorticoid receptor (GR), proven to be expressed on murine uNK cells (65). In addition to the lack of steroid receptors, uNKs also miss the classical LH/CG receptor. Thus, hCG was suggested to induce human uNK cell proliferation through the mannose receptor (66). Regarding the function of uNK cells, they have been shown to contain high amounts of perforin but only display low cytotoxic activity. Several studies indicated that P4 and its mediator PIBF inhibit human NK cell activity via a block of degranulation (67–70). In agreement, E2 increased human and murine NK cell number but reduced their cytotoxicity (71,

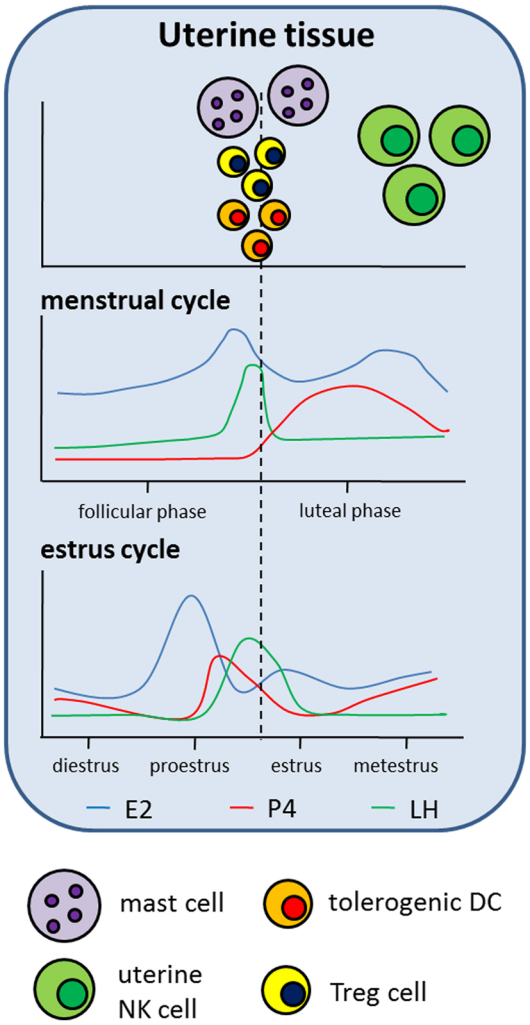

FIGURE 2 | Hypothetical scenario presenting the influence of hormonal changes taking place during the reproductive cycle on the distribution of innate and adaptive immune cell populations in the uterus. The upper part of the scenario displays the accumulation of different immune cell populations in uterine tissue correlated with the different phases of the human menstrual cycle and the murine estrus cycle. The lower part of the scenario displays hormonal changes taking place during the reproductive cycles, both in humans and mice.

72). By contrast Kitaya and colleagues as well as Kurashige and colleagues revealed that neither P4 nor E2 had significant effects on the proliferation, cytolytic activity, and cytokine secretion of human endometrial NK cells (73, 74). This was also true for AFP (73). In contrast to these results, it was shown that hCG and LH applied to virgin mice resulted in an enhanced NK cell activity (75, 76) suggesting that pregnancy hormones differently regulate NK cell function. Moreover, contradictory results between humans and mice regarding an influence of pregnancy hormones on different immune cell populations depict the limitations of animal models in understanding mechanisms unique to human pregnancy. Finally, hCG not only influences NK cells but is also produced and secreted by them during pregnancy (77). Hormonal effects on NK cells are summarized in **Figures 1** and **2**.

EFFECT OF PREGNANCY HORMONES ON MAST CELLS

Mast cells (MCs) are best known for their effector function in allergic diseases. After binding of allergen-specific IgE to its receptor (FcεRI), preformed and newly synthesized mediators stored within the MCs are released to induce inflammatory immune responses (78). Beside this well-documented function of MCs, recent data suggest MCs as critical regulators of adaptive immune responses (79). Additionally, we recently uncovered a critical role for MCs in pregnancy success. To study the importance of MCs in murine pregnancy, we took advantage of a MC-deficient mouse model. We found uterine MCs (uMCs) to have a unique phenotype. uMCs increase in number every time a female becomes receptive and rapidly expand after pregnancy occurs. In the absence of MCs implantation is severely impaired and spiral artery remodeling has shown to be insufficient. This is suggested to result in fetuses that are growth-retarded (80). We also proposed a role for uMCs in trophoblast survival, placentation, and fetal growth. MC salutary role in murine pregnancy is mediated at least in part by Galectin-1 (80). Oscillations in the number of uMCs during the reproductive cycle in humans (81) and mice (82) seem to be, at least partially, hormone regulated. Several studies demonstrated that P4 and E2 influence rat and mouse MC density in different tissues, including mammary glands and uterine tissue (82–85). We additionally suggested a function for both hormones in the recruitment of murine MCs from the periphery into the uterus as well as an impact on MC activity (86). In agreement, several studies confirmed a major effect of P4 and E2 on rat, mouse, and human MC activation (83, 87–90). By contrast, two other studies did not observe alterations in the number of granulated or degranulated rat and mouse MCs after P4 or E2 treatment (91, 92). Hunt and colleagues revealed that E2 influences the expression of iNOS and TNF-α in murine uMC (93). It can be assumed that the observed effects of P4 and E2 on MCs are in part of direct nature as MCs from different species have been proven to express P4 and E2 receptors (85–87, 89, 94). Studies analyzing the impact of hCG on MCs are rare to find in the literature. One study investigated the histamine content in MCs in a model of ovarian hyperstimulation induced in rabbits. However, hCG application seemed not to change the histamine content in MCs (95). Hormonal effects on MCs are summarized in **Figures 1** and **2**.

EFFECT OF PREGNANCY HORMONES ON DENDRITIC CELLS

As professional antigen-presenting cells, DCs are at the interphase between the innate and the adaptive immune system; hence their activation and modulation is critical for the outcome of the immune response. Dependent on their activation status, DCs either secrete pro- or anti-inflammatory cytokines, thereby inducing immune responses or suppressing them, respectively. During normal pregnancy, the majority of human and murine decidual DCs presents an immature (tolerogenic) phenotype and mainly produce IL-10, thus contributing to a fetus-friendly local environment (96, 97). In line with this, spontaneous abortions in humans and mice are associated with an increased number of mature, IL-12-producing DCs (98, 99). Moreover, the importance

of DCs for a proper decidualization and implantation has been nicely shown by Plaks and colleagues in a mouse model (100). Data regarding the influence of pregnancy-associated hormones on DC function are widespread and inconsistent. DCs derived from bone-marrow precursors or monocytes as well as DCs from spleen or decidua have been shown to react differentially to hormonal stimulation. This may offer a possible explanation how the endocrine system supports the pregnant immune system in tolerating the semi-allogeneic fetus while at the same time being aware of pathogens; these cells being pleiotropic and it should not come as a surprise that they have the machinery to respond differently depending on the situation. DCs are highly susceptible to hormonal stimulation by expressing receptors for P4, E2, hCG, and LH (101–103). Hormonal stimulation of activated bone-marrow derived DCs (BMDCs) resulted in the majority of studies in an impaired up-regulation of MHCII molecules and co-stimulatory molecules associated with a reduced capability to secrete pro-inflammatory cytokines (101, 104). This would imply that upon hormonal stimulation they acquire a rather pregnancy-friendly phenotype. The concrete impact of P4 on IL-10 secretion by activated rat and mouse BMDCs was differentially discussed and has to be further evaluated (101, 104, 105). In line with the hormonal-mediated induction of a tolerogenic phenotype in activated BMDCs, their T-cell stimulatory capacity was reduced. This suggests that hormone-treated DCs have a pregnancy-protective effect by suppressing alloreactive T cell responses (101, 104, 105). However, results obtained after the addition of P4, E2, and hCG to monocyte-derived DCs (moDCs) from human peripheral blood are different from the results obtained after hormonal stimulation of BMDCs. Here, two studies showed that combinations of P4, E2, and hCG did not change the expression of maturation markers of moDCs or their T cell stimulatory capacity (106, 107). Segerer and colleagues demonstrated, at least for hCG, a pregnancy-positive effect on HLA-DR expression associated with a significant reduction in the ability to stimulate T cells (108). Of great interest is the fact that most of the published studies observed a significant up-regulation of IL-10 production by human DCs after treatment with pregnancy hormones (106, 107, 109). Uemura and colleagues additionally proposed an induction of T cell differentiation into Th2 (107), a phenotype that is reportedly pregnancy-friendly. Analysis of hormonal effects on splenic DCs has been performed in several mouse models, including models of autoimmune diseases and pregnancy. In a murine model for multiple sclerosis, namely experimental autoimmune encephalomyelitis (EAE), E2 treatment has shown to be protective. Here, E2 did not affect the expression of activation markers and co-stimulatory molecules of DCs but inhibit their ability to stimulate T cell proliferation and secretion of Th1 and Th2 cytokines. The reduced T cell stimulatory capacity was suggested to be due to an increased expression of indoleamine 2,3-dioxygenase (IDO) in DCs after E2 treatment (110, 111). Accordingly, hCG was also proven to up-regulate IDO in DCs in a murine model of autoimmune diabetes (112). As for E2, it was reported that P4 seems to affect the ratio of Th1-promoting DEC-205$^+$ DCs and Th2-promoting 33D1$^+$ DCs during pregnancy, favoring the dominance of 33D1$^+$ DCs. The need of 33D1$^+$ DCs for pregnancy success results from the observation that depletion of this specific DC subset during the perinatal period in mice caused substantial fetal loss probably mediated through Th1 up-regulation via transient IL-12 secretion (113). We have recently investigated the influence of hCG and LH on the number and phenotype of peripheral and local DCs in a murine model of disturbed tolerance to the semi-allogeneic fetus. We found that the *in vivo* application of both hormones prevented fetal rejection and this was associated with a reduced number of total and mature DCs both in the periphery and in decidua. Furthermore, we proved that hCG-treated decidual DCs had an elevated capacity to induce regulatory T (Treg) cells (103). This confirms the pregnancy-protective impact of both gonadotropins via modulation of adaptive immune responses. Effects of AFP on different DC subsets during pregnancy are almost unknown. Evidence for an AFP-mediated induction of tolerogenic DCs came from a tumor model where AFP has been demonstrated to induce tolerogenic DC capable of suppressing tumor-specific CD8$^+$ cytotoxic T lymphocytes within the tumor (114). Hormonal effects on DCs are summarized in **Figures 1** and **2**.

EFFECT OF PREGNANCY HORMONES ON B CELLS

B lymphocytes are allocated to the adaptive immune system and they are best known for their capability to secrete antibodies. However, B cells do more than producing antibodies. They efficiently present antigens and modulate the function of T cells and DCs by producing cytokines (115, 116). During pregnancy, various B cell subsets are proposed to differently affect pregnancy outcome [reviewed in Ref. (117)]. Basically, B cells can be divided in two main populations, namely B1 and B2 B cells. These two populations differ in their developmental origin, surface marker expression and function (118). B1 B cells are then further subdivided in B1a and B1b B cells based on the expression of the surface marker CD5 (119). Interestingly, B1a B cells produce a specific type of antibody, the so-called natural antibodies (120). Due to their poly-reactive nature, natural antibodies are suggested to induce autoreactivity and thus are involved in the onset of autoimmune diseases (121). Even during pregnancy, B1a B cells producing natural antibodies are proposed to have detrimental effects on pregnancy outcome. We recently showed that the number of B1a B cells significantly decrease in the third trimester of normal pregnant women while remain elevated in pre-eclamptic patients (122). As patients suffering from pre-eclampsia have augmented serum hCG levels compared to normal pregnant women, we assumed that hCG may be responsible for the increased B1a B cell number. This assumption is further underlined by the fact that almost all B1a B cells express the LH/CG receptor and expand *in vitro* upon hCG stimulation (122). It was however shown that hCG inhibited antibody formation of murine B cells (123, 124). However, these two mentioned studies did not focus their analysis on B1a B cells but in total B cells. It is tempting to speculate that hCG may influence cell phenotype and antibody production differently in different B cell subsets. In contrast to the detrimental effect proposed for natural antibodies on pregnancy outcome, asymmetric antibodies (AABs), due to their structural anomaly, favor pregnancy success by reducing alloreactive immune responses (125). AABs increase during

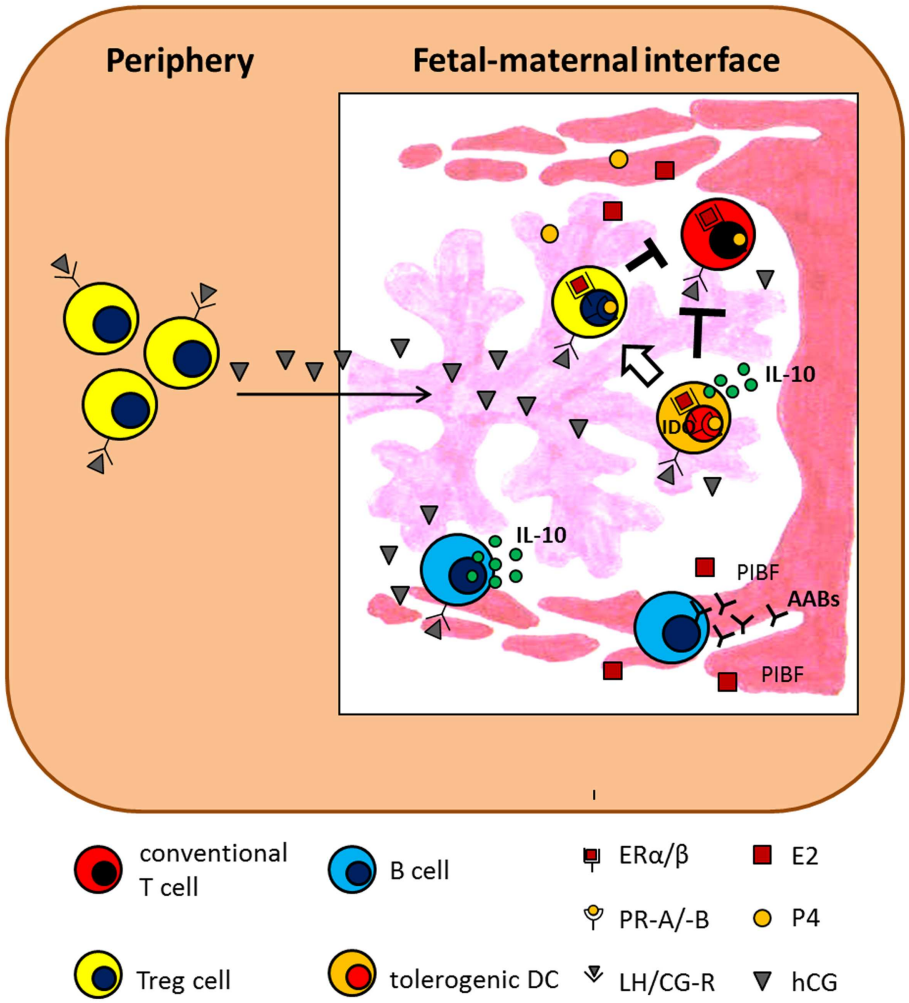

FIGURE 3 | Hypothetical scenario presenting the influence of pregnancy-associated hormones on adaptive immunity. The scenario suggests several mechanisms by which E2, P4, and hCG influence adaptive immune cells and thereby support pregnancy success. AABs, asymmetric antibodies; ER, estrogen receptor; IDO, indoleamine 2,3-dioxygenase; IL-10, interleukin-10; LH/CG-R, luteinizing hormone/chorionic gonadotropin receptor; PIBF, progesterone-induced blocking factor; PR, progesterone receptor.

pregnancy and their lack is associated with pregnancy failure in humans (126, 127). Secretion of AABs seems to be, at least partially, hormone regulated. P4 but not E2 provoke AAB secretion (128). Hereby, P4 mediates its function via induction of PIBF (129). Beside an influence on antibody formation and secretion, pregnancy hormones also regulate B cell development and cytokine secretion. Administration of E2 alone, or in combination with P4, preferentially suppressed IL-7 responding cells and their progeny in bone-marrow (130). In contrast to P4 and E2, human B cell stimulation by hCG seems to be highly dependent on hCG doses and purity of hCG preparations (131–133). Recently, we proposed another pregnancy-protective effect of hCG, namely its capacity of increasing the regulatory function of human regulatory B cells (Breg or B10) shown to contribute to fetal survival. Breg express the LH/CG receptor and increase their IL-10 production in response to hCG treatment (134). Hormonal effects on B cells are summarized in **Figure 3**.

EFFECT OF PREGNANCY HORMONES ON T CELLS

T cells are key regulators of both the antibody and the cell-mediated arms of the adaptive immune system and can be divided into two subcategories, CD4 expressing T helper cells and cytotoxic T cells expressing CD8. According to their cytokine secretion pattern, they are usually further subdivided in Th1, Th2, and Th3 cells although it is well known that this classification is oversimplified as further subsets as Th9 and Th17 has been described. T cells mediate their function either by direct cell–cell contact or indirectly by the secretion of cytokines defining the local environment as a pro-inflammatory or anti-inflammatory one. Normal pregnancy is associated with a pro-inflammatory Th1 profile at early and late pregnancy stages being important for a proper blastocyst implantation and initiation of labor, respectively. At midgestation, an anti-inflammatory Th2 profile guarantees tolerance of the foreign fetal antigens. Imbalances in cytokine profiles were associated with human pregnancy complications (135), suggesting a major

part for T cells in fetal tolerance by regulating the local cytokine milieu. Steroid hormones are reportedly involved in modulating cytokine secretion by different T cell subsets. P4 and E2 are proposed to influence the Th1/Th2 balance favoring a Th2 predominance at the fetal–maternal interface in humans and mice (136–144). Hormonal effects are mainly mediated via their classical receptors expressed by human and murine T cells (16, 103, 143, 145, 146). However, Chien and colleagues provide evidence that P4 may act through non-classical steroid receptors to cause immune modulation and suppression of T cell activation during pregnancy (147). In addition to regulate the cytokine secretion of T cells, steroid hormones and their mediators (e.g., glycodelin A) induce apoptosis of effector T cells (148–150) and increase the expression of pregnancy-protective molecules such as the leukemia inhibitory factor (LIF) known to modulate immune responses during early human and murine pregnancy (151, 152). In line with this, hCG is proposed to support fetal survival by modulating murine T cell activity and function (153, 154). More precisely, Khil and colleagues demonstrated that hCG prevented the development of the disease in a murine model of autoimmune diabetes. Here, hCG treatment efficiently suppressed IFN-γ production, but increased IL-10 and TGF-β production in splenocytes. hCG application also suppressed TNF-α production (155). In contrast, AFP apparently has no direct effect on T lymphocytes. T cells stimulated with AFP retain their full capacity to respond in mixed lymphocytes culture (MLC) and cell-mediated lympholysis. However, AFP provokes major changes in the functional status of monocyte-enriched, MLC-stimulating cell population. This monocyte-enriched population induces T suppressor cells while at the same time, suppresses generation of cytotoxic T cells (156–158).

Pregnancy-associated hormones not only affect conventional T cells but have also been shown to support the generation and function of Treg cells. Treg cells represent a specialized subset within the T cell compartment with unique properties. During pregnancy, they are fundamentally involved in the suppression of alloreactive immune responses and, thus their absence results in worse pregnancy outcome both in humans (159) and mice (160). The presence of Treg cells in the uterus before pregnancy seems to be very relevant as well. A diminished endometrial expression of their major transcription factor Foxp3 is associated with infertility in women (161) while depletion of Treg cells in a murine model derived in a hostile uterine microenvironment hindered the implantation of the embryo (162). During the reproductive cycle, Treg cells fluctuate (162–164) and this was suggested to be hormone-driven (165). This could be interpreted as hormones preparing the mother and particularly the tissue for pregnancy. Already in the 80s hCG was assumed to induce human and murine suppressor T lymphocytes (166–168). Nowadays, steroid hormones and gonadotropins are proposed to regulate several aspects of Treg cell biology, including generation, expansion, migration, and suppressive function both in humans and mice (103, 145, 169–176). However, some studies provide evidence that P4 and E2 induce a reduction of Treg cells or did not provoke changes in the number of Treg cells (177–179). Thus, these contradictory findings may depend on the markers used to define Treg cells, the time point of analysis and the model used. Hence, more research is needed to understand at which time point and through, which mechanisms pregnancy hormones influence Treg cell function. Hormonal effects on T cells are summarized in **Figures 2** and **3**.

THERAPEUTIC POTENTIAL OF PREGNANCY-ASSOCIATED HORMONES IN ASSISTED REPRODUCTIVE TECHNIQUES

Immunological disorders have been suggested as one of the major reasons for unexplained infertility, implantation failures, and recurrent pregnancy loss. It has been discussed that alteration in the number and function of immune cell populations during the reproductive cycle and in early pregnancy stages may result in the inability to become pregnant and in worse pregnancy outcome. Women undergoing assisted reproductive techniques (ART) often present recurrent implantation failures after *in vitro* fertilization (IVF) and embryo transfer (ET). Hormonal treatment with steroid hormones and gonadotropins before and after ART was shown to support successful implantation and thereby improve pregnancy rates (26, 180–182). Based on the data discussed here, it can be speculated that hormonal treatment in ART counteract immunological disorders and may therefore, at least partially explain improved pregnancy rates. By affecting immune cell populations and their products, hormones are suggested to influence the local environment and thereby support an optimal implantation of the embryo (3). Lukassen and colleagues showed that hormonal stimulation for IVF treatment positively affected the $CD56^{bright}/CD56^{dim}$ ratio in the endometrium by a relative decrease in the cytotoxic $CD56^{dim}$ $CD16^{+}$ NK cell number. Moreover, the same authors observed an increase in B cells and macrophages and these effects were restricted to the endometrium and could not be observed in peripheral blood (183). By doing so, hormones may contribute to tissue remodeling and angiogenesis resulting in a proper placentation and fetal nourishment. The positive effect of hCG on ART is underlined by a study of Mansour and colleagues who showed that intrauterine injection of hCG before ET in patients undergoing IVF significantly improved implantation and pregnancy rates (180). Unfortunately, the authors did not analyze the number and activity of Treg cells although augmented pregnancy rates after IVF have been associated with elevated Treg cell numbers in peripheral blood (184). Interestingly, based on our observation that hCG efficiently attracts Treg cells to trophoblasts (145), the Egyptian IVF-ET Center conducted a clinical trial investigating the effect of intrauterine injection of hCG on endometrial Treg cells. Upcoming results will prove whether hCG treatment around implantation time may increase endogenous endometrial Treg cell levels and thereby favor the implantation process.

CONCLUSION

Altogether, an exhaustive analysis of the literature indicates a crucial role for sex hormones on a variety of immune cell populations building up a complex network of interactions between the endocrine and the immune system. Irritations in this fine regulated balance may result in implantation failure and undesired pregnancy outcome. Thus, further investigations on the immune modulating functions of pregnancy-associated hormones will improve our understanding of endocrine–immune interactions before and during pregnancy and may help to develop selective strategies in the treatment of infertility and pregnancy complications.

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Innate and adaptive immune interactions at the fetal–maternal interface in healthy human pregnancy and pre-eclampsia

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Maternal immune tolerance of the fetus is indispensable for a healthy pregnancy outcome. Nowhere is this immune tolerance more important than at the fetal–maternal interface – the decidua, the site of implantation, and placentation. Indeed, many lines of evidence suggest an immunological origin to the common pregnancy-related disorder, pre-eclampsia. Within the innate immune system, decidual NK cells and antigen presenting cells (including dendritic cells and macrophages) make up a large proportion of the decidual leukocyte population, and are thought to modulate vascular remodeling and trophoblast invasion. On the other hand, within the adaptive immune system, $Foxp3^+$ regulatory T cells are crucial for ensuring immune tolerance toward the semi-allogeneic fetus. Additionally, another population of $CD4^+HLA\text{-}G^+$ suppressor T cells has also been identified as a potential player in the maintenance of immune tolerance. More recently, studies are beginning to unravel the potential interactions between the innate and the adaptive immune system within the decidua, that are required to maintain a healthy pregnancy. In this review, we discuss the recent advances exploring the complex crosstalk between the innate and the adaptive immune system during human pregnancy.

Keywords: pregnancy, pre-eclampsia, T regulatory cells, decidual, NK cells, $CD4^+HLA\text{-}G^+$, dendritic cells

INTRODUCTION

Pregnancy presents a significant challenge to the maternal immune system. In humans, the maternal immune system must tolerate the semi-allogeneic fetus throughout the 9 months of pregnancy. The remarkable nature of this phenomenon was recognized by Peter Medawar in the 1950s (1), whose work on skin graft rejection in genetically different individuals, led him to perceive this apparent immunological paradox. At the time, he proposed that three factors contribute to this phenomenon: (1) the anatomical separation between the mother and the fetus, (2) the reduced antigenic property of the fetus, and (3) the immunological inertness of the maternal immune system.

These proposals have significantly influenced subsequent research in the field. Indeed, it is now well-known that fetal cells are largely separated from the maternal immune system, with the point of contact being fetal extravillous trophoblast (EVT) cells, which have poor antigenic properties owing to the lack of expression of classical MHC class I (except HLA-C) and MHC class II molecules (2). However, as fetal–maternal microchimerism is a well-recognized occurrence during human pregnancy, fetal cells frequently induce maternal immune activation (3, 4) as evidenced by the detection of anti-fetal HLA antibodies in maternal serum during pregnancy (5, 6). Additionally, although direct MHC presentation of fetal antigens by fetal cells generally does not occur, fetal antigens can be processed and presented by maternal antigen presenting cells (APCs) at the fetal–maternal interface (7). Indeed, various different subsets of maternal immune cells are present at the fetal–maternal interface, which is the decidua, the mucous membrane (endometrium) of the pregnant uterus. In fact, up to 50% of the cells in the decidua are maternal immune cells (8). The decidua is therefore, an important site where the maternal immune system encounters fetal antigens and must develop tolerance mechanisms.

Not surprisingly, many of the pregnancy-related disorders such as recurrent miscarriages and pre-eclampsia are thought to be due to the breakdown of this immune tolerance (6, 9, 10). In pre-eclampsia, whilst the clinical manifestations such as hypertension and proteinuria are thought to be due to endotheliopathy secondary to insufficient placentation (11, 12), the shallow fetal trophoblast invasion is likely related to partial breakdown of maternal–fetal immune tolerance (9).

In this review, we will explore the role of decidual innate and the adaptive immune cells in facilitating tolerance to the fetus. In particular, we will highlight some of the recent advances documenting the interaction between these cells, drawing comparisons between healthy human pregnancy and pre-eclampsia.

INNATE IMMUNE CELLS AT THE FETAL–MATERNAL INTERFACE

DECIDUAL ANTIGEN PRESENTING CELLS DURING PREGNANCY

Antigen presenting cells are likely to be important players in the mediation of immune tolerance in the decidua. In mice, a previous

study has shown that maternal APCs take up apoptotic debris from the fetal/placental cells and present fetal antigens to maternal T cells. As the major histocompatibility antigens (classical MHC I and II antigens) are suppressed on fetal trophoblast cells to evade maternal immune recognition, antigen presentation of fetal minor histocompatibility antigens by maternal APCs is an important route for immune recognition (7). Therefore, exploring the characteristics of the decidual APCs and their interaction with decidual T cells is of great importance in the understanding of fetal–maternal immune tolerance.

DECIDUAL DENDRITIC CELLS IN HEALTHY PREGNANCY AND PRE-ECLAMPSIA

Study of decidual dendritic cells (dDCs) has been difficult, not only because isolation of decidual cells including dDCs can be technically demanding, but also because phenotypic definition of DCs is controversial as there is no single specific marker for DCs. In this particular section, we refer primarily to the lineage negative HLA-DR$^+$ classical DCs. Using lineage negative and HLA-DR$^+$ as combination marker for dendritic cell (DC), Gardener et al. found that dDC comprises ~1% of the total decidual cell isolates in first trimester decidua (13). These DCs were CD11c$^+$, CD1a$^-$, and CD123$^-$, indicating a myeloid rather than plasmacytoid origin. Interestingly, they showed that these DCs were DC-SIGN$^-$, compared to CD14$^+$ "macrophages," which were DC-SIGN$^+$. These results were further explored in a later study by Ban et al., who showed that first trimester lineage negative and HLA-DR$^+$ dDCs predominantly expressed BDCA1 and BDCA3 surface antigens, corresponding with different subsets of myeloid DCs (14).

Overall, due to the difficulty in decidual mononuclear cell isolation and the rarity of dDCs, functional studies on these DCs are scarce. In a study by Kammerer et al., the authors demonstrated a small population of mature CD83$^+$ DCs as well as CD1a$^+$ DCs in human first trimester decidua, by both immunohistochemistry and flow cytometry (15). They went on to show that the CD83$^+$ cells are potent stimulators in mixed lymphocyte reactions comparable to mature peripheral blood monocyte-derived DCs. Another study by Laskarin et al. showed that CD1a$^+$ DC isolated from decidua stimulated NK cell activity and proliferation better than decidual CD83$^+$ DCs (16). Their experiments were done *in vitro*, however, and there was no demonstration of CD1a$^+$ or CD83$^+$ DC interaction with decidual NK cells *in situ*. An earlier study demonstrated that lineage− and HLA-DR$^+$ DCs in first trimester human decidua were mostly of myeloid origin, but produced less IL-12 compared to their peripheral counterparts. They also showed that dDCs were more likely to prime CD4 cells into a Th2 phenotype compared to their peripheral counterparts (17). The authors concluded that such polarization of the immune response toward Th2 has potential roles in averting Th1-mediated rejection of the fetus.

Studies of decidual DC functions in mice are more definitive. Selective ablation of CD11c$^+$ decidual DCs leads to failure of decidualization and embryo implantation (18), highlighting the potential role of dDCs in the initiation of successful pregnancy. Another study demonstrated that during murine pregnancy, decidual CD11c$^+$ DCs fail to migrate to draining lymph nodes due to absent lymphatic vessels and CCL21 (ligand for lymphoid homing CCR7) expression in the murine decidua and therefore do not significantly contribute to anti-fetal T cell responses (19). However, it is important to note that in contrast to mice, lymphatic vessels are abundant and CCL21 is expressed within the human decidua (20, 21), which therefore might facilitate decidual DC migration in humans. Furthermore, whilst these studies shed light on the function of CD11c$^+$ DCs in mice, it is difficult to know whether these CD11c$^+$ cells are comparable to the lineage negative, HLA-DR$^+$CD11c$^+$ DCs in human decidua. Nevertheless, at least in mice, decidual CD11c$^+$ DCs appear to be important for the initiation of pregnancy and maintenance of immune tolerance.

So far, few studies have examined the role of decidual DCs in pre-eclampsia. Huang et al. found that there were increased numbers of CD83$^+$ and DC-SIGN$^+$ APCs in the pre-eclamptic decidua (22). Scholz et al. partially confirmed this finding showing increased numbers of DC-SIGN$^+$ cells in the decidua of patients affected by HELLP syndrome, a severe form of pre-eclampsia (23). However, it is important to note that DC-SIGN$^+$ APCs in particular, are likely a different group of cells distinct from lineage negative HLA-DR$^+$CD11c$^+$ classical myeloid DCs (discussed above), as highlighted in subsequent sections.

DECIDUAL MACROPHAGES IN HEALTHY PREGNANCY AND PRE-ECLAMPSIA

Macrophages are specialized phagocytic cells of the innate immune system and they are present in every organ of the body in one form or another. Macrophages, like DCs, are part of the mononuclear phagocyte system consisting of committed bone marrow precursors, peripheral blood monocytes and DCs, as well as tissue macrophages and DCs (24). Whilst many have attempted to separate macrophages from DCs based on phenotype and function, significant controversy exists as to whether these cells are indeed distinct from one another (25).

CD14$^+$ decidual macrophages (dMacs) comprise about 10–20% of decidual CD45$^+$ leukocyte population (26). Their phenotype has been characterized in several studies. In a study of human CD14$^+$ dMacs, Heikinnen et al. (27) observed that compared to the peripheral blood monocytes, dMacs expressed lower level of co-stimulatory molecule CD86. This coupled with the expression of indoleamine 2,3-dioxygenase (IDO), known to have an immunosuppressive effect on T cells, led them to conclude that dMacs have an "immunosuppressive" phenotype. Notably however, their data showed that dMacs expressed higher level of HLA-DR, as well as the co-stimulatory molecule CD80, compared to peripheral blood monocytes. Another study by Repnik et al. (28) confirmed the expression of HLA-DR, CD80, and CD86 on dMacs. They further showed that expression of these markers were higher earlier in the gestation, implying greater dMac activation at the time of implantation. A more recent study examined dMac in the first trimester using gene micro-array analysis. The authors found that compared to peripheral blood macrophages, dMacs have a gene expression profile, which biases toward alternatively activated macrophages or M2 phenotype, which suggests that dMacs are likely immunosuppressive (29).

In a study of dMac function, Mizuno et al. (30) showed that dMacs have antigen presentation capacity, but are less stimulatory and produce less IL-1 than peripheral blood monocytes in

mixed lymphocyte reactions. The suppressive activity of dMac has also been supported by other studies (31). The cytokine profile of dMac was also examined by Heikkinen et al., showing that term decidual $CD14^+$ dMac spontaneously produced significantly more IL-10 than peripheral blood monocytes *ex vivo*. In addition, these macrophages were less able to differentiate into mature DCs *in vitro* under polarizing conditions, possibly owing to their production of IL-10 (27). Thus, it is likely that dMacs are a special subset of APCs specialized in tolerance induction. In addition, there is evidence that dMacs are also involved in vascular remodeling (5, 32) and parturition in the peripartum period (33, 34).

In a large study with 33 pre-eclamptic patients and 66 controls, Rieger et al. examined decidual leukocyte populations using flow cytometry (35). They did not find any difference in HLA-DR, dendritic cell specific intercellular adhesion molecule 3 (ICAM3) grabbing non-integrin (DC-SIGN), or CD14 expression within $CD45^+$ cells between healthy pregnancy and pre-eclampsia. In a smaller study, Schonkeren et al. compared the distribution and phenotype of $CD14^+$ dMacs between preterm control pregnancies and preterm pre-eclampsia (36). Using sequential or two-color immunohistochemistry, they found reduced CD163/CD14 ratio [CD163 being a marker of alternatively activated macrophage or M2 (37)], increased DC-SIGN/CD14 ratio, and reduced IL-10 expression in preterm pre-eclamptic pregnancies, which may suggest a more pro-inflammatory phenotype of dMacs in pre-eclampsia. More recently, we examined decidual $CD14^+$ APCs in more detail during healthy pregnancy and pre-eclampsia using multi-color flow cytometry (38). However, in this study, we focused on the distinct subset of $CD14^+$DC-$SIGN^+$ APCs, which is discussed below.

DECIDUAL $CD14^+$DC-$SIGN^+$ APCs IN HEALTHY PREGNANCY AND PRE-ECLAMPSIA

Dendritic cell specific ICAM3 grabbing non-integrin is an ICAM3 receptor, where ICAM3 is an adhesion molecule. DC-SIGN, also known as CD209, is important for the initiation of DC and T cell interaction (39). Despite its name, DC-SIGN may be expressed by a variety of APCs other than classical lineage negative HLA-DR^+ DCs, including $CD14^+$ macrophages (40). Nevertheless, in monocyte-derived DCs, DC-SIGN is one of the markers upregulated in maturing DCs in mice (33) and humans (39). Therefore, whilst the expression of DC-SIGN is not DC specific, it probably marks myeloid cells, which are on the DC differentiation pathway (i.e., immature DCs).

In the human decidua, Kammerer et al. found that a significant percentage of $CD14^+$HLA-DR^+ APCs expressed DC-SIGN in the first trimester decidua (41). These $CD14^+$DC-$SIGN^+$ cells did not express CD83, but expressed CD4. Interestingly, the authors found these cells to be unique to the decidua in pregnancy and not in normal non-pregnant endometrium. In addition, these cells show a high proliferative rate and good antigen uptake, but poor stimulatory activity in MLR. Importantly, these cells have a veiled appearance typical of immature DCs on immunohistochemistry and can be matured *in vitro* with a cocktail of inflammatory cytokines into $CD83^+$ mature DCs, with decreased CD14 and DC-SIGN expression, as well as potent stimulatory activity in MLR. The authors concluded that these $CD14^+$DC-$SIGN^+$ cells are likely to be precursors of DCs and may play an important role in mediating fetal–maternal immune tolerance. Repnik et al. also confirmed DC-SIGN expression in decidual $CD14^+$ APCs and showed that DC-SIGN expression peaked in the second trimester (28). Obviously, decidual $CD14^+$DC-$SIGN^+$ APCs would be included in studies examining dMacs in view of their CD14 expression. Such studies include recent work by Svensson et al., who showed that $CD14^+$ dMacs can be divided into two distinct groups based on ICAM3 expression, with the $ICAM3^-$ group expressing DC-SIGN and markers of alternative (M2) macrophage activation (CD163, CD206, neuropilin) (42). They further showed that the phenotype of these DC-$SIGN^+$ dMacs may be replicated *in vitro* (with similar gene expression profile) in the presence of M-CSF (and/or GM-CSF) plus IL-10. Another study divided first trimester $CD14^+$ dMacs into $CD11c^{hi}$ and $CD11c^{lo}$ cells corresponding to DC-$SIGN^-$ and DC-$SIGN^+$ cells, respectively (43). Using gene expression profiles, the authors here showed that neither of the $CD11c^{hi}$ or $CD11c^{lo}$ macrophages corresponds to *in vitro* differentiated M1 or M2 macrophages exactly, though $CD11c^{hi}$ macrophages were skewed toward maternal peripheral blood monocytes and shared common genes with synovial macrophages from rheumatoid arthritis patients. In the same study, the authors showed that $CD11c^{hi}$ dMac produced significantly more TNFα, IL-6, and paradoxically IL-10 compared to $CD11c^{lo}$ macrophages. On the other hand, there was a slight trend toward increased TGFβ secretion by $CD11c^{lo}$ cells.

Collectively, these studies confirm that the human decidua harbors two distinct populations of $CD14^+$ APCs, one which is $CD11c^{lo}$DC-$SIGN^+CD206^+CD163^+$neuropilin$^+ICAM3^-$ and is likely immunoregulatory and important for tolerance induction, the other which is $CD11c^{hi}$DC-$SIGN^-CD206^-CD163^-$ neuropilin$^-$ $ICAM3^+$ and probably pro-inflammatory and important for tissue remodeling. In our recent study, we examined term decidual $CD14^+$DC-$SIGN^+$ APCs in detail using multi-color flow cytometry. We show that decidual $CD14^+$DC-$SIGN^+$ APCs expressed significantly higher amount of tolerogenic molecules (HLA-G and ILT4), lymphoid homing molecule (CCR7), as well as antigen presentation apparatus (HLA-DR, CD80, CD86), but less CD14 than $CD14^+$DC-$SIGN^-$ cells (38). This suggests that decidual $CD14^+$DC-$SIGN^+$ APCs may be further along the differentiation pathway than their DC-$SIGN^-$ counterparts and that these cells possess enhanced tolerogenic properties. Both of these observations are consistent with the previously described studies (42, 43). The tolerogenic properties are likely induced by IL-10, which is known to upregulate HLA-G and ILT4 (44, 45). Interestingly *in vitro*, we were able to differentiate peripheral blood monocytes into $CD14^+$DC-$SIGN^+$HLA-G^+ILT4^+ APCs by adding IL-10 to the DC polarizing protocol (with GM-CSF and IL-4) (46). In the context of the blurred border between macrophages and DCs, and given that the phenotype of decidual DC-$SIGN^+$ APCs may be replicated *in vitro* with both DC or macrophage polarizing protocols, we suggest that decidual $CD14^+$DC-$SIGN^+$ APCs are likely an intermediate cell type on the continuum of macrophage/DC differentiation under the influence of IL-10. Whether decidual $CD14^+$DC-$SIGN^-$ APCs are completely distinct from, or on the same developmental continuum as, $CD14^+$DC-$SIGN^+$ APCs is

Table 1 | Differences between decidual DC-SIGN$^+$ and DC-SIGN$^-$ APCs.

	DC-SIGN$^+$	DC-SIGN$^-$
LINEAGE MARKER		
CD14 (38)	Intermediate	High
CD4 (41)	Intermediate	Low
ANTIGEN PRESENTATION APPARATUS (38)		
HLA-DR	High	Intermediate
CD80	High	Low
CD86	High	Intermediate
ADHESION MOLECULES		
CD11c (43)	Low	High
ICAM3 (42)	Low	High
CCR7 (38)	High	Low
M2 MARKERS (42)		
CD163	High	Low
Neuropilin	High	Low
TOLEROGENIC MOLECULES (38)		
HLA-G	High	Low
ILT4	High	Intermediate
CYTOKINE PRODUCTION (43)		
TNFα, IL-6	Low	High
IL-10	Low	High
TGFβ	High	Intermediate
% CD14+ cells (first trimester) (43)	~70%	20–30%
In vitro differentiation	M-CSF ± GM-CSF + IL-10 (42); GM-CSF+IL-4+IL-10 (46)	Unknown
In vitro differentiation to DC (41)	Yes	Probably
Likely function	Immune regulation	Tissue remodeling

currently unknown. However, the later hypothesis is supported by the fact that CD14$^+$DC-SIGN$^-$ APCs are closer to peripheral blood monocytes (43) and possess less antigen presentation apparatus. The differences between decidual CD14$^+$DC-SIGN$^+$ and CD14$^+$DC-SIGN$^-$ APCs are summarized in **Table 1**.

Interestingly in pre-eclampsia, we found an increased percentage of DC-SIGN$^+$ APCs within the CD14$^+$ population, however, pre-eclamptic decidual CD14$^+$DC-SIGN$^+$ APCs expressed significantly less HLA-G and ILT4 compared to the same cells in healthy pregnancy, suggestive of reduced tolerogenic capacity. We speculate that this phenotypic difference may be related to the reduced placental IL-10 levels in pre-eclamptic pregnancies (47).

In summary, there are several different types of APCs present in the decidua, including lineage negative HLA-DR$^+$CD11c$^+$ classical DCs, mature CD83$^+$ DCs, CD1a$^+$ DCs, and CD14$^+$DC-SIGN$^-$ dMac, which may have developed from peripheral blood monocytes and are probably precursors to decidual CD14$^+$DC-SIGN$^+$ APCs. Their potential relationships and differences in healthy pregnancy and pre-eclampsia are summarized in **Figure 1**.

DECIDUAL NK CELLS IN HEALTHY PREGNANCY AND PRE-ECLAMPSIA

Decidual NK cells (dNK) are the most abundant maternal leukocytes in the decidua, especially in the first trimester, making up 70% of the maternal CD45$^+$ leukocyte population. (48) The dNK cells are distinct from majority of peripheral blood NK cells, in that they are large, granular, and are CD56^{hi} and CD16$^-$ (8). The origin of these cells is unclear, although some have proposed possible recruitment of a subset of peripheral blood CD56^{hi} NK cells into the decidua (49). Interestingly, during early pregnancy, dNK accumulate as a dense infiltrate around the trophoblast cells, but they progressively decrease in number from mid-gestation onward (50). This timing seems to implicate that dNK cells may be involved in modulating trophoblast invasion and vascular remodeling. Indeed, dNK have been shown to produce vascular endothelial growth factor C (VEGFC), placental growth factor (PIGF), and angiopoietin 2 (ANG2).

Decidual NK cells may also be important in modulating the degree of trophoblast invasion, as they are seen in close proximity to the invading trophoblasts in the decidua. Certainly, dNK have been shown to express killer inhibitory receptor (KIR) (51), CD94/NKG2A (52), and ILT2 (53), which recognize HLA-C and HLA-G, respectively, expressed on trophoblast cells. The HLA-C–KIR interaction is thought to be important in the pathogenesis of pre-eclampsia. As HLA-C is dimorphic and KIR polymorphic, it has been shown that certain combinations of maternal KIR and fetal HLA-C lead to an increased risk of pre-eclampsia, possibly through modulation of trophoblast migration, implying that HLA-C–KIR interaction is important in placentation (54, 55). However, NK cell KIR and HLA-C mismatch clearly does not explain all cases of pre-eclampsia, since only 30% of pre-eclamptic pregnancies have the at-risk maternal KIR phenotype (KIR AA) (54). HLA-G–ILT2 interaction on NK cells on the other hand, has been shown to increase dNK secretion of inflammatory and proangiogenic factors such as IL-1β, IL-6, TNF, and IL-8 (56). NK cells themselves are also susceptible to modulation by the decidual cytokine milieu. Indeed, dNK cells are thought to be the mediator of fetal demise in IL-10-deficient mice treated with LPS, which conversely can be rescued by administration of IL-10 (57). This suggests that IL-10 may modulate dNK cell cytotoxicity.

Collectively, these data suggest that dNK cells are important for modulation of trophoblast invasion and decidual vascularization in pregnancy. However, the recognition and tolerance of paternal allo-antigens via APC presentation during pregnancy, clearly requires the participation of other limbs of the immune system, such as the adaptive immune cells.

ADAPTIVE IMMUNE CELLS AT THE FETAL–MATERNAL INTERFACE

The adaptive immune system distinguishes itself from the innate immune system by its antigen specificity and immunological memory. Therefore, the fact that pre-eclampsia is essentially a disease of primigravida and subsequent pregnancies with the same partner protect against pre-eclampsia (58, 59), supports the involvement of the adaptive immune system. Within the lymphocyte subsets, CD4$^+$Foxp3$^+$ regulatory T (Treg) cells in particular have been the subject of many studies, with their pivotal role in pregnancy now firmly established. Th17 cells, the

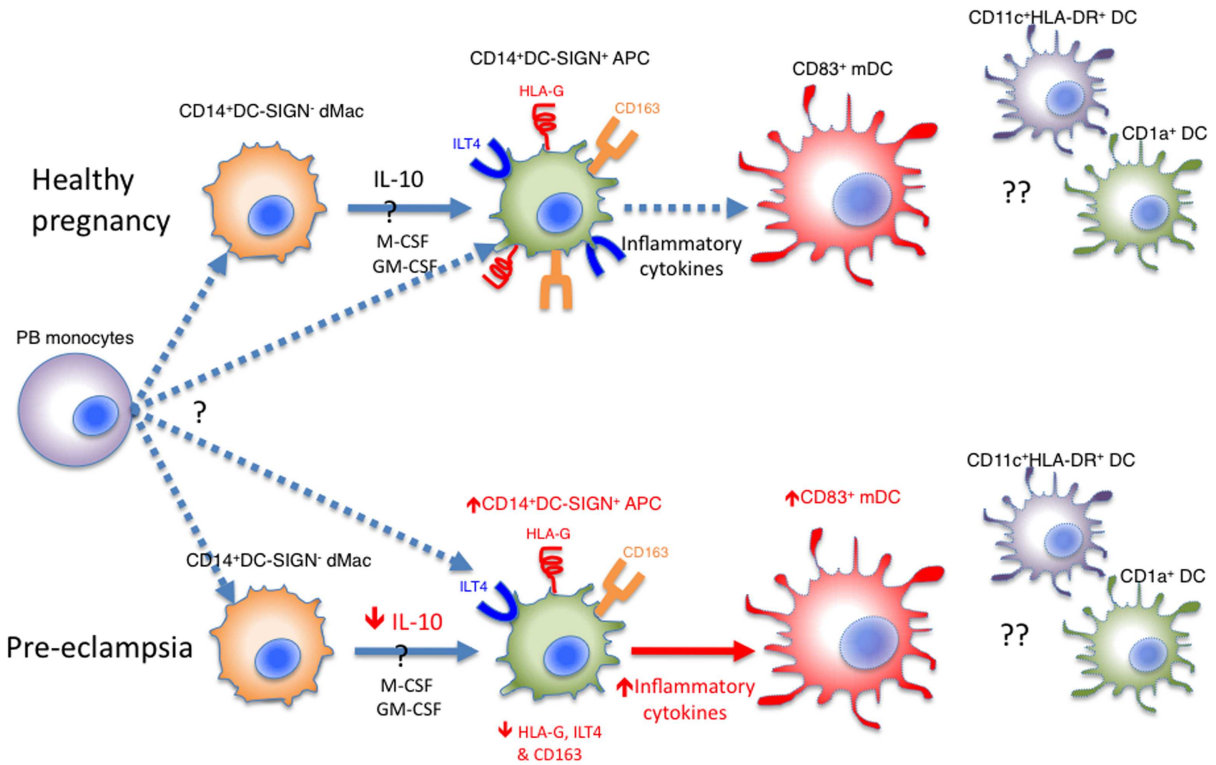

FIGURE 1 | Various APCs in the human decidua. Whether decidual CD14$^+$DC-SIGN$^-$ dMacs and CD14$^+$DC-SIGN$^+$ APCs are derived independently from peripheral blood (PB) monocytes is unknown. Alternatively, CD14$^+$DC-SIGN$^-$ dMacs may be on a continuum of DC differentiation, where local IL-10, M-CSF, and GM-CSF drive their development into CD14$^+$DC-SIGN$^+$ APCs expressing HLA-G and ILT4. These APCs may be matured into CD83$^+$ mature DCs (mDCs) under the influence of inflammatory cytokines, which in healthy pregnancy is minimal. In pre-eclampsia, both CD14$^+$DC-SIGN$^+$ APCs and mDCs increase, probably driven by increased inflammatory cytokines in this disease. This is coupled with reduced HLA-G and ILT4 expression by CD14$^+$DC-SIGN$^+$ APCs, likely due to reduced local IL-10 levels. Whilst CD11c$^+$HLA-DR$^+$ and CD1a$^+$ DCs have been identified in human decidua, their roles and relationship with CD83$^+$ DCs are unclear.

pro-inflammatory antagonist of Treg cells have also become a focus of studies in the last few years. More recently, CD4$^+$HLA-G$^+$ suppressor T cells have also been implicated for their potential role in healthy pregnancy and pre-eclampsia. Whilst other cells within the adaptive immune system such as Th1, Th2, gamma-delta T cells, and CD8$^+$ T cells also play a role in fetal–maternal immune tolerance (60–63), our focus in this review will be on Treg, Th17, and CD4$^+$HLA-G$^+$ suppressor T cells.

Treg CELLS IN HEALTHY PREGNANCY AND PRE-ECLAMPSIA

Foxp3$^+$ Treg cells are a unique subset of suppressive CD4$^+$ T helper cells indispensable for immune tolerance to self- and foreign-antigens in humans and mice (64–67). Several authors have shown that in pregnancy, there is an expansion of peripheral blood Treg cell pool in both humans (68, 69) and mice (70). The study by Somerset et al. showed that Treg cell population seems to peak in the second trimester and thereafter decreases to slightly above normal levels at delivery of the conceptus. Some have suggested that this expansion of Treg cell is not allo-antigen driven at least in the mice, as both syngeneically and allogeneically pregnant mice show expansion of the Treg cell population (70). However, the authors did not show a direct comparison of Treg cell percentage between syngeneic and allogeneic pregnancies. In contrast, Zhao et al. showed through direct comparison, that the percentage of peripheral blood Treg cells in allogeneic pregnancy is higher compared to syngeneic pregnancy, suggesting that the expansion of Treg cell is at least partially allo-antigen driven (71). Since then, other studies have demonstrated the fetal antigen-specific nature of maternal Treg cells during pregnancy (72, 73), further supporting the role of fetal allo-antigen in Treg cell expansion. Therefore, whilst other factors such as pregnancy-related hormones can also contribute to Treg cell expansion (74–76), it is likely that fetal allo-antigen stimulation is the primary driving force.

Given the decidua is the fetal–maternal interface and the likely place of fetal antigen encounter, it is not surprising that the proportion of Treg cells is even greater in the decidua during pregnancy compared to the peripheral blood (77, 78). The question is whether these Treg cells are recruited from the peripheral blood or induced locally. Currently in humans, despite some controversy, the only marker that differentiates thymus-derived natural Treg (nTreg) cells from peripherally induced Treg (iTreg) is Helios, where Helios$^+$ Treg cells are nTreg cells, which have acquired Treg cell phenotype in the thymus, whereas Helios$^-$ Treg cells have differentiated in the peripheral tissues/lymph nodes from naïve T

cells (79). Based on this premise, we found that the proportion of Helios$^-$ iTreg cells was significantly higher in the decidua compared to the peripheral blood (38). Interestingly, our results also indicate that the previously described peripheral blood expansion of Treg cells associated with healthy pregnancy is accounted for by the expansion of iTreg cells, and not nTreg cells. This suggests that in healthy pregnancy, iTreg cells are induced locally in the decidua/draining lymph nodes most likely in response to fetal allo-antigens. This observation is consistent with the murine studies previously discussed (72, 73), as well as the fact that iTreg cells are thought to facilitate tolerance to foreign- (in this case fetal) and self-antigens, whereas nTreg cells are primarily involved in self-tolerance (67, 80, 81).

Functionally, *in vivo* experiments in the murine model have shown that Treg cells are important for fetal–maternal immune tolerance. Aluvihare et al. showed that adoptive transfer of whole T cell populations to T cell-deficient pregnant mice did not result in fetal rejection, whereas transfer of T cells depleted of CD25$^+$ Treg cells led to fetal demise, especially in allogeneic pregnancies (70). This was confirmed by another method, where PC61 monoclonal antibody against CD25 was used to deplete Tregs in murine syngeneic and allogeneic pregnancy. The authors found that fewer fetuses in allogeneic pregnancies survived to term whereas syngeneic pregnancies were not affected by Treg cell depletion (82). This indicates that Treg cells are critically required for allogeneic but not syngeneic pregnancy.

Whereas total Treg cell depletion leads to almost complete fetal rejection (70, 82), depletion of the iTreg, but not nTreg, compartment in CNS1 (conserved non-coding sequence 1) deficient mice (CNS1 being critical for iTreg development) leads to partial fetal resorption (~10%) and abnormal spiral artery formation in allogeneic murine pregnancies (83). This phenotype is reminiscent of the human disease – pre-eclampsia, where IUGR (intrauterine growth retardation) and abnormal spiral artery remodeling with shallow placentation is a key pathological feature (84), being mindful of the caveat that there are significant differences between human and murine pregnancies (85).

Nevertheless, our investigations did show that the blunted peripheral blood Treg cell expansion in pre-eclampsia (69, 86, 87) is primarily due to the failure of iTreg cell expansion, whereas nTreg cells are not affected. Interestingly in the pre-eclamptic decidua, whilst we did not find a reduction in total Treg cell percentage, there was a significant reduction in the percentage of Helios$^-$ iTreg cells compared to healthy pregnancy (38). These observations suggest that in pre-eclampsia, there is impaired expansion of iTreg cells in the decidua, with compensatory nTreg cell recruitment to avert fetal rejection. Collectively, these data from murine and human studies suggest that iTreg and nTreg cells collaborate to maintain fetal–maternal immune tolerance, such that complete lack of Treg cells leads to fetal rejection, whereas specific iTreg cell deficiency results in poor placentation and fetal growth restriction.

Th17 CELLS IN HEALTHY PREGNANCY AND PRE-ECLAMPSIA

Th17 cells are a subset of CD4$^+$ T helper cells, which secrete the pro-inflammatory cytokines IL-17, IL-22, regulated by the transcription factor RORγt (88). Importantly, both iTreg and Th17 cells are derived from naïve CD4$^+$ T cells under the influence of TGFβ (89) in a concentration-dependent manner, with high levels of TGFβ favoring iTreg cell induction and low concentrations favoring development of Th17 cells (88, 90). Additionally, the presence of the pro-inflammatory cytokine, IL-6 is crucial for skewing T cell differentiation toward Th17 phenotype (91).

We have previously shown that the percentage of peripheral blood Th17 cells decreases in healthy pregnancy, in stark contrast to the expanding iTreg cell population. Whereas in pre-eclampsia, the percentages of peripheral blood Th17 and iTreg cell subsets remain comparable to the non-pregnant state. This leads to an increased Treg: Th17 ratio in healthy pregnancy, which is blunted in pre-eclampsia (69). These results were later replicated in other studies (92, 93). Collectively, these observations are congruous with the raised serum IL-6 level in pre-eclampsia compared to healthy pregnancy (94), which may contribute to the increased Th17 cells compared to normal pregnancy. Additionally soluble endoglin, a circulating TGFβ glycoprotein receptor capable of inhibiting TGFβ signaling, is elevated in pre-eclampsia (95). This would likely reduce the degree of TGFβ signaling, hence favoring Th17 cell differentiation. Therefore, current evidence indicates that in healthy pregnancy, there is a preferential differentiation of iTreg cell over Th17 cells, which is deranged in pre-eclampsia, possibly related to altered systemic levels of various factors such as IL-6 and soluble endoglin (**Figure 2**).

Currently, few studies have examined Th17 cells in the human decidua. A study by Mjosberg et al. attempted to examine the proportions of Th1, Th2, Treg, and Th17 cells in early pregnancy decidua, however, in this study, the authors used chemokine receptors as surrogate markers for Th1, 2, and 17 cells, which is less than ideal, they concluded that there is near absence of Th17 cells in early healthy pregnancy decidua (96). Wang et al. found increased peripheral blood and decidual Th17 cell percentage in women with unexplained recurrent miscarriages compared to healthy pregnancy. In their samples, the percentage of Th17 cells in the decidua appears to be comparable or even slightly lower than in

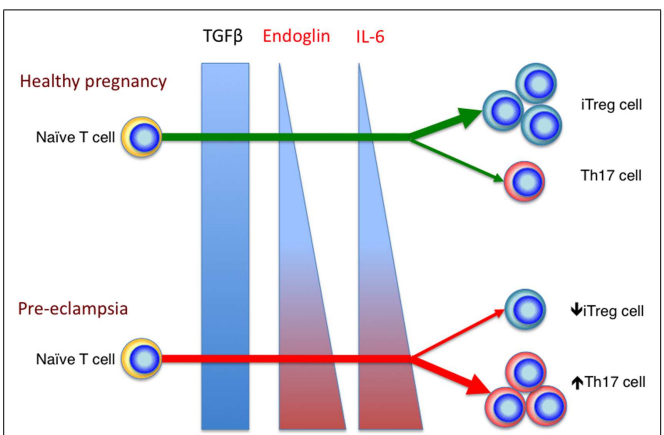

FIGURE 2 | Reciprocal development of iTreg and Th17 cells in healthy pregnancy and pre-eclampsia. In the relative absence of IL-6 and endoglin, TGFβ signaling enhances iTreg, but not Th17 cell differentiation in healthy pregnancy. In pre-eclampsia, the elevated level of endoglin could reduce TGFβ signaling, which in combination with the increased IL-6 level, would deter iTreg cell induction, but drive Th17 differentiation.

peripheral blood in both study groups (97). This is in contrast to another study, which showed higher percentages of Th17 cells in the decidua in healthy pregnancy (98). Therefore, current evidence demonstrates that Th17 cells are present in the decidua, although their prevalence is controversial. Furthermore, no study to date has examined the presence and prevalence of decidual Th17 cells in pre-eclampsia. It would be interesting to see whether there is an increase in decidual Th17 cells in pre-eclampsia, which would add to the body of evidence implicating local immune dysregulation in this disease.

$CD4^+HLA\text{-}G^+$ T CELLS IN HEALTHY PREGNANCY AND PRE-ECLAMPSIA

HLA-G is an atypical MHC class I molecule first discovered on human trophoblasts (99, 100). It exerts immunosuppressive effects on various immune cells, including APCs, NK cells, and T cells (101–103). Therefore, it is not surprising that it is expressed at immune privileged sites such as the decidua (99), the cornea (104), and thymic medulla (105). Interestingly, distinct subsets of $HLA\text{-}G^+$ T cells (both $CD4^+$ and $CD8^+$) are present at low levels in the peripheral blood of healthy donors (106). These cells are immunosuppressive but do not express Foxp3. They mediate suppression in a HLA-G and IL-10-dependent manner (107). Some evidence suggests that these cells originate from the thymus (106), however others have also shown that activated T cells can also "acquire" HLA-G from HLA-G expressing APCs, through the process of trogocytosis (108). Trogocytosis is a process by which membrane fragments including surface molecules are transferred from one cell to another in a contact-dependent manner (109). These HLA-G expressing T cells are similarly immunosuppressive (108, 110).

We and others have shown that the percentage of peripheral blood $CD4^+HLA\text{-}G^+$ T cells is significantly increased in healthy pregnancy, which is even more pronounced within the decidua, where up to 20% of the $CD4^+$ T cells are $HLA\text{-}G^+$ (46, 111). These $CD4^+HLA\text{-}G^+$ T cells are more mature and activated than their $HLA\text{-}G^-$ counterparts, they are $Foxp3^-$ but are immunosuppressive (46). Importantly, in pre-eclampsia there is impaired expansion of these $CD4^+HLA\text{-}G^+$ T cells in both the peripheral blood and the decidua. This is in keeping with the reduced serum HLA-G and placental HLA-G level in this disease, and reinforces the dysregulated adaptive immune responses in pre-eclampsia.

INNATE AND ADAPTIVE INTERACTION AT THE FETAL–MATERNAL INTERFACE

The evidence and discussions presented so far have focused on individual cell populations and their role in fetal–maternal immune tolerance, however, the immune system clearly does not work in isolation, but rather like an intricate, changing network. It is therefore important to investigate the interactions between the various immune cells, whether innate or adaptive at the fetal–maternal interface. In the following sections of this review, we will focus on the current available evidence in this regard and attempt to present a unifying concept, as well as future research directions for innate and adaptive immune interactions within the decidua.

DECIDUAL NK CELL CROSSTALK WITH INNATE AND ADAPTIVE IMMUNE CELLS

As discussed previously, dNK cells are important for modulating fetal trophoblast invasion and vascular remodeling. However, emerging evidence also suggests that dNK cells interact and modulate other maternal immune cells. Kammerer et al. first noted that dNK cells are closely associated with decidual $DC\text{-}SIGN^+$ APCs. They further demonstrated that this interaction occurs through ICAM3 (expressed on NK cells) and DC-SIGN interaction, although it was unclear what this interaction involves (41).

A later study demonstrated that dNK cells modulate decidual $CD14^+$ macrophages (dMac) to expand Treg cells *in vitro* (112). In this study, Vacca et al. demonstrated that interaction between dMac and dNK cells led to release of IFNγ by dNK cells, the IFNγ in turn induces upregulation of IDO in dMac. Importantly, IDO is known to contribute to immune suppression at the fetal–maternal interface (113, 114). It works by catabolizing tryptophan into L-kynurenine, which results in impaired T cell activation and favors Treg cell induction (115). Indeed, Vacca et al. showed that the IDO induction was important for subsequent Treg cell expansion by dMacs cultured with dNK, along with other factors such as TGFβ and CTLA-4 engagement. Notably, CTLA-4 engagement of APC co-stimulatory B7 molecules has also been shown to upregulate IDO expression in APCs (116). This could provide a continuous positive reinforcement loop, where the expanded CTLA-4 expressing Treg cells further enhance APC IDO expression. Interestingly, L-kynurenine inhibited the ability of peripheral blood NK cells, but not dNK cells to secrete IFNγ, which may explain how this negative feedback prevents peripheral blood NK cells from modulating dMacs in the same way. Although the authors did not clarify, these "dMacs" are probably decidual $DC\text{-}SIGN^+$ APCs, which interact with $ICAM3^+$ dNK cells via DC-SIGN. It is also important to note that the authors did not clearly demonstrate *de novo* Treg cell induction under these conditions, since the starting peripheral blood $CD3^+$ population would contain nTreg cell population. Nevertheless, the interaction between dNK and dMac appears to favor Treg cell proliferation and expansion through IFNγ-induced upregulation of IDO.

Interestingly, a more recent study showed that dNK production of IFNγ may be important for averting Th17 differentiation at the fetal–maternal interface (55). Here, the authors found that $CD56^{hi}CD27^+$ dNK cells are particularly primed to secrete IFNγ, which has been shown to inhibit Th17 differentiation via STAT1 activation (117). They went on to show that in the murine model, deletion of NK cells led to increased Th17 cell accumulation in the decidua and increased fetal loss. *In vitro*, dNK cell-derived IFNγ significantly inhibited Th17 cell differentiation, an effect that was reversed by IFNγ neutralizing antibodies. Finally they showed that there is reduced $CD56^{hi}CD27^+$ dNK cell:Th17 cell ratio in the decidua of women with recurrent miscarriages, accompanied by reduced IFNγ secretion by dNK cells in these women. These results suggest that a special subset of $CD56^{hi}CD27^+$ dNK cells may be important for limiting Th17 cell differentiation and inflammation in normal pregnancy via IFNγ. It is intriguing to contemplate whether this inhibition of Th17 cell differentiation may

paradoxically promote iTreg cell development, since both develop along the same pathway and reciprocally inhibit one another (84, 118). Furthermore, whether similar, but perhaps milder, pathophysiology may be found in pre-eclampsia is also of interest and requires further investigations.

Thus, these recent results highlight that NK cells are able to influence both decidual APCs and T cells through their secretion of IFNγ to promote immune tolerance.

APC AND T CELL INTERACTION AT THE FETAL–MATERNAL INTERFACE

Antigen presenting cells play important roles in shaping T cell responses and differentiation; T cells in turn also modulate APC function. In our recent study, we showed by immunohistochemistry that decidual DC-SIGN$^+$ APCs are closely associated with Foxp3$^+$ Treg cells (38). In fact the number of DC-SIGN$^+$ APCs correlated significantly with Foxp3$^+$ Treg cells in healthy pregnancy, but interestingly not in pre-eclampsia, suggesting a dysregulated relationship between these cells in this disease. We went on to show that decidual CD14$^+$DC-SIGN$^+$ APCs from healthy pregnant, but not pre-eclamptic women induced iTreg cells significantly more efficiently than CD14$^+$DC-SIGN$^-$ APCs. This suggests that there is an intrinsic defect in decidual CD14$^+$DC-SIGN$^+$ APCs in pre-eclampsia. This is consistent with the reduced expression of the tolerogenic molecules HLA-G and ILT4 in this cell subset in pre-eclampsia. These results are also consistent with Vacca et al.'s study described in the previous section (112), and reinforce that decidual DC-SIGN$^+$ APCs are an important population of cells in human pregnancy, which likely present fetal allo-antigens and induce local iTreg cells. Importantly, in contrast to CD14$^+$DC-SIGN$^-$ APCs, decidual CD14$^+$DC-SIGN$^+$ APCs express CCR7 (38), which suggests that they may also migrate to uterine draining lymph nodes and induce iTreg cells there.

The unresolved question is how these CD14$^+$DC-SIGN$^+$ APCs induce iTreg cells. Vacca et al.'s study suggests that TGFβ and IDO may be required for this process (112). This raises another intriguing question, as to whether the elevated endoglin levels in pre-eclampsia may impair TGFβ signaling and impede iTreg cell induction whilst promoting Th17 differentiation. It is important to note however, that our experiments were done *in vitro*, removed from the *in vivo* environment where endoglin may play a role. Therefore, our data indicates that there may be an intrinsic defect in CD14$^+$DC-SIGN$^+$ APCs in pre-eclampsia. Perhaps in pre-eclampsia, decidual CD14$^+$DC-SIGN$^+$ APCs secrete less TGFβ, or have reduced IDO expression?

The abundance of immunosuppressive CD4$^+$HLA-G$^+$ T cells in the decidua raises the question regarding how these cells have developed. Since HLA-G could be transferred from cell to cell via trogocytosis (108, 110, 119), we reasoned that these CD4$^+$ T cells could have acquired HLA-G from any of the HLA-G expressing cells in the decidua, including fetal EVT and maternal CD14$^+$DC-SIGN$^+$ APCs. To this end, we showed that *in vitro*, decidual CD14$^+$DC-SIGN$^+$ APCs, but not JEG3 cells (an EVT-like cell line), were able to induce CD4$^+$HLA-G$^+$ T cells from naïve T cells (46). This is consistent with the fact that T cell trogocytosis is facilitated by the formation of immunological synapse with TCR–MHC engagement (120–122), since EVTs and JEG3 cells do not express MHC II. We further showed that the acquisition of HLA-G from T cells occurred via trogocytosis, since PE labeled HLA-G was passed from the APC to responding T cells. Therefore, we suggest that in the human decidua, T cells activated by fetal allo-antigens may be "silenced" by their acquisition of HLA-G, indeed the HLA-G expressing T cells in the decidua exhibit an activated phenotype (46). Importantly in pre-eclampsia, there is significant reduction of CD4$^+$HLA-G$^+$ T cells, which may be secondary to the reduced HLA-G expression by decidual CD14$^+$DC-SIGN$^+$ APCs in this disease.

Based on these recent data, it appears that decidual CD14$^+$DC-SIGN$^+$ APCs are a unique and important population of cells, which play central roles in regulating local immune responses by their interactions with dNK cells and resident CD4$^+$ T cells. As described previously, decidual CD14$^+$DC-SIGN$^+$ APCs may have developed under the influence of local IL-10 (42, 46), which is probably derived from dNK and CD14$^+$DC-SIGN$^-$ dMac (41).

Therefore, IL-10 may be a central cytokine driving the differentiation of decidual tolerogenic APCs and suppressor T cells. Interestingly, IL-10 is dispensable for murine pregnancy in the germ free environment, but crucial when LPS is present (57), suggesting that IL-10 is important for controlling inflammation at the fetal–maternal interface. This is not dissimilar to the gut where bacteria colonized, but not germ free mice, with IL-10 deficiency develop severe enterocolitis (123, 124), and humans with defects in IL-10 signaling pathway develop early onset inflammatory bowel disease (125, 126). In the gut, it has been shown that it is the pathogenic T cells that cause disease in the IL-10-deficient mice (81). Furthermore, recent evidence suggests that IL-10 may enhance Treg cell function and augment suppression of pathogenic Th17 cells (127, 128). It remains to be seen whether IL-10 plays similar roles in pregnancy. Nevertheless, it is clear that IL-10 is a crucial regulatory cytokine at mucosal surfaces in the presence of inflammation and foreign antigens. Indeed pre-eclampsia, rather than being an overt rejection of the fetus, is more like a pro-inflammatory state with flaws in the regulatory mechanisms, including reduced IL-10, altered decidual DC-SIGN$^+$ APCs, and impaired suppressor T cell (iTreg cells and CD4$^+$HLA-G$^+$ T cells) differentiation; further fueled by enhanced pro-inflammatory effectors such as raised IL-6 level and enhanced Th17 cell differentiation.

CONCLUDING REMARKS

The fetal–maternal interface is an important frontier for immunology, as it represents the junctional point between two immunologically distinct individuals. Within this mucosal surface, the various maternal innate and adaptive immune cells must work together to ensure tolerance toward invading fetal cells and foreign fetal antigens. Whilst several APCs are present in the decidua, CD14$^+$DC-SIGN$^+$ APCs are unique at this interface and play central roles in regulating T cell responses, by inducing Foxp3$^+$ iTreg cells and CD4$^+$HLA-G$^+$ suppressor T cells. Some evidence suggests that IL-10 may be an important factor in these processes. Certainly in pre-eclampsia, where there is relative deficiency of IL-10, these tolerance mechanisms are impaired. Whether CD14$^+$DC-SIGN$^+$ APCs also influence decidual Th17 cell differentiation remains unknown and requires further investigations.

On the other hand, decidual NK cells, on top of their role in modulating trophoblast invasion and vascular remodeling, are able

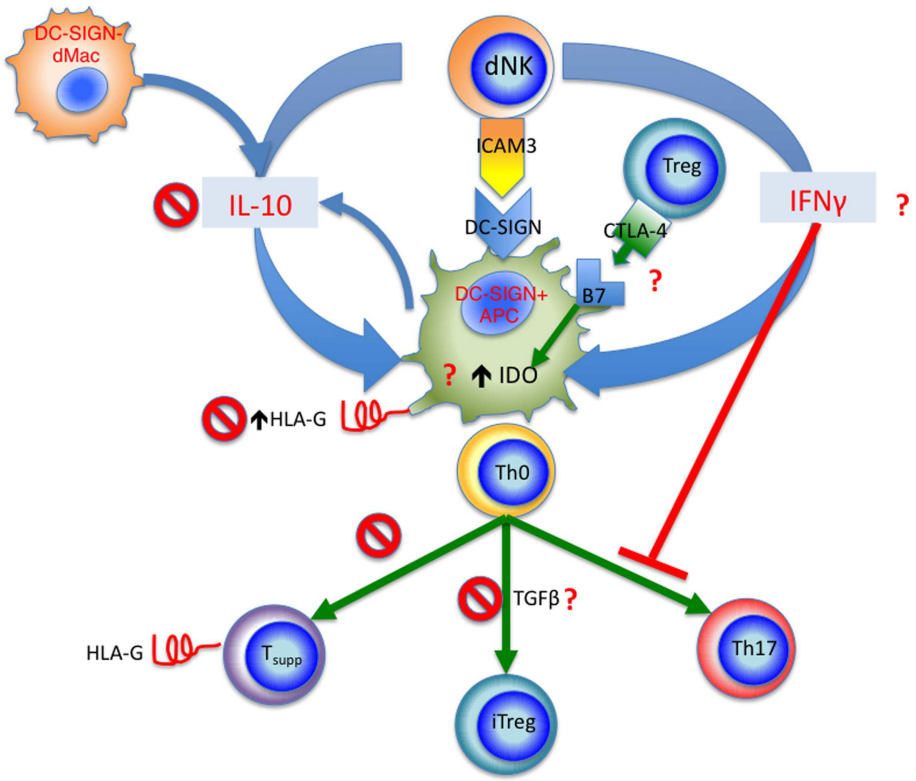

FIGURE 3 | Summary of proposed innate and adaptive interactions in human pregnancy. Decidual DC-SIGN$^+$ APCs appear to be a central player in these interactions. dNK cells interact with DC-SIGN$^+$ APCs via ICAM3, this leads to release of IFNγ, which in turn upregulates IDO in DC-SIGN$^+$ APCs, as well as inhibiting Th17 cell differentiation. The tolerogenic DC-SIGN$^+$ APCs function to induce iTreg cells from naïve CD4$^+$ T cells (Th0), Treg cells in turn regulated DC-SIGN$^+$ APCs via CTLA-4 and B7 (CD80 and CD86) interaction, further increasing IDO expression. IL-10 from dNK cells and DC-SIGN$^-$ dMacs acts to upregulate HLA-G expression by DC-SIGN$^+$ APCs; the HLA-G is then passed onto activated T cells via trogocytosis, resulting in accumulation of CD4$^+$HLA-G$^+$ T suppressor cells. In pre-eclampsia, several check points are affected as marked by the "No symbol." These include reduced IL-10 level, reduced HLA-G expression by DC-SIGN$^+$ APCs, reduced generation of CD4$^+$HLA-G$^+$ T suppressor cells, and reduced iTreg cell induction. Other yet unknown mechanisms may also be affected in pre-eclampsia marked by the red question mark, including IFNγ production by dNKs, CTLA-4 expression by Treg cells and interaction with B7 molecules, as well as IDO expression and TGFβ production by DC-SIGN$^+$ APCs.

to regulate decidual Th17 differentiation by their production of IFNγ. Interestingly, their production of IFNγ also modulate decidual CD14$^+$DC-SIGN$^+$ APCs by upregulating their IDO expression to enhance Treg cell expansion. Whether these processes are affected in pre-eclampsia remain to be seen. These interactions are summarized in **Figure 3**. Clearly, there are likely many other innate and adaptive interactions involving different cell types, which work to foster the delicate balance of immune tolerance at the fetal–maternal interface. Disturbance of the quality and quantity of these interactions likely contribute to the pathogenesis of pre-eclampsia, which like so many diseases of the modern era, is a disease of immune dysregulation.

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Monocytes and macrophages in pregnancy and pre-eclampsia

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Preeclampsia is an important complication in pregnancy, characterized by hypertension and proteinuria in the second half of pregnancy. Generalized activation of the inflammatory response is thought to play a role in the pathogenesis of pre-eclampsia. Monocytes may play a central role in this inflammatory response. Monocytes are short lived cells that mature in the circulation and invade into tissues upon an inflammatory stimulus and develop into macrophages. Macrophages are abundantly present in the endometrium and play a role in implantation and placentation in normal pregnancy. In pre-eclampsia, these macrophages appear to be present in larger numbers and are also activated. In the present review, we focused on the role of monocytes and macrophages in the pathophysiology of pre-eclampsia.

Keywords: pregnancy, pre-eclampsia, monocytes, macrophages, decidua, placenta

INTRODUCTION

Preeclampsia is one of the leading complications of pregnancy, characterized by hypertension and proteinuria and developing in the second half of pregnancy (1, 2). Preeclampsia is suggested to be a two stage disease: the first stage being poor placentation (3). The second stage is the production of pro-inflammatory factors by the diseased placenta, which activates the systemic inflammatory response, leading to the signs of pre-eclampsia (3).

During normal pregnancy, the circulation of peripheral blood through the placenta results in direct or indirect contact of maternal immune cells with the placenta. This may activate circulating immune cells, especially monocytes (4, 5). In pre-eclampsia, due to production of pro-inflammatory factors from the placenta (6–9), monocytes are even further activated and together with activation of other inflammatory cells, such as granulocytes and endothelial cells, finally induce the full blown syndrome of pre-eclampsia (3).

At the maternal–fetal interface, from the beginning of a healthy pregnancy, there is an increase of innate immune cells, such as macrophages and NK cells (10). These macrophages and NK cells may have a local immune function, however, they also appear to be important for placental development by promoting trophoblast recruitment, spiral artery remodeling, and angiogenesis (11). The present review will focus on macrophages at the maternal–fetal interface. In normal pregnancy, most of the macrophages at the maternal–fetal interface are M2 macrophages, i.e., immunomodulatory macrophages (11). In pre-eclampsia, there appear to be increased numbers of M1 macrophages, suggesting a role for these macrophages in the poor placental development in pre-eclampsia.

Monocytes and macrophages may thus play an important role in healthy pregnancy as well in the pathophysiology of pre-eclampsia. Further insight into the role of these cells in these conditions, may lead to a better understating of the inflammatory response in normal pregnancy and in pre-eclampsia. Therefore, the present paper will review the systemic and local changes in the decidua in monocytes and macrophages and their subsets during healthy human pregnancy and pre-eclampsia. Examples from animal models will also be included.

MONOCYTES AND MACROPHAGES AND THEIR SUBSETS

MONOCYTES

Monocytes arise from precursors in the bone marrow and comprise about 5–10% of the circulating blood leukocytes. They circulate in the blood for a few days before migrating into tissues to become macrophages or dendritic cells (12). They have important functions in homeostasis, tissue repair, and inflammation (12). It has recently become clear that circulating monocytes are a heterogeneous population (12). In humans, the monocyte subsets can be distinguished based on the expression of CD14, the lipopolysaccharide (LPS) receptor. The main subset (comprising about 90–95% of the monocytes) is a subset expressing high levels of CD14, but lacking CD16 (FcγR-III) expression. Since this is the main subsets and until recently thought to be the only subset, this subset is usually called "classical subset". The second subset of monocytes is characterized by low expression of CD14 together with CD16. This subset is often called the non-classical subset. More recently, a third, intermediate subset of monocytes has been defined, called the intermediate subset (13). This subset is characterized by high expression of CD14 in combination with expression of CD16 and is a separate subset of monocytes. It has been suggested that classical monocytes arise from the bone marrow and mature into non-classical monocytes via intermediate monocytes (13, 14). These subsets differ in many respects, including expression of adhesion

molecules and chemokine receptors and function [reviewed in Ref. (12, 13)]. Classical monocytes are professional phagocytes that can generate reactive oxygen species (ROS) and produce cytokines in response to toll-like receptor dependent activation by f.i. LPS. Non-classical monocytes are weak phagocytes and do not generate ROS, but are more efficient producers of pro-inflammatory cytokines after TLR dependent activation (12). This subset has been shown to have a longer half-life and localize to both resting and inflamed tissue (12). They crawl on the luminal side of the endothelium and survey endothelial cells and tissues for damage and infection (13). Upon damage or infection, they may rapidly invade the tissue and initiate the inflammatory response (15). Non-classical monocytes have been shown to be increased in various inflammatory diseases (13, 16, 17).

MACROPHAGES

Macrophages are located in all body tissues, where they are important in detecting, ingesting, and processing foreign material, dead cells, and other debris (12). Monocytes are macrophage precursors (12); monocytes can be recruited into tissues, to replenish steady state macrophages or can be recruited in inflammatory conditions (12), where they mature into macrophages (or dendritic cells) (12). Macrophages play an important role in the innate and adaptive immune responses to pathogens and are important mediators of inflammatory processes (12). However, they also have anti-inflammatory properties, as they are also involved in the resolution of the inflammation (12). Indeed, several macrophage subsets with distinct functions have been described. Broadly, they can be classified into two groups: M1 or classically activated macrophages, and M2 or alternatively activated macrophages (18). These subsets differ in receptor, cytokine, and chemokine expression and in effector function (18). M1 macrophages are microbicidal and inflammatory, M2 macrophages are immunomodulatory, which can induce tolerance and the resolution of inflammation, and are only weak microbicidal (18). It has been suggested that these two populations may be extreme ends of polarization and that macrophages may actively switch their phenotype, depending on the environment (19).

There is debate on the fate of the different monocyte subsets; it is unclear whether tissue macrophages are derived from a specific monocyte subset or from either subset randomly (12). It has been suggested that classical monocytes preferentially differentiate into M1 macrophages, while the non-classical monocytes preferentially differentiate into M2 macrophages during inflammation (20). However, various studies have shown that such a strict distinction between differentiation of classical and non-classical monocytes may not be very likely and that it may depend on the model and the inflammatory stimulus whether a monocyte differentiates into an M1 or M2 macrophage (20, 21).

MONOCYTES IN PREGNANCY

During normal pregnancy, the female immune system has to adapt to the presence of the semi-allogeneic fetus. Many changes in the peripheral circulation have been observed, both in the specific and innate immune response. In the specific immune response, a decreased Th1/Th2 ratio has been observed in both T cells (22–24) as well as in NK cells (23, 25). These changes may be associated with changes in regulatory T cells (26, 27) and Th17 cells (27). It has been suggested, that to compensate for such changes in the specific immune response, also the innate immune response has to adapt to pregnancy. This has most often been shown by increased numbers of circulating monocytes and granulocytes, resulting in increased number of total leukocytes during pregnancy (28–30). Here, we will discuss changes in monocytes during healthy pregnancy and in pre-eclampsia.

Although it has been known for a long time that leukocyte numbers increase during pregnancy, at that time this was not recognized as a sign of generalized inflammation in pregnant women. With the introduction of new techniques, most importantly, flow cytometry, function and activation status of leukocytes monocyte could be examined by measuring expression of markers of activation and production of intracellular cytokines. Moreover, the flow cytometric analysis did not require isolation of cells from whole blood, as measurements could be done in whole blood. This represents the *in vivo* situation much better, since isolation of leukocytes from blood may activate these cells (31). Using the whole blood method, Sacks et al. (32) showed phenotypical activation of monocytes during pregnancy, by showing increased expression of the activation markers CD11b, CD14, and CD64 on monocytes from pregnant women as compared with monocytes from non-pregnant women. Afterward, these results have been confirmed by others (33–35).

The monocytes are also functionally changed in pregnant women. This has, for instance, been demonstrated by measuring the production of oxygen free radicals (32), which is increased in pregnant women. Although some authors have shown increased cytokine production by non-stimulated monocytes from pregnant women vs. non-pregnant women (34), others could not confirm this finding and only observed cytokine production by stimulated monocytes (8, 30). Whether stimulated cytokine production of pregnant monocytes is increased or decreased as compared to non-pregnant women seems to depend on the stimulus. After stimulation with only LPS cytokine production by monocytes from pregnant women was decreased as compared with cytokine production by monocytes from non-pregnant women (30, 36, 37). However, after stimulation of monocytes with both LPS and IFNγ, monocytes of pregnant women showed increased cytokine production as compared with monocytes from non-pregnant women (38). Although these findings seem contradictory, they can be explained as follows: decreased cytokine production of monocytes from pregnant women following LPS stimulation is a sign of activation of monocytes, since activated monocytes become tolerant to LPS (39). IFNγ, however, abrogates LPS tolerance (40). Therefore, if LPS tolerance is abrogated by IFNγ during pregnancy, monocytes produce increased amounts of cytokines during pregnancy. The above mentioned studies have been performed in the third trimester of pregnancy and based on all above mentioned data, it is now generally accepted that monocytes are activated during pregnancy. However, little is known about monocyte activation during the course of pregnancy. However, gradually developing monocyte activation may occur during the course of pregnancy, since one paper showed progressive phenotypical activation of monocytes from the first trimester to the third trimester (34).

MONOCYTE SUBSETS IN PREGNANCY

In the studies presented above, monocytes have been characterized by CD14 expression, indicating that mainly classical monocytes have been studied in pregnancy. Recently, we conducted a study in which we identified the three subtypes of monocytes in pregnant women (41). We showed a decreased number of classical monocytes and an increased number of intermediate monocytes in healthy pregnancy. These results are in line with the suggestion that pregnancy is an inflammatory condition, since in other inflammatory diseases, this intermediate subset has also been shown to be increased (42, 43). Our data, however, were not in line with data of Al-ofi et al. (44), who showed increased numbers of classical monocytes and decreased numbers of non-classical monocytes in pregnant vs. non-pregnant women. The reason for this difference is unclear, but may be due to differences in experimental methods. Further studies are warranted to evaluate whether the subsets respond differently to stimulation in pregnant and non-pregnant women.

MONOCYTES AND PARTURITION

Parturition is associated with an inflammatory response (45). At the end of gestation, the number of leukocytes in the uterine tissue are increased (46). Also in the peripheral circulation just before delivery, further phenotypical activation of monocytes in comparison with earlier in pregnancy has been shown (47), indicating further activation of these cells just before delivery. In line with this suggestion, more recently, Vega-Sanchez et al. (48) showed differences in cytokine production of monocytes between pregnant women in labor and pregnant women not in labor. A role for activated monocytes in parturition can also be deduced from data from pre-term labor, where increased expression of activation markers by monocytes has been observed compared with healthy pregnancy (49).

MONOCYTES IN PRE-ECLAMPSIA

It has now been well-established that during pre-eclampsia, the innate immune system is even further activated as compared with normal pregnancy (50). Activation of monocytes has been demonstrated by increased expression of inflammation associated adhesion molecules such as CD11b, ICAM-1, and CD14 (5, 32, 51, 52). However, monocytes are not only phenotypically activated, they also produced increased amounts of oxygen free radicals as compared to normal pregnancy (32) and their cytokine production also differed as compared to monocytes from normal pregnant women (38, 53–56). As for normal pregnancy, the above mentioned studies did not take into account the presence of monocyte subsets and monocytes are generally defined as CD14 positive. In our recent study, we observed decreased numbers of classical monocytes and an increased numbers of intermediate monocytes in women with pre-eclampsia as compared with normal pregnant women (41). Although Al-ofi et al. also showed decreased numbers of classical monocytes, in contrast to our study, they showed increased numbers of non-classical monocytes in pre-eclamptic women compared with healthy pregnant women (44). As explained above, this may be due to different techniques used, but may also be due to a different selection of patient groups (we exclusively included early onset pre-eclamptic women, while Al-ofi et al. included a more heterogeneous group of pre-eclamptic women).

POSSIBLE MECHANISMS OF MONOCYTE ACTIVATION IN PREGNANCY AND PRE-ECLAMPSIA

The exact mechanisms involved in the activation of monocytes during pregnancy and pre-eclampsia remain unknown. The most obvious suggestion is that the placenta is involved. Peripheral monocytes circulate through the placental circulation and come into close contact with the semi-allogeneic villous syncytiotrophoblast (**Figure 1**). This may activate monocytes. This notion is supported by the fact that monocytes become activated during their passage through the placenta (5). It is, however, unsure whether this activation of monocytes occurs due to direct contact, since several soluble placental products, such as cytokines (57), placental microparticles (58), fetal DNA (59), released into the maternal circulation, may also activate monocytes (60).

Many factors may be involved in further activation of monocytes during pre-eclampsia. Factors may be derived from the stressed placenta, such as anti-angiogenic factors (61), placental microparticles (62), or ATP (9), which are released at increased amounts from the pre-eclamptic placenta. These factors may activate the monocytes. Also upregulation of various pro-inflammatory cytokines, such as TNFα, IL-1β, IL-18, in the placenta of pre-eclamptic women has been observed (63–65). On the other hand, decreased levels of the anti-inflammatory cytokine IL-10 have been observed in the placenta of pre-eclamptic women (66, 67). These increased levels of pro-inflammatory cytokines in the pre-eclamptic placenta may be responsible for the increased

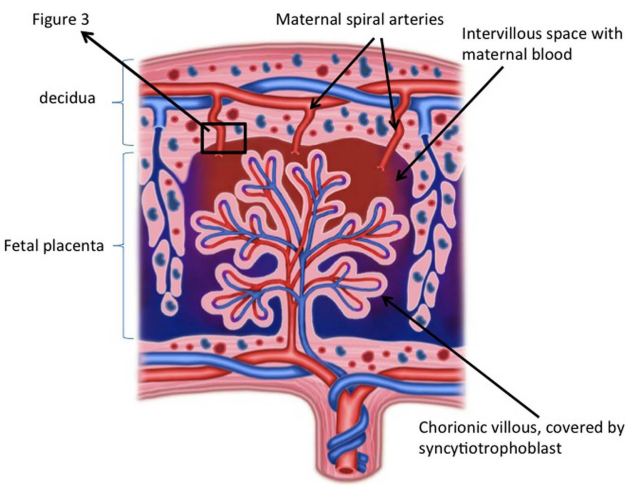

FIGURE 1 | Schematic overview of the placenta. The placenta consists of a fetal part and a maternal part. In the fetal part of the placenta, chorionic villi, covered with syncytiotrophoblast, bath in maternal blood of the intervillous space. Direct or indirect contact (via soluble factors) of monocytes with the syncytiotrophoblast may results in monocyte activation. The maternal part of the placenta consists of decidua in which remodeled spiral arteries are present, which take maternal blood to the intervillous space. In the decidua fetal trophoblast, and maternal macrophages and NK cells are present and necessary for immune regulation and spiral artery remodeling ©ilusjessy – Fotolia.com.

circulating levels of these cytokines in pre-eclamptic patients (68, 69). These cytokines may also activate the monocytes. Since monocytes themselves are potent producers of cytokines, the activation of monocyte by placental factors and cytokines may in turn result in a vicious circle of monocytes activation and cytokine production leading to persistent increased monocyte activation in pre-eclampsia.

It appears to be important for induction of pre-eclamptic signs how monocytes are activated. In pregnant rats, hypertension and proteinuria can only be induced after infusion with *E coli* LPS (70), not after infusion of LPS from *Porphyromonas gingivalis* (71), despite the fact that monocytes are activated by this LPS (72). This may explain why certain infections, such as urinary tract infections or periodontitis, may increase the risk of pre-eclampsia, while other infections, such as CMV or malaria do not increase the risk for pre-eclampsia (73). Apparently, the immune response, and specifically monocyte activation is different in different infections. Differences may amongst others relate to differences in cytokine production between states of monocytes activation, since we have previously shown that activation of monocytes with *E coli* LPS or *P. gingivalis* LPS resulted in different cytokine production (36).

MONOCYTES DURING PREGNANCY AND EXPERIMENTAL PRE-ECLAMPSIA IN ANIMALS

Although it is now generally accepted that during pregnancy monocytes are activated and that they are even further activated during pre-eclampsia, whether this is the cause or consequence of pre-eclampsia still remains to be shown. It is difficult to study the role of monocytes in pregnancy and pre-eclampsia in human subjects. Therefore, animal models are needed. A good animal model to study innate immune responses in pregnancy is the rat. Although not completely similar, like humans, rats have a hemochorial placenta, showing deep trophoblast invasion into the uterine wall (74) indicating that fetal–maternal interactions may be similar in rat and human pregnancy. Therefore pregnancy-induced changes in the immune response may also be similar to human pregnancy. Indeed, similar phenotypical and functional activation of monocytes during the course of pregnancy have been observed in rats as compared with humans (75, 76). Moreover, in accordance with human pregnancy, we found decreased numbers of classical monocytes and increased numbers of non-classical monocytes during pregnancy in this species (41).

Various rat models have suggested that activation of monocytes, by LPS, ATP, or TNFα during pregnancy, induced pre-eclampsia-like signs (70, 77, 78). Interestingly, such pre-eclampsia-like syndromes were only induced in pregnant rats, not in non-pregnant rats (70, 77). The pathophysiology of the LPS and ATP induced pre-eclampsia was characterized by a pregnancy-specific inflammatory response, characterized by persistent general (75, 76, 79) and glomerular (79, 80) inflammation, in which monocytes play a major role. In the ATP model, we have shown that, similar to human pre-eclampsia, non-classical monocytes are increased and activated by ATP, suggesting an important role for this subset in pre-eclampsia. Together, these animal studies support the hypothesis that activation of monocytes in pregnancy may result in pre-eclampsia-like signs, such as hypertension and proteinuria.

Based on the above data on monocytes during pregnancy and pre-eclampsia, we suggest that factors that arise from the healthy placenta during pregnancy induce phenotypical activation of monocytes and induce increased maturation toward non-classical monocytes. These factors may also affect endothelial cells directly (**Figure 2A**). During pre-eclampsia, the stressed placenta starts to produce various pro-inflammatory factors, which further activate the monocytes and further increased monocyte maturation toward non-classical monocytes. Monocyte activation results in monocyte cytokine production. Via a vicious circle, these cytokines may further activate the monocytes themselves as well as the

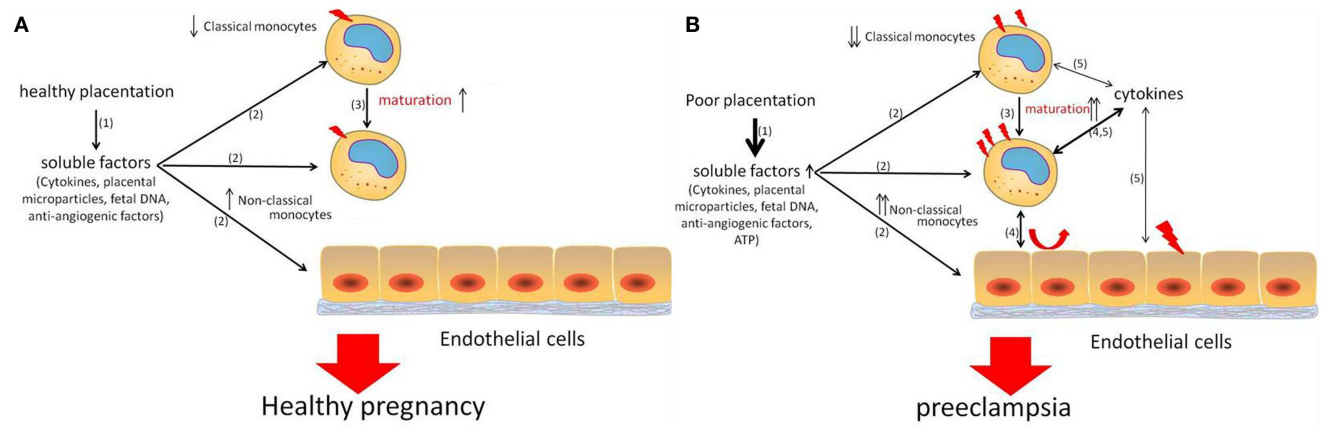

FIGURE 2 | Schematic overview of the role of monocytes during healthy pregnancy (A) and pre-eclampsia (B). During healthy pregnancy, placental factors (1) activate monocytes (2) and may affect endothelial cells (2) and induce increased maturation of classical monocytes toward non-classical monocytes (3). During pre-eclampsia, more and other soluble factors are produced from the stressed placenta (1), resulting in further activation of monocytes and endothelial cells (2) and further maturation of classical monocytes toward non-classical monocytes (3). Numbers non-classical monocytes are increased and they may play an important role in this inflammatory process, since they are known to produce increased numbers of cytokines upon activation (4). These cytokines further activate the monocytes (5) as well as endothelial cells (5). This vicious circle of activation of monocytes and endothelial cells finally results in the symptoms of pre-eclampsia, such as hypertension and proteinuria.

endothelial cells, finally resulting in the signs of pre-eclampsia, such as proteinuria and hypertension (**Figure 2B**).

DECIDUAL MACROPHAGES

Macrophages are already present in the non-pregnant endometrium, although in low numbers (81). Since their numbers fluctuate during the menstrual cycle (81, 82), it seems likely that these are under hormonal control (83). After fertilization, the number of uterine macrophages increase, due to expression of various chemokines (84) and during pregnancy macrophages are abundantly present in the decidua at all times of pregnancy (85). During pregnancy, they comprise about 20–30% of all decidual leukocytes (86, 87). The number of decidual macrophages may vary with gestational age being highest in the first and second trimester (88). Macrophages in the decidua are usually associated with spiral arteries and glands as well as with extravillous trophoblast (86, 89), but are also found in the myometrium (85). When the presence of macrophages in the decidua was first discovered, it was suggested that these cells were recruited as the result of an immune response to the semi-allogeneic fetus (90). However, it is now generally accepted that macrophages, and other immune cells present in the decidua, are necessary for successful implantation (85). Various studies have focused on specific functions of macrophages in the decidua and it has been suggested that the decidual macrophages have various roles during pregnancy, mainly in placentation (91), but they may also play a role in protecting the fetus against intrauterine infection (92).

DECIDUAL MACROPHAGES IN EARLY PREGNANCY

Most of the studies on macrophages in the decidua have been performed in early pregnancy. At this time of pregnancy, macrophages are located near the spiral arteries during trophoblast invasion and spiral artery remodeling (86, 89). The role of macrophages in spiral artery remodeling was further emphasized by the fact that they are present even before the presence of extravillous trophoblast (93). At that time, disruption and disorganization of vascular smooth muscle cells and endothelial cells was also observed (93). This suggests that macrophages may be important in the very early phases of spiral artery remodeling, preparing the spiral arteries for further remodeling by trophoblast cells (93). Their suggested role in vascular remodeling is in accordance with the findings of production of factors associated with angiogenesis and tissue remodeling by these cells (94, 95). Indeed macrophages, which were MMP 9 positive, and which were shown to have phagocytotic capacities were found to infiltrate spiral arteries during remodeling (96). Macrophages have also been shown to be important for clearance of apoptotic cells in the decidua (97). Apoptosis is an important process during spiral artery remodeling and trophoblast invasion. During these processes, apoptotic trophoblast cells (98) as well apoptotic cells in the vascular wall that is being remodeled have been observed (93). By engulfment of the apoptotic cells, macrophages prevent the release of pro-inflammatory substances from the apoptotic cells into the decidua [reviewed in Ref. (97)].

Decidual macrophages have mainly been classified as M2-like macrophages, i.e., immunomodulatory macrophages (99). Although they express many markers of M2 macrophages, such as CD206, CD163, and DC-sign (100–102), they appeared not to be typical M2 macrophages, since they are not induced by Th2 cytokines, such as IL4, but by M-CSF and IL-10 (102). These data are in line with the abundant presence of M-CSF and IL-10 in the decidua (103–105). The M2 phenotype is most likely due to hypermethylation of genes encoding markers of classical activation and hypomethylation of genes encoding markers for non-classical activation (106). Next to the typical M2 cytokine gene expression, these decidual macrophages also showed gene expression for inflammatory cytokines such as IL-6 and TNFα (102, 107). The production of pro-inflammatory cytokines by decidual macrophages may also be explained by the presence of two macrophage subpopulations in the early decidua (107). One of these subsets may be a more pro-inflammatory subset, since this subset expressed genes associated with inflammation. The other subset, which was higher in number, expressed genes associated with extracellular matrix formation, networking, communication, and growth (107).

Apart from the putative role of M-CSF and IL-10 in induction of M2 macrophages in the decidua, other factors have also been suggested to be involved in inducing/maintaining the M2 phenotype in decidual macrophages. Decidual macrophages have been shown to express inhibitory receptors immunoglobulin like transcript (ILT)2 and ILT4 (108). These receptors can bind to HLA-G expressed on invading extravillous trophoblast (108), after which a negative signal is delivered to the macrophages, resulting in tolerance to the trophoblast and the induction of anti-inflammatory cytokines. It has also been suggested that the engulfment of the apoptotic cells induced an immunosuppressive and anti-inflammatory phenotype of the macrophages (97). Not only the phagocytosis of apoptotic cells, but also the phagocytosis of trophoblast cell debris at the maternal–fetal interface may be associated with the M2 phenotype of macrophages (109–111). In addition, as it has been suggested that non-classical monocytes preferentially differentiate into M2 macrophages (20), it may be speculated that the increased numbers of non-classical monocytes in the circulation during pregnancy (41), results in increased invasion of these cells into the decidua to become M2 macrophages.

DECIDUAL MACROPHAGES IN LATE PREGNANCY

Macrophages are present in the decidua throughout pregnancy until the end of pregnancy, although their numbers may decrease at the end of pregnancy (88). The exact role of decidual macrophages at the end of pregnancy remains to be established, it seems, however, likely that they are still involved in immunoregulation and clearance of apoptotic cells. Indeed, many of the macrophages present in the decidua at the end of pregnancy, appeared to be M2 macrophages (112). The potential protective effect of M2 macrophages for the fetus was recently shown by van Schonkeren et al., who showed the presence of an inflammatory lesion in placentae from women who underwent egg donation (113). This lesion consisted of maternal cells, expressing high levels of CD14 and CD163, suggesting the presence of M2 macrophages. These lesions appeared to protect against pre-eclampsia (113).

DECIDUAL MACROPHAGES IN PRE-ECLAMPSIA

Preeclampsia is associated with defective trophoblast invasion and spiral artery remodeling: while in healthy pregnancy, spiral artery remodeling extends into the myometrium, in pre-eclampsia, spiral artery remodeling can only be found in the decidua (3). Unfortunately, not very many studies focused on macrophages in the decidua in pre-eclampsia. Most of the studies in pre-eclampsia were obviously performed after delivery of the placenta. Some of the studies reported decreased numbers of macrophages in the decidua of pre-eclamptic patients (114, 115). Most of the studies, however, found increased numbers of macrophages in pre-eclamptic patients (112, 116–118). These data may not necessarily be conflicting, since not only different methods were used (Williams and Burk performed a flow cytometric study, while the other studies were immunohistochemical studies), also different antibodies were used. Increased numbers of macrophages in the decidua of pre-eclamptic patients appears to be in line with increased presence of macrophage chemotactic factors, such as M-CSF, IL-8, and MCP-1 (119–121) in pre-eclamptic patients. Not only numbers of macrophages were found to be different in pre-eclamptic patients, macrophages may also be differently activated in pre-eclampsia (120–122). This may be in line with the presence of increased pro-inflammatory cytokines (63–65) and decreased anti-inflammatory cytokines in the placenta of pre-eclamptic women (66, 67). More recently, it has been shown that in the decidua of pre-eclamptic women decreased numbers of M2 macrophages are present (112). Differences in macrophage numbers may be regional, since increased numbers of macrophages were found around the spiral arteries of pre-eclamptic patients (120, 121). The presence of macrophages in the spiral arteries may be associated with the development of acute artherosis (120). Acute artherosis is a process mainly seen in poorly remodeled spiral arteries at the end of pregnancy, characterized by the presence of subendothelial CD68 positive foam cells (123). Its presence is associated with adverse fetal and maternal outcome (124).

The question remains whether the increased presence of macrophages in the decidua of pre-eclamptic women is the cause or the result of pre-eclampsia. This question is difficult to answer, due to the difficulties of obtaining material from early decidua of women who later in pregnancy developed pre-eclampsia. However, recently we have shown that in early decidua from women who later developed pregnancy-induced hypertension (PIH) (including pre-eclampsia) CD68 mRNA expression was increased (125), suggesting increased numbers of macrophages in the early decidua of women who later develop hypertension in pregnancy. Moreover, the CD206/CD68 mRNA ratio was decreased in PIH women, suggesting that decreased numbers of M2 macrophages are present in the decidua of women who later develop pregnancy-induced hypertension (125). The increased numbers of macrophages, with decreased numbers of M2 macrophages may thus already be present before the onset pre-eclampsia and therefore suggest a role for macrophages in defective trophoblast invasion and spiral artery remodeling. Recent *in vitro* data showed that macrophages migrate toward invading trophoblast (126), while other groups have shown that activated macrophages *in vitro* are able to inhibit trophoblast invasion and spiral artery remodeling (127, 128). *In vivo*, data have shown that there is a reciprocal presence of trophoblast cells and macrophages

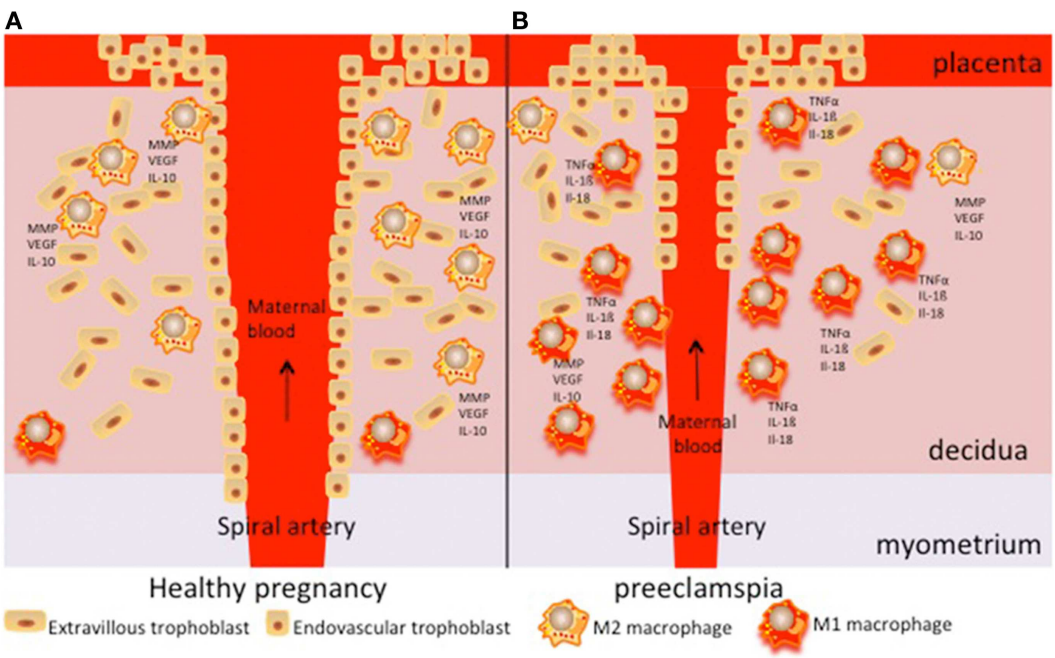

FIGURE 3 | Schematic overview of the role of decidual macrophages in pregnancy (A) and pre-eclampsia (B). During normal pregnancy, M2-like macrophages are present around spiral arteries and play a role in remodeling of these arteries by producing various factors associated with angiogenesis and tissue remodeling (such as MMP and VEGF). They also play a role in immunomodulation, for instance by producing IL-10. During pre-eclampsia, increased numbers of M1-like macrophages are found. They may produce pro-inflammatory cytokines, such as TNFα, IL-1β, or IL-18.

in the spiral arteries of both healthy and pre-eclamptic women (121). Therefore the increased numbers of macrophages in and around spiral arteries of pre-eclamptic women (121) may inhibit spiral artery remodeling.

Since it is difficult to study the role of macrophages in pre-eclampsia in humans, animal models may help in understanding critical questions. Studying whether trophoblast invasion and spiral artery remodeling is associated with macrophages in animal models for pre-eclampsia may shed light on the question whether increased numbers of macrophages in the decidua are the cause or the result of pre-eclampsia. In an animal model for pre-eclampsia induced by multiple doses of LPS in pregnant rats, decreased trophoblast invasion and spiral artery remodeling after LPS was associated with increased numbers of macrophages. We studied this subject and showed increased invasion of activated macrophages in the mesometrial triangle (the equivalent of the placental bed in humans) before defective trophoblast invasion and spiral artery remodeling (129). This appears to be in line with the sparse human data and suggests a role for activated macrophages in the pathophysiology of pre-eclampsia.

M2-like macrophages are thus abundantly present in the decidua of healthy pregnant women. They are observed in the presence of spiral arteries and extravillous trophoblast cells and may play a role in spiral artery remodeling by producing factors associated with angiogenesis and tissue remodeling, such as MMPs and VEGF (**Figure 3A**). During pre-eclampsia, increased numbers of decidual macrophages are observed, which may be of the M1 phenotype and therefore produce pro-inflammatory cytokines (**Figure 3B**). These activated macrophages may affect spiral arteries and may induce acute artherosis, affecting the placental blood circulation.

SUMMARY

Monocytes and macrophages play important roles in pregnancy and pre-eclampsia. Monocyte activation and increased numbers of non-classical monocytes, is important for normal pregnancy. Monocyte derived macrophages, especially M2-like macrophages (which may be derived from non-classical monocytes) in the decidua in healthy pregnancy play an important role in blastocyst implantation, trophoblast invasion, and spiral artery remodeling as well as in defense against infection and in immunomodulation (**Figure 4**). During pre-eclampsia, decreased spiral artery remodeling results in increased production of soluble factors (or different factors), inducing further activation of both classical and non-classical monocytes and further maturation toward non-classical monocytes. These placental factors as well as the activated monocytes also induce activation of endothelial cells. Activated monocytes (both classical and non-classical monocytes) may invade into the decidua, resulting in increased numbers of M1-like macrophages in the decidua of pre-eclamptic women (**Figure 4**). The M1-like macrophages may affect the spiral arteries, by for instance inducing acute artherosis. This may further affect the placental blood circulation and stress the placenta.

Unfortunately, most studies on monocytes and macrophages in pre-eclampsia have been performed during pre-eclampsia. Although we do believe that monocytes and decidual macrophages do play a role in inducing the maternal symptoms of pre-eclampsia, it is relatively unknown whether monocytes and decidual macrophages do also play a role in the aberrant spiral artery remodeling early in pregnancy. The question thus remains as to what induces the aberrant spiral artery remodeling? Future studies should therefore not only focus on the three monocyte subsets in pregnancy and pre-eclampsia, but also on the relationship between the circulating monocyte subsets and macrophages in the decidua. Moreover, since data on macrophages in the decidua in and before pre-eclampsia are relatively scarce future studies should therefore also focus on macrophage function and phenotype in and before pre-eclampsia.

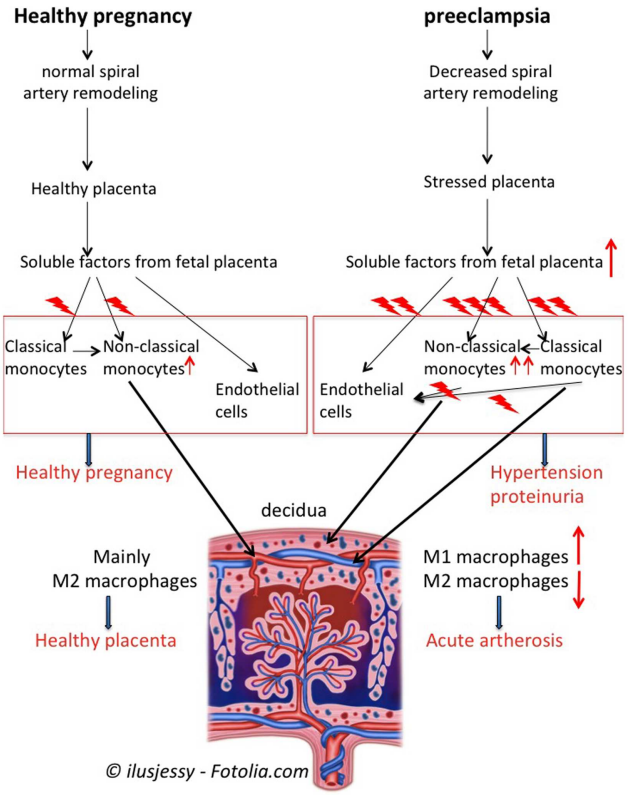

FIGURE 4 | Summary of monocytes and macrophages in pregnancy and pre-eclampsia. In healthy pregnancy, soluble factors from the villous trophoblast activate circulating monocytes, induce maturation of classical monocytes toward non-classical monocytes and affect endothelial cells. Non-classical monocytes will invade into the decidua to become M2-like macrophages to support healthy placentation and immunomodulation. During pre-eclampsia, decreased remodeling of the spiral arteries will results in a stressed placenta, which produces increased amounts or different soluble factors as compared with healthy pregnancy. The soluble factors will further activate the monocytes, induce further maturation of classical monocytes toward non-classical monocytes and activate endothelial cells. Activated monocytes, by f.i. producing cytokines, further affect monocytes and endothelial cells. This vicious circle of monocyte and endothelial cell activation results in the maternal symptoms of pre-eclampsia, i.e., hypertension and proteinuria. Moreover, activated classical and non-classical monocytes may invade into the decidua to develop into M1-like and M2-like macrophages, resulting in increased numbers of M1-like macrophages in the pre-eclamptic decidua. The M1-like macrophages may affect the spiral arteries resulting in f.i. acute atherosis, thereby further affecting the placental blood circulation.

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Regulatory T-cells in pregnancy: historical perspective, state of the art, and burning questions

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In this review, we first revisit the original concept of "suppressor T-cells" in pregnancy, put it in a historical perspective, and then highlight the main data that licensed its resurrection and revision into the concept of "regulatory T-cells" (Tregs) in pregnancy. We review the evidence for a major role of Tregs in murine and human pregnancy and discuss Treg interactions with dendritic and uterine natural killer cells, other players of maternal–fetal tolerance. Finally, we highlight what we consider as the most important questions in the field.

Keywords: suppressor T-cells, NK cells, cancer tolerance, Treg, evolution of the immune system

ON THE RISE AND FALL OF SUPPRESSOR T-CELLS IN (REPRODUCTIVE) IMMUNOLOGY

The history of immunosuppression in pregnancy started in the 1970s, just after the discoveries of Gershon and Kondo (1), when transplantation and tumor immunologists devoted much work to suppressor T-cells (Ts) and suppression. Since 1953, pregnancy has been viewed as "Nature's allograft" (2), the maternal immune system being in direct contact with a semi-allogenic organism, deeply engrafted and invasive, without, however, any sign of rejection. Medawar conceived three possible explanations for such a paradox: (i) the uterus is an immunologically privileged site, (ii) the fetus is an antigenically immature body, and (iii) there is a non-specific immune depression of the mother, global or at the maternal–fetal interface. As none of these three hypotheses later proved to be correct, the search for an active phenomenon started.

Likewise, as recalled by Trowsdale and Betz (3), the discovery of infectious tolerance by Gershon and Kondo awakened the search for pregnancy-induced Ts. It was first shown that multiple syngeneic pregnancies of C57BL/6 female mice induced tolerance to the male-specific H-Y antigen, as females showed delayed rejection of male skin grafts expressing H-Y (4). A few years later, Simpson et al. reported that multiparity induced a state of tolerance transferable by T-cells (5), and we demonstrated that allo-multiparity evoked systemic T-cell tolerance or hypo-responsiveness to paternal alloantigens (6). Importantly, there were no reports showing that Ts could be involved in tolerance to the first allopregnancy, leaving the question of maternal tolerance to fetal alloantigens unanswered (except for a single unpublished but well-known study by Baines, presented at the 1981 Bannf meeting).

The question of the specificity of a putative suppressive phenomenon (or cells) was of importance, but inadequately answered or even addressed, as Waldmann pointed out: "For example, on the issue of "antigen specificity," many of the early claims of antigen-specific suppression lacked the discipline cultivated by the classical serologists, in not performing criss-cross experiments. In other words, to claim antigen specificity in a population of cells or extracts thereof, one had to show that A-type T-cells primed to B would suppress responses to B but not C, but also (and critically) that A T-cells primed to C would suppress responses to C and not B. This might easily have misled them into concluding specificity on insufficient data!" (7).

In contrast, in their elegant human studies, Engleman et al. reported that Ts induced by allopregnancy, and their soluble factors, were specific for both stimulator cells and responder cells in mixed lymphocyte reactions (MLRs) (8, 9). They took advantage of the rather rare existence of two multiparous twins, A and A′, married to B and C. Ts from A suppressed an MLR of A and A′ against stimulator lymphocytes from B, but not C; conversely, Ts from A′ suppressed an MLR of A and A′ against stimulator lymphocytes from C, but not B. None of the A or A′ T-cells could suppress B anti-C or C anti-B MLR, nor those of an unrelated E female against B or C.

Despite intense research in the field, the concept of Ts became shaky in the early 1980s, mostly because the absence of a specific marker for Ts prevented study of the functionality of pure populations of cells. The main data supporting the existence of Ts and suppression were for a long time their linkage to an "I–J" or "I–C" sub-region of the class II murine MHC loci, which were supposed

to be coding for Ts as well as antigen-specific and non-specific soluble suppressor factors. The "*coup de grace*" to the concept came from the demonstration that these regions did not exist (10, 11). For several years, the concept of suppression became politically incorrect, with very few scientists "saying the *S*. . . word in public," to quote Green (12).

Nevertheless, without always explicitly mentioning Ts, several studies continued to point to a form of regulation of maternal immune status by T-cells during pregnancy. Some of them were done in the now classic CBA x DBA/2J murine model of spontaneous immune abortion, in which it was shown pre-immunization with BALB/c splenocytes had a protective effect and was transferable by T-cells (13, 14). To further investigate the mechanisms underlying this protection, nine recombinant inbred strains between BALB/c and DBA/2 were used for pre-immunization. Only three strains behaved as BALB/c. However, when peripheral lymphocytes from pre-immunized CBA females were used as putative regulatory cells in a CBA anti-BALB/c MLR, there was no correlation between the presence of "suppression" and abortion rates, suggesting that local intrauterine immunoregulation is the determinant of success or failure of allopregnancy (15). Immunoregulation was also supported by (i) reports that hypo-responsiveness or tolerance to paternal antigens was repeatedly demonstrated in multiple allopregnancy, with several studies pointing to an important role of the seminal plasma (16–19) and (ii) the "Th1/Th2" paradigm, e.g., a dominance of the production of Th2 cytokines by the pregnant CBA/J (20–22) as well as the earlier demonstration that in "responder" mice the allopregnancy-induced anti-paternal alloantibody response is dominated by IgG1 (23).

ON THE REBIRTH OF SUPPRESSOR T-CELLS AS JUST "REGULATORY" T-CELLS

In 1995, Sakaguchi and colleagues showed that elimination of $CD25^+CD4^+$ T-cells elicits multi-organ autoimmunity, which could be prevented by reinjection of the same cells (24, 25). These properties would qualify $CD25^+CD4^+$ as Ts. Yet, the trauma induced by the I–J story was probably so strong that they were given the more "benign" denomination of regulatory T-cells (Tregs), though they were cells endowed with suppressive activity.

The presence of the CD25 marker on the surface of these cells enabled their negative or positive selection, and thus demonstration of their suppressive activity in various *in vivo* and *in vitro* settings. However, as CD25 is not only constitutively expressed by Tregs, but is also transiently expressed on activated T-cells, another quantum leap for the biology of Tregs was the discovery of a more specific marker, Foxp3, the master regulator of Treg development and function (26). The understanding that mice and human beings with a genetic defect in Foxp3 developed multi-organ autoimmune diseases (27) sealed the case for the discovery of the long-sought suppressor cells of immune responses.

ON REGULATORY T-CELLS AND MATERNAL–FETAL TOLERANCE IN MICE

Treg DEPLETION INDUCES ABORTION IN MURINE PREGNANCY

These discoveries impacted reproductive biology, with the resurrection of the concept of T-cell-dependent immunoregulation. We now know that Tregs are rapidly recruited to uterus-draining lymph nodes and activated during the first day after embryo implantation (28). These Tregs have the phenotype of activated/memory Treg subsets and are, at least in part, self-Ag specific (28).

The functional importance of this recruitment has been highlighted by transfer/depletion experiments. Aluvihare et al. first noted that Tregs increased markedly in all lymphoid organs of C57BL/6 females mated with CBA males. Importantly, a similar increase was observed whether syngeneic or allogeneic matings were performed, suggesting that this was an alloantigen-independent phenomenon. The cells obtained from B6 mice allopregnant of CBA were able to suppress *in vitro* an MLR of B6 responder T-cells stimulated by CBA cells. However, and rather surprisingly, third party stimulators, MLRs, were not tested for sensitivity to suppression. The authors also transferred lymphocytes from BALB/c females, either allopregnant from a C57BL/6 male or syn-pregnant, into a nude BALB/c mouse subsequently mated with a C57BL/6 male. Such a pregnancy proceeded normally if the whole lymphocyte population was transferred, but the transfer of lymphocytes depleted of $CD25^+$ cells resulted in a high rate of fetal resorptions, and T-cells massively infiltrated the implantation sites. Interestingly, (i) both T-cells from syn- and allopregnant mice were abortifacient for allopregnancy when depleted of $CD25^+$ T-cells and (ii) none of these two CD25-depleted populations caused pregnancy problems in BALB/c syngeneic matings (29). These results indicate that allospecific effector T-cells are responsible for fetal rejection, but also that these allospecific effector T-cells do not require prior exposure to MHC. Importantly, it should be noted that the experimental setting is based on the transfer of T-cells into a lymphopenic mouse, devoid of B- and T-cells. This induces a major non-specific homeostatic proliferation and activation of the transferred T-cells, and thus the setting does not fully reflect immune regulation during physiological pregnancy.

We demonstrated that Tregs are involved in maternal–fetal tolerance using a more physiological setting by directly depleting/inhibiting $CD25^+$ cells *in vivo* in pregnant mice, without any further cell manipulation (30). We showed that treatment with anti-CD25 antibodies did not affect syn-pregnant BALB/c mice, but induced fetus resorption in BALB/c allopregnant females. Incidentally, it should be noted that in all the experiments reported, it was not tested whether elimination of Tregs affected primarily, or exclusively, male ($H\text{-}Y^+$) fetuses – see Kahn and Baltimore (31).

Treg EXPANSION/ACTIVATION OR TRANSFER REDUCES ABORTION IN MURINE PREGNANCY

Zenclussen and co-workers have extensively used the CBA x DBA/2J model of naturally occurring murine spontaneous abortion (32–35), initially described by us in 1983 (13). The authors claimed that they were able to "completely prevent" abortion in CBA x DBA/2J mice by transferring Tregs from alloimmunized mice, reporting also "no abortion" at all in the controls CBA x BALB/c and CBA x CBA (32–34). They also deduced antigen specificity from the "complete protection against abortion" (0%) obtained by transferring Tregs from BALB/c-mated CBA/J females, but not those from C57/BL6-mated CBA/J females. Furthermore, transfer of Tregs from the CBA/J x CBA/J mating combination was also protective, which is rather surprising in terms of antigen

specificity (35). These results are puzzling since every mammal species (murine strains included) have a strain-specific abortion rate (see, for example, the records of the Jackson laboratory), depending notably on genetic chromosome anomalies, most of them occurring as a consequence of meiosis.

More recently, the same authors showed that Treg-transferred CBA/J females treated with anti-IL-10 – but not anti-TGF-ß – prior to mating with DBA/2J males had an increased abortion rate (36). In this line, we have reported that anti-IL-10 treatment selectively affects CBA x DBA/2J mating, but not other mating combinations (22).

We investigated whether *in vivo* Treg expansion/activation could improve successful pregnancy rates. We observed that Treg stimulation, either directly by low-dose IL-2 or indirectly by Fms-related tyrosine kinase 3 ligand, led to normal pregnancy rates in CBA x DBA/2J abortion-prone mice (28).

Conversely, high doses of intravaginal interferon have been shown to be abortifacient and/or anti-implantation not only because of their classic effects in conjunction with TNF but also by reducing Tregs and IL-17 at the implantation site (37).

Treg CHANGES DURING THE ESTROUS CYCLE

A further case for an important role of Tregs in pregnancy is the observation that the uterus "prepares" itself for pregnancy by specific cyclic accumulation of Tregs (38, 39).

Kallikourdis et al. studied changes in the numbers of T-cells in the uterus together with the expression levels of chemokines known to induce Treg migration. A rise for CCL3, CCL4, CCL22, and CX3CL1 was noted from diestrus to estrus. If mating led to pregnancy, only CCL4 remained high. In fact, there was a direct correlation between uterine CCL4 expression and $Foxp3^+$ T-cells. Moreover, from estrus to gravid uterus, $CCR5^+$ cells rose from 50% to more than 70%. The authors concluded that since "alloantigen-experienced effector Tregs" express CCR5, CCL4 might be responsible for the retention of these cells in the gravid uterus (39).

Hormonal changes may be drivers for Treg changes. In particular, estrogen has been shown to induce expansion of $Foxp3^+$ cells (40, 41), including in the (pregnant) uterus (42). Analyses of Treg suppressive activity in wild-type, estrogen receptor knockout (ERKO), and programed death-1 (PD-1) KO mice, revealed that (i) estrogen induces PD-1 in $CD4^+Foxp3^+$ cells and (ii) PD-1 expression as well as Treg suppressive activity was reduced in estrogen receptor KO mice. Pre-treatment of PD-1 KO mice with estrogen led to a partial recovery of Treg suppression without enhancing Foxp3 expression. Yet, PD-1 is likely not the only pathway controlling Treg activity, since Treg function is also partly restored by estrogen in PD-1 deficient animals (43). Thus, both PD-1-dependent and PD-1-independent pathways could be involved in estrogen-mediated Treg suppressive activity. Estrogen has also been shown to directly influence Treg expression of IL-10 (44).

An increase of Tregs in mice at day 2 of pregnancy has been described, except for the CBA x DBA/2J mating combination (45), which led to the conclusion that Tregs do not depend on hormonal levels. This is in disagreement not only with the aforementioned reports but also with the data of Mao et al., who showed an increase in Tregs in mid-pregnancy, which is at least in part progesterone-dependent and correlates with an increase in IL-10 production by Tregs (46).

The human chorionic gonadotropin (hCG) has been reported to attract Tregs locally in the murine uterus (47, 48). Similarly, as mentioned before, the luteinizing hormone (LH) has been reported to completely prevent abortion in the classic CBA x DBA/2J murine model of immune abortion, which correlated with increased Treg numbers both locally and at the periphery (45).

THE INFLUENCE OF MATING/SEMINAL FLUID ON Tregs

Events occurring early during pregnancy seem to influence future Treg expansion/function. Using several murine models, Robertson's group demonstrated that mating itself is important for successful pregnancies, with the seminal plasma driving the immediate and preparing the future expansion of uterine and, likely, systemic Tregs. This induces a (transient) "tolerance-like" state to paternal alloantigens in mice. Moreover, the authors showed that seminal fluid contains both TGF-β and prostaglandin E, which potently induces Tregs (49, 50).

ON REGULATORY T-CELLS IN HUMAN PREGNANCY

In human beings, Saito's group identified decidual $Foxp3^+$ Tregs in uterine biopsies (51, 52). Robertson's group showed the presence of Foxp3 mRNA in the uterus of normal women by qRT-PCR in endometrial biopsies obtained during the mid-secretory phase of the menstrual cycle. Interestingly, they found that Foxp3 mRNA levels decrease two-fold in patients with primary unexplained infertility compared with fertile women (53). However, they could not correlate this result with endometrial cytokine levels (TGF-β1, TGF-β2, TGF-β3, IFN-γ, IL-2, IL-4, IL-5, IL-10 and IL-12p40, IL-1α, IL-1β, IL-6, LIF, GM-CSF, and TNF-α) (53).

Fainboim and colleagues monitored Tregs in the menstrual cycle of fertile and infertile women (54) and showed a periodic modulation of Tregs. Treg levels peaked in the late follicular phase, which correlated with serum estradiol, and decreased markedly in the luteal phase. Interestingly, they also showed that in patients with recurrent spontaneous abortions (RSAs), Tregs were low and changes in Treg numbers in the follicular or luteal phase were not significant. Treg numbers in women with RSAs were very similar to the numbers observed in post-menopausal women (54). Furthermore, when these Tregs were tested for their suppressive capacity, a higher number of cells was required to obtain the same level of suppression as Tregs from fertile women, suggesting that, in RSA patients, Tregs are functionally defective (54).

Likewise in mice, the influx of Tregs in the decidua is not only dependent on the hormonal levels in the environment but is also linked to the intercourse, which temporarily increases their number (50). In RSA, the reduction of Tregs appears not to be related to the reduced levels of IL-6 and rIL-1α mRNAs. On the contrary, the relative abundance of mRNAs encoding for LIF, GM-CSF, IFN-γ, IL-1β, IL-4, IL-5, IL-10, IL-12p40, TNF-α, TGF-β1, TGF-β2, and TGF-β3 remained unaltered regardless of the fertility status (53, 55). In this context, IL-27 has recently been suggested to regulate Tregs, IL-17, and IL-10 expression (56).

Besides hormones and cytokines, trophoblasts can also recruit and induce Tregs. The high levels of TGF-β produced by trophoblasts both induce and recruit $CD4^+$ peripheral Tregs (pTregs)

in vitro. Trophoblasts can also activate some $CD8^+$ regulatory cells, which are independent of MHC class I, have a restricted TCR repertoire, and co-express the mucosal markers CD103 and CD101 (57). In the blood of pregnant women, they rapidly expand, suggesting a potential role for these cells *in vivo*. Despite extensive evidence of their role in regulating immune responses – see for example (58–60) – the role of the $CD8^+$ Treg subset in pregnancy or embryo implantation is still poorly understood.

Regulatory T-cells may also be involved in pre-eclampsia (PE), together with regulatory NK T-cells (52, 61, 70, 71), as reported by several authors (62–65), except for Paeschke et al. (66). Furthermore, it has been suggested that there might be an imbalance between $CD4^+CD25^{hi}Foxp3^+$ and $CD4^+CD25^-Foxp3^+$ Treg subsets in PE (61). Recently, however, not only Tregs but also $HLA\text{-}G^+CD4^+$ T-cells have been suggested to play a role (65).

ON REGULATORY T-CELL SPECIFICITY IN PREGNANCY

The studies discussed so far point to an interesting problem: what is the specificity of Tregs mobilized for successful pregnancy? This question was recently addressed by Rowe and colleagues, who showed that pregnancy primes the selective accumulation and activation of maternal Tregs with fetal specificity (67). The authors employed transgenic mice that expressed a surrogate fetal antigen, the $I\text{-}A^b$ $2W1S_{55–68}$ peptide. They found that pregnancy-induced maternal $CD4^+Foxp3^+$ cells specific for $I\text{-}A^b$ $2W1S_{55–68}$, a peptide that expressed CD44 and rapidly accumulated during mid-gestation. These cells persisted at levels increased approximately 10-fold through day 100 post-partum. The same maternal Tregs with fetal specificity expanded at an accelerated rate during secondary pregnancy with the same partner. Using the Foxp3-DTR model (68), the authors also demonstrated that the expanded cells were pTregs and that partial ablation of Tregs in Foxp3-DTR/WT mice resulted in reduced fetal abortion rates compared with primary pregnancy (67).

In pregnant mice, a reduced number of paternal antigen-specific T-cells (69), likely due to peripheral clonal deletion (70), and a reduced responsiveness to tumors bearing the same paternal antigen (T-cell awareness of pregnancy), was demonstrated using a transgenic mouse model and a weakly antigenic tumor allograft challenge. This was interpreted as implying that multiple tolerogenic mechanisms are at play at the same time. T-cell phenotype and responsiveness to tumors was restored after delivery (69).

This questions the antigen specificity of pregnancy-induced Tregs. In the aforementioned system, it has been shown using MHC tetramer that Tregs are not themselves Ag specific, but mediate antigen specificity by locally anergizing the highly specific effector T-cells (67, 69–73).

However, the existence of "true" antigen specificity of Tregs involved in maternal–fetal tolerance is claimed in several studies in human beings (54, 74) and in mice (31, 35, 36, 75). In the classic CBA x DBA/2J murine resorption model, Treg function has been shown to be elicited by the paternal-specific "protective" peptide (76). Similarly, the data of Kahn and Baltimore in an elegant transgenic system support specificity in the regulation of anti H-Y responses (31).

In contrast, we find in the very same model that Treg expansion is driven, at least in part, by recognition of self-specific antigens (28).

This apparent contradiction could be solved if in the uterus and draining lymph nodes two different Treg subsets were mobilized at implantation and later throughout pregnancy, one being self-specific, the other being fetus-Ag or MHC-specific. As reviewed by Marrack et al. in "T-cells and their eons-old obsession with MHC" (77), T-cells could be both antigen- and MHC-specific and thus self-biased. The different loops created by the germline-encoded and non-germline portions of the TCR may contact the MHC proteins and the peptide bound on the MHC, respectively. This idea comes from the observation that there are many TCR variable elements that form specific patterns to contact a particular site on the MHC. Mutations in these sites affect the ability of T-cells to react with the MHC. Interestingly, these similar elements were found in evolutionarily distant species, such as sharks and human beings, suggesting that they evolved to allow TCR to react with MHC proteins.

ON REGULATORY T-CELLS IN EVOLUTION: THE DEVELOPMENT OF THYMIC AND PERIPHERAL Tregs AND THEIR ROLE IN MATERNAL–FETAL TOLERANCE

Placentae appeared very early, and reappeared at various stages during evolution. Velvet worms – onychophora – are placental viviparous, as are sharks and other fishes, some dinosaurs and reptiles, too. Placentation in eutherian mammals came later as the first mammals, the monotremes, are oviparous. The placental mammals emerged 165–80 million years ago (the oldest known eutherian fossil so far being 160 million years old, the *Juramaia sinensis* (78), which fits with most DNA clock analysis of the separation between eutherians and marsupials. The first well-documented placental eutherian is the 65-million-year-old *Maelestes gobiensis* (79).

The first viviparous mammals, in between dinosaurs and mammals, were faced with the development of a sophisticated adaptive immune system, a challenge not previously present. Marsupials escaped the threat of fetus rejection just before it appeared by using the marsupial pouch to house the quasi-fetus newborn. The development of placentation in eutherians involved a series of suppressive mechanisms. Only a few of them have been demonstrated to be crucial, including those involving Tregs.

Thymic Tregs (tTregs) differentiate in the thymus following up-regulation of Foxp3 as a consequence of their expression of self-antigens highly reactive TCRs. pTregs generate in the periphery upon stimulation with high-affinity cognate TCR ligands in the presence of TGF-β and retinoic acid (80–83). The observation that CNS1 – an intronic *Foxp3* enhancer containing Smad3 – and retinoic acid receptor (RAR)-binding sites facilitate TGF-β-dependent Foxp3 induction and pTreg cell differentiation, but is dispensable for tTreg generation, suggests that the biological functions of these two Treg cell subsets are distinct (84).

Samstein and co-workers generated CNS1-deficient mice (85), which lack only pTreg cells but not tTregs. They observed that pregnancy in these mice resulted in a high abortion rate in allogeneic, but not syngeneic matings. Moreover, ablating tTregs in the CNS1-deficient mice did not enhance allopregnancy abortion (85).

Hence, they concluded that pTregs are necessary for successful pregnancy while tTregs are dispensable.

The CNS1 non-coding sequence does not exist in other phyla, such as non-mammals, and in mammals is present only in eutherians, but not in marsupials. Thus, Samstein and co-workers concluded that "the mechanism of extrathymic differentiation of pTreg cells may have been gained during evolution to reinforce tolerance to paternal alloantigens presented by the fetus during the increasingly long gestation period in placental mammals" (85). However, the authors also reported a defect in spiral artery formation in mice lacking pTregs, which open other possibility than just tolerance for the role of pTregs in pregnancy.

We believe that the unique role of pTregs should be balanced by the fact that there exist yet no models of a pure tTreg depletion, which could demonstrate the role – or absence of role – of this subset in maternal–fetal tolerance. Furthermore, two gestational periods should be considered: the embryo implantation period and later fetus development. We showed that the immediate response of the immune system to embryo implantation is mediated by activated/memory self-specific Tregs, hence tTregs. It is thus possible that tTregs initiate a tolerance state that is later maintained with the recruitment of pTregs. We believe that both tTregs and pTregs have been selected during evolution primarily for the purpose of establishing maternal–fetal tolerance in eutherians (28, 85).

ON SIMILARITIES BETWEEN REGULATORY T-CELL RESPONSES TO FETAL AND TUMOR GROWTH

As often mentioned in the literature since the dawn of Reproductive Immunology, there are striking similarities between malignant processes and pregnancy (86). In a tumor model, we observed that tumor emergence elicits a brisk Treg response that precedes and preempts the response of effector T-cells. This Treg response is detectable as soon as days 2–3 post-tumor cell implantation or emergence and is mediated by self-antigen-specific $CD44^{hi}CD62^{low}$ activated/memory Tregs (87). We recently reported striking similarities in the Treg response to embryo implantation, with the same recruitment of self-antigen-specific $CD44^{hi}CD62^{low}$ activated/memory Tregs detectable within 2 days post-implantation (28).

However, the parallel is not complete. Pre-immunization against an artificial paternal antigen (HA in our case) only marginally increased fetal loss, whereas pre-immunization with the HA antigen resulted in 100% eradication of HA-expressing tumors. However, mixing Treg depletion with pre-immunization drastically increased fetal loss (28).

Furthermore, the immunological paradox of pregnancy, whereby the maternal immune system tolerates the presence of the semi-allogenic fetus, has historically been associated with the early work on immunological tolerance to transplantation. However, even though Tregs play a role in the control of allogeneic responses to solid or cell grafts (including allogeneic cancer cells) (88) and have demonstrated therapeutic potential in this setting (89), these grafts are always rejected in the absence of specific intervention. This highlights the uniqueness of the immune responses in the allogeneic maternal/fetal tolerance setting.

We hypothesized that the similarities in the Treg response to tumor or embryo implantation suggest that protection of cancer cells by Tregs became the price paid for an efficient protection of embryos (28).

ON OTHER IMPORTANT CELLS

The decidua is populated by several immune cell types, which coexist together with stroma cells and trophoblasts. Among them, dendritic cells (DCs) and uterine NK (uNK) cells are highly abundant. During the female estrus cycle and throughout pregnancy, the number of these cells undergoes dramatic chances, as do, likely, their reciprocal interactions. The concept of decidual cell–cell interactions is relatively new and arises from an important feature of immune cells, their ability to migrate, which confers them dynamic properties. The introduction of intravital two-photon microscopy made it possible to study the dynamic behavior of immune cells and their interactions, in a spatio-temporal dimension. However, information about immune cell dynamics at the maternal–fetal interface remains limited, while abundant in other models such as cancer, infection, or inflammation [reviewed in Ref. (90)]. T-cells are relatively rare in the uterus of both pregnant and non-pregnant human beings and mice (91, 92). However, despite their paucity, Tregs are critical for normal pregnancy. The secret of their pivotal role could thus reside in the dynamic interactions they establish within the decidua.

UTERINE NK CELLS

Uterine NK have long been considered the most important cell type for the success of pregnancy due to their abundance in the decidua. Moreover, as increasing evidence points to the importance of other leukocytes, such as Tregs, the functional relationship between uNK cells and the other immune cells has come into focus.

Uterine NK cells differ from NK cells in other sites of the body. Mature uNK contain numerous granules (rich in perforin, granzymes, granulysis) (93), but, unlike peripheral blood NK cells, uNK cells are only weakly cytotoxic *in vitro* and do not kill trophoblasts *in vivo*. They seem to both differentiate and proliferate in the uterus, but also migrate from the periphery (94, 95). In mice, uNK increases upon implantation in concomitance with trophoblast invasion of the endometrium and subsequent decidualization (96) and they peak at mid-gestation. Mouse uNK cells have been shown to localize in the mesometrium to form a characteristic ring-shaped structure around the spiral arteries characterized by the presence of highly proliferative cells, called the mesometrial leukocyte aggregate of pregnancy, MLAp (97). Even though models of artificial decidualization have shown that uNK differentiation depends on hormonal changes (98), rather than on trophoblast invasion, mouse uNK cells do not express progesterone receptor (96).

Early implantation sites in mice deficient for NK, T-, and B-cells showed abnormal decidual and mid-gestational myometrial structures and no spiral artery modifications (99–101). Noteworthy, despite these defects, litters of normal size were born (102–105), except in the Tge26 (100, 101) mice, which display a reproductive deficit. Bone marrow transplantation of NK^+ T^-B^- pools before mating restored the defects suggesting a major role of uNK cells more related to vascularization than to tolerance.

In this line, human pregnancy-associated disorders, such as PE, still birth, and fetal growth restriction, all display deficits in

spiral artery formation and are characterized by a "shallow invasion" of the uterine wall (106, 107). uNK cells produce several angiogenic factors (108–110) such as IFN-γ, and Croy and colleagues have shown that artery remodeling is strictly dependent on IFN-γ produced by NK cells in the uterus (111, 112). However, in human beings, the levels of IFN-γ during pregnancy are rather low although spiral artery remodeling remains crucial (113, 114). Trophoblasts express a characteristic combination of HLA-C, HLA-G, and HLA-E MHC class I molecules (115) and correct spiral artery remodeling has been correlated to allo-recognition of trophoblasts cells by uNKs.

UTERINE DENDRITIC CELLS

Together with uNK cells, DCs represent the most abundant cell type in the uterus. They are known as potent antigen-presenting cells (APC). DCs have been reported to recognize foreign antigens present on sperm cells upon mating (49, 50, 116), but most likely also recognize alloantigens expressed by the invasive trophoblasts during implantation and decidualization. Interestingly, upon implantation, DCs re-localize to different areas of the decidua (117). In particular, Erlebacher and co-workers have suggested that the decidua works as a barrier that impedes DCs to efficiently prime T-cells in the lymphoid organs, to minimize the immune response to paternal alloantigens (118). Furthermore, they described how DCs remain entrapped in the uterus and are unable to carry antigens to the lymph nodes due to the lack of lymphatic vessels, which, in the mouse uterus, are confined exclusively to the myometrium (118, 119). Importantly, they demonstrated the spatio-temporal regulation and the extent of antigen-specific T-cell priming during pregnancy (120). They mated wild-type

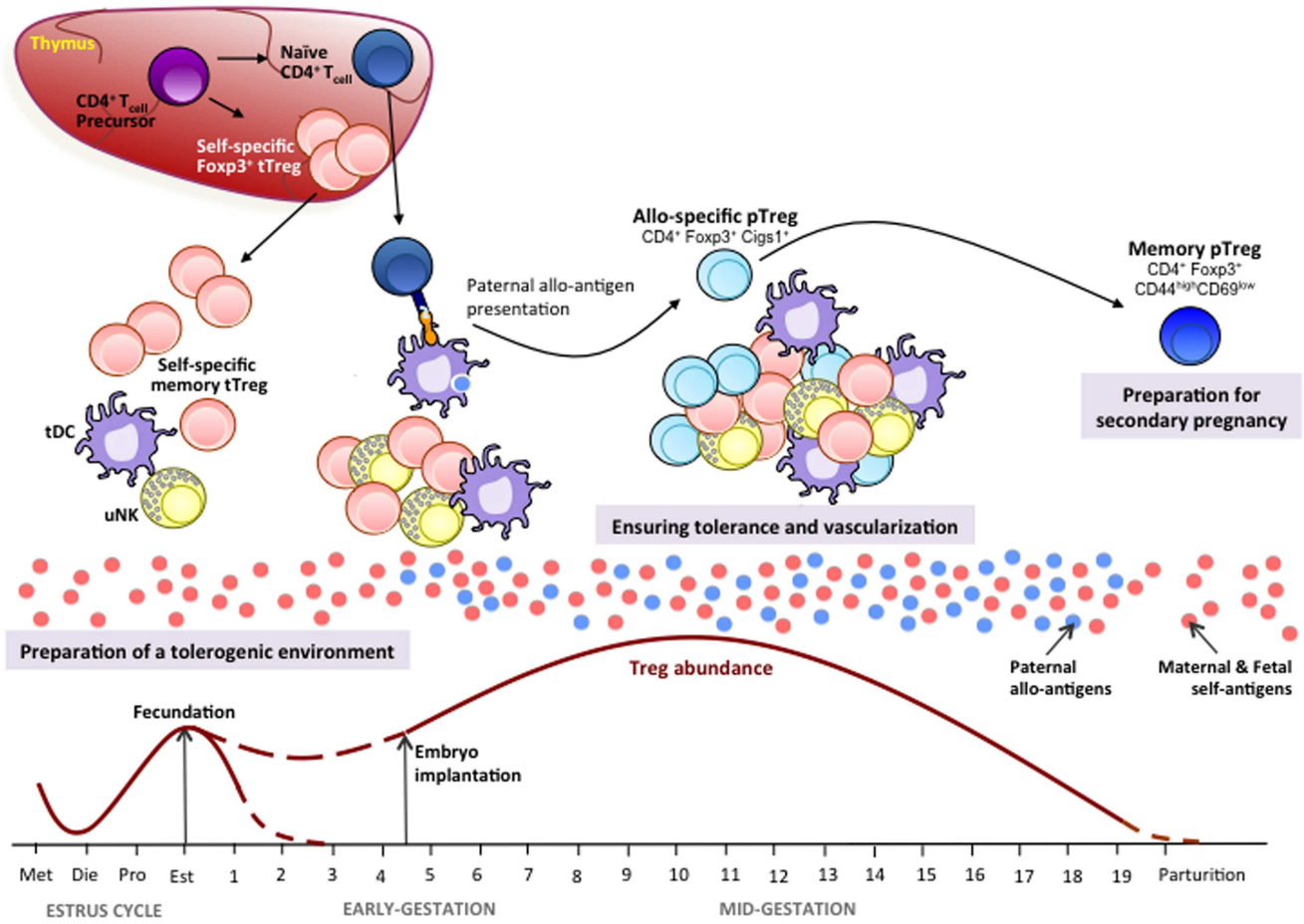

FIGURE 1 | Tregs in mouse pregnancy. Thymic Tregs (tTregs) recognizing maternal/fetal self-antigens differentiate in the thymus from the CD4+ T-cell precursors by up-regulating Foxp3 expression. During the estrus cycle, there is an increase in tTregs in the periphery and the uterus where, together with tolerogenic dendritic cells (tDC) and uterine NK (uNK) cells, they prepare a uterine tolerogenic environment for pregnancy under a hormonal control. During the estrus phase, recruitment of tTregs at ovulation is maximum in order to prepare a tolerogenic uterine environment for a potential embryo implantation. During pregnancy, self-antigen-specific activated/memory tTregs mount a first-line tolerogenic response (28). Later, the first fetal/paternal alloantigens generated by fetal cells trigger an immune response to paternal alloantigens (85). Alloantigen presentation through tDCs favors the conversion of naïve CD4+ T-cells in induced peripheral Tregs (pTregs) by up-regulating Foxp3 and its Cigs1 enhancer gene expression (84). The clonal expansions of allospecific pTregs together with the proliferation of tTregs, uNKs, and tDCs during the mid-gestation periods ensure the maintenance of immune tolerance to the fetus and allow vascularization to guarantee a steady supply of nutrients and oxygen to the fetus for a proper growth and development. Generation of memory pTregs specific for paternal antigens will contribute to tolerance induction to the same fetal/paternal alloantigen exposure in case of a secondary pregnancy with the same paternal antigens. The tTregs, uNKs, and tDCs cross-talk is yet poorly defined.

females with Act-mOVA males, where OVA expressed by the conceptus mimics a paternal-specific antigen. Only DCs of maternal origins presented OVA-MHC in the LNs and induced OVA-specific T-cell expansion at mid-gestation, suggesting that the LNs are the primary site of alloantigen-presentation.

Finally, uterine DCs have also been proposed to perform trophic functions. Plaks and co-authors induced fetal loss by depleting DCs before implantation (121) using the CD11c-DTR transgenic mouse model (122). The absence of DC-derived angiogenic factors hampered vessels' formation, and affected normal implantation and decidualization. Taken together, these results indicate that during mouse pregnancy, DCs prime T-cells and play a trophic function by ensuring correct vessel formation.

ON Treg CROSS-TALKS

Similar to Tregs, uNK cell numbers vary during the estrus cycle. Recent results from Rudensky's group have highlighted a defect in spiral artery formation in mice lacking pTregs (85). Absence of pTregs determines fetal demise in their model. These results pose an interesting question: is there co-operation between uNK cells and Tregs to ensure correct spiral artery modification?

Moreover, Rowe and colleagues have recently shown that maternal Tregs specific for paternal alloantigens expand > 100 folds during pregnancy (67). These cells persist after delivery and, because of their antigen-specific memory, expand faster than naïve Tregs in subsequent pregnancies, possibly contributing the well-known "lymphoid recall flare" in second pregnancy (123). Relating these results to the human situation, the authors suggest that their observations might explain why the rates of pregnancy complications, such as PE, decrease in subsequent pregnancies. Taken together, both in human beings and mouse, uNK cells and Tregs seem to affect spiral artery formation with important consequences for fetal survival. Moreover, Tregs can also suppress NK cells. uNK cells in turn might control Treg recruitment to the pregnant uterus.

Furthermore, DC maturation in the pregnant uterus is thought to support expansion of antigen-specific Tregs that finally protect the fetus from abortion (49). Thus, DCs have been proposed to exert a dual role in promoting tolerance to paternal alloantigens, limiting their own priming-activity, also in response to signals in the microenvironment, and priming the few Tregs present in the decidua.

Finally, the known cross-talk between NK cells, DCs, and Tregs may be operating locally in the uterus during pregnancy (124).

ON BURNING QUESTIONS

Since the discovery of Tregs 30 years ago, our knowledge about immune tolerance has dramatically improved. The data summarized above suggest their important role in conserved mechanisms that establish and maintain immune tolerance during early pregnancy (**Figure 1**).

We believe that most important points for the field that remain unanswered or controversial are: (i) the antigen specificity of Tregs involved, which could be elucidated by TCR deep-sequencing, (ii) the respective functional role of the Treg subsets involved (i.e., tTreg, pTreg, etc.), (iii) the localization and functional cross-talk of Tregs with uNK and uDCs, which could be studied by intravital imaging and novel transgenic mice (125–127), and (iv) the link between Treg responses to embryo and tumor cell implantation.

A better understanding of these mechanisms will be pivotal in identifying more effective therapeutic targets for the treatment of pathological conditions related to pregnancy (128, 129) and, more generally, to diseases in which the immune balance is perturbed.

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Stimulation of monocytes by placental microparticles involves toll-like receptors and nuclear factor kappa-light-chain-enhancer of activated B cells

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Human pregnancy is accompanied by a mild systemic inflammatory response, which includes the activation of monocytes circulating in maternal blood. This response is exaggerated in preeclampsia, a placental-dependent disorder specific to human pregnancies. We and others showed that placental syncytiotrophoblast membrane microparticles (STBM) generated *in vitro* from normal placentas stimulated peripheral blood monocytes, which suggest a contribution of STBM to the systemic maternal inflammation. Here, we analyzed the inflammatory potential of STBM prepared from preeclamptic placentas on primary monocytes and investigated the mode of action *in vitro*. STBM generated *in vitro* by placental villous explants of normal or preeclamptic placentas were co-incubated with human peripheral blood monocytes. In some cases, inhibitors of specific cellular functions or signaling pathways were used. The analysis of the monocytic response was performed by flow cytometry, enzyme-linked immunoassays, real-time PCR, and fluorescence microscopy. STBM derived from preeclamptic placentas up-regulated the cell surface expression of CD54, and stimulated the secretion of the pro-inflammatory interleukin (IL)-6 and IL-8 in a similar, dose-dependent manner as did STBM prepared from normal placentas. STBM bound to the cell surface of monocytes, but phagocytosis was not necessary for activation. STBM-induced cytokine secretion was impaired in the presence of inhibitors of toll-like receptor (TLR) signaling or when nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation was blocked. Our results suggest that the inflammatory reaction in monocytes may be initiated by the interaction of STBM with TLRs, which in turn signal through NF-κB to mediate the transcription of genes coding for pro-inflammatory factors.

Keywords: human pregnancy, inflammation, STBM, monocytes, NF-κB, TLR

INTRODUCTION

Preeclampsia is a multi-symptom disorder of the second half of pregnancy, which affects 2–7% of pregnant women worldwide (1). Despite extensive research, the etiology of this pathologic pregnancy condition remains unclear. The current research suggests that, in patients destined to develop preeclampsia, pregnancy is associated with an increased maternal inflammatory reaction, which will lead to placental stress and ultimately result in the mother's systemic endothelial dysfunction and the large array of life-threatening symptoms, which characterize the disorder (2–4). Interestingly, normal pregnancy also induces a physiologic systemic inflammatory response toward term, however in a much milder form as found in preeclampsia (5).

On the other hand, it is well-acknowledged that the placenta plays a crucial role in the development of the systemic maternal symptoms of preeclampsia. Over the years, evidence for abnormal development, function, and tissue turnover of the placenta has accumulated (6–8). We and others have suggested that placenta-derived syncytiotrophoblast membrane microparticles (STBM), which are shed into maternal blood in higher amounts in preeclampsia as compared to normotensive pregnancies (9), may play an active role in stimulating the mother's inflammatory response (10–12). *In vitro*, STBM interfere with human umbilical vein endothelial cell proliferation and survival or with the relaxation of artificially pre-constricted small subcutaneous fat arteries (13–16). In addition, STBM induce a strong pro-inflammatory response in donor-derived human peripheral polymorphonuclear leukocytes (17) and in human peripheral blood monocytes (18, 19). In monocytes, the production of the

Abbreviations: 6AQ, 6-amino-4-(4-phenoxyphenylethylamino)quinazoline; MACS, magnetic cell separation system; MyD88, myeloid differentiation primary response gene 88; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; PA, perillyl alcohol; STBM, syncytiotrophoblast membrane microparticles; STBM-NP, STBM derived from normal placentas; STBM-PE, STBM derived from preeclamptic placentas; TLR, toll-like receptor.

pro-inflammatory cytokines tumor necrosis factor (TNF) α, interleukin (IL)-12, IL-6 and of the chemokine IL-8 is increased, whereas the cells also adopt a cell surface expression pattern with up-regulation of the adhesion molecule CD54, which is very reminiscent of the pattern exhibited by peripheral blood monocytes freshly harvested from pregnant women (20). Compared to non-pregnant controls, circulating monocytes from healthy pregnant women display an inflammatory phenotype, which is hallmarked by an enhanced phagocytic activity, elevated basal production of reactive oxygen species (21), and increased production of pro-inflammatory mediators (22). In preeclampsia, peripheral monocytes are more extensively activated when compared to their counterparts in normal pregnancy, with a further increase in the production of IL-1β, IL-6, and IL-8 (23). These latter features concur with our previous results indicating that STBM generated from normal placentas had the potential to stimulate monocytes in a dose-dependent manner (19). Therefore, we suggested that the progressive monocytic activation in the maternal peripheral blood during pregnancy might be due to the steady increase in the amount of placental microparticles as gestational age advances. We also proposed that the excessive monocytic activation in preeclampsia might be correlated with the elevated circulatory STBM concentrations existing in this pregnancy condition. However, evidence that microparticles shed from preeclamptic placentas similarly stimulate monocytes is still scarce. Interestingly, plasma-derived microparticles from preeclamptic women activate endothelial cells *in vitro*, in the presence of monocytes, to a higher extent than microparticles isolated from normotensive women (24). This study did however not identify the specific subgroup of microparticles, which affected the co-cultures of endothelial cells with monocytes and did not detail the monocytic contribution to endothelial cell activation. Therefore, in the present study, we prepared STBM *in vitro* by explant cultures of preeclamptic placentas and investigated their effect on human peripheral blood monocytes.

MATERIALS AND METHODS

IN VITRO GENERATION OF STBM

This study was approved by the local ethical committee (Cantonal Institutional Review Board of Basel, Switzerland). In all cases, written informed consent was received. Human term placentas from uncomplicated pregnancies and placentas from cases with preeclampsia were collected in the Department of Obstetrics and Gynecology, University Hospital of Basel, within 1 h following elective or secondary cesarean section. Explants from villous tissue were set in culture in a controlled atmosphere (37°C, 20% oxygen/5% carbon dioxide) as described previously (19). STBM were isolated from the culture supernatants by a three-step centrifugation procedure at 4°C, namely 1000 × *g* for 10 min, 10,000 × *g* for 10 min, and 60,000 × *g* for 90 min. The microparticle-containing pellet was washed with PBS, re-suspended in PBS/5% sucrose and stored at −20°C until use. The protein content of the STBM was quantified with the Advanced Protein Assay Reagent (Cytoskeleton Inc., Denver, CO, USA) and STBM were standardized for protein concentrations as indicated in the figure legends.

ISOLATION OF HUMAN MONOCYTES

Forty milliliters of venous blood from healthy male donors, which were collected in EDTA-containing tubes, were centrifuged on Histopaque (Sigma, Saint Louis, MO, USA) density gradient according to the manufacturer's instructions. Peripheral blood mononuclear cells (PBMCs) were washed twice with PBS supplemented with 2 mM EDTA and the residual erythrocytes were lysed with the red blood cell lysis solution (Qiagen, Valencia, CA, USA). Monocytes were isolated through negative selection by means of the Human Monocyte Isolation Kit II and a magnetic cell separation system (MACS), according to the manufacturer's instructions (Miltenyi Biotec Inc., Auburn, CA, USA).

CO-CULTURE OF MONOCYTES AND STBM

Monocytes were cultured in a final concentration of 5×10^5 cells/ml in RPMI-1640 (Gibco, Grand Island, NY, USA) supplemented with 10% FCS (Amimed, Allschwil, Switzerland), 4 mM glutamine (Gibco), and 100 U/ml penicillin/streptomycin (Gibco). Cells were either left untreated or co-incubated with different amounts of STBM as mentioned in the figure legends, or stimulated with lipopolysaccharide (LPS) from Gram-negative bacteria (Sigma) as positive control. In some experiments, monocytes were pre-treated for 15 min with the phagocytosis inhibitor cytochalasin B (Sigma), or the NF-κB inhibitors 6-amino-4-(4-phenoxyphenylethylamino) quinazoline (Calbiochem, San Diego, CA, USA) and perillyl alcohol (PA) (Sigma), or for 24 h with a peptide inhibiting myeloid differentiation response gene (MyD) 88 homodimerization, before addition of STBM. Co-cultures were incubated during 4 h (RNA analysis), 12 h (cytokine analysis after the inhibition of MyD88 homodimerization), or 16 h (flow cytometry and cytokine analysis) at 37°C in 20% oxygen/5% carbon dioxide. At the end of the culture, the cells and the culture supernatants were separately harvested for further analysis. Cell viability was confirmed with the Cell Proliferation Reagent WST-1 (Roche Diagnostics GmbH; Mannheim, Germany).

FLOW CYTOMETRY

Monocytes were first incubated with 200 μg/ml of purified human IgG (Sigma) in PBS supplemented with 2 mM EDTA and 1% FCS for 5 min at 4°C to prevent unspecific binding through Fc receptors. Incubations with specific antibodies were carried out for 15 min at 4°C with ready-to-use concentrations of FITC-conjugated antibodies against CD14 (BD Pharmingen, San Jose, CA, USA) and PE-conjugated antibodies against CD54 (BD Pharmingen) or APC-conjugated antibody against CD11a (BD Pharmingen). Labeled cells were washed with PBS and re-suspended in PBS supplemented with 2 mM EDTA and 0.5% BSA. The data was acquired on a Dako Cyan flow cytometer (Beckman Coulter, Fullerton, CA, USA) and analyzed with the Summit software.

ENZYME-LINKED IMMUNOSORBENT ASSAY

The secreted levels of IL-8 and IL-6 were measured in duplicates using the respective DuoSet® enzyme-linked immunosorbent assay (ELISA) development kits (R&D Systems Inc., Minneapolis, MN, USA), according to the manufacturer's protocol. Plates were read at 450 nm with a wavelength correction set at 562 nm, in the

Spectramax 250 microplate reader (Molecular Devices, Sunnyvale, CA, USA) and analyzed with Softmax Pro software (Molecular Devices).

FLUORESCENT MICROSCOPY

Purified monocytes were incubated in 1 μM carboxyfluorescein succinimidyl ester (CFSE) (kindly provided by Prof. G. Spagnoli, Department of Biomedicine, University Hospital of Basel) in the dark at 37°C for 10 min. The staining reaction was stopped with 2 ml of complete RPMI-1640 culture medium. Cells were washed three times with PBS and re-suspended in complete RPMI-1640 culture medium. STBM were diluted 1:10 in diluent C and incubated with 5 μM PKH26, using the PKH26 Red Fluorescent Cell Linker kit (Sigma). After 5 min of incubation at RT, the staining was stopped with 2 ml FCS (Amimed, Allschwil, Switzerland). STBM were washed with PBS and re-suspended in PBS/5% sucrose. The CFSE-labeled monocytes (5×10^5 cells/ml) were co-cultured with 100 μg/ml PKH26-labeled STBM for 16 h at 37°C. Cells were then harvested, washed with PBS, and transferred by cyto-centrifugation onto a glass slide (Shandon, Frankfurt, Germany). Slides were dried at RT in the dark, fixed with 4% formaldehyde (Sigma) for 30 s, counterstained with 0.01% DAPI/Glycerol (Fluka Chemie GmbH, Buchs, Switzerland), and immediately examined with an Axioplan 2 imaging fluorescent microscope using the appropriate filters (Carl Zeiss, Zürich, Switzerland).

RNA EXTRACTION AND REVERSE TRANSCRIPTION

Monocytes were washed with ice-cold PBS and lysed in 500 μl Trizol reagent (Gibco). After centrifugation at 12,000 × *g* at 4°C, the aqueous phase was collected and 0.5 volume of ice-cold EtOH was added. RNA was then isolated using the RNAeasy Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. The concentration of RNA was determined by spectrophotometry (NanoDrop Technologies, Wilmington, DE, USA). One hundred sixty seven nanograms of RNA were reverse-transcribed using 500 ng random primers included in the Reverse Transcription System (Promega Corporation, Madison, WI, USA). The reaction was performed on a TRIO-Thermoblock (Biometra, Goettingen, Germany) under the following conditions: 10 min at 37°C, 60 min at 45°C, 5 min at 95°C, and 15 min at 4°C.

REAL-TIME PCR

The human NF-κB signaling pathway RT^2 profiler PCR array is a commercially available real-time PCR based assay (SABiosciences, Frederick, MD, USA), which profiles the expression of 84 key genes involved in the NF-κB signaling transduction. The plates were received pre-coated with forward and reverse primers of the respective genes. For one 96-well plate, 102 μl of cDNA was mixed with 1275 μl of 2× SuperArray RT^2 qPCR master mix and filled up with H_2O to the final volume of 2550 μl. Twenty-five microliters of the experimental cocktail were pipetted into each well of the PCR array. The PCR was run with the following PCR cycling program on ABI PRISM® 7000 Sequence Detection System (Applied Biosystems Inc., Forster City, CA, USA): 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. The data were analyzed with the delta–delta C_t method using the online software provided on the company's webpage and expressed as fold change ($2^{-\Delta\Delta C_t}$) relative to the untreated cells.

STATISTICAL ANALYSIS

To calculate the significance of differences between the experimental groups, the Mann–Whitney test was performed using SPSS 15.0 (Statistical Package for the Social Sciences; Chicago, IL, USA). $p < 0.05$ was considered to be statistically significant.

RESULTS

STBM GENERATED BY EXPLANT CULTURES OF PREECLAMPTIC PLACENTAS ACTIVATE HUMAN PERIPHERAL BLOOD MONOCYTES IN A SIMILAR WAY AS STBM DERIVED FROM NORMAL PLACENTAS (STBM-NP)

Since only a minor fraction of all microparticles circulating in the blood of healthy pregnant women are shed from the placenta (25), STBM are usually generated *in vitro*. Recently, we established short-term explant cultures of villous tissue to produce STBM-NP (14). Here, this method was applied on placentas, which were collected from women with preeclampsia.

To investigate if the microparticles generated from the preeclamptic placentas (STBM-PE) altered the expression profile of the inflammatory markers CD54 and CD11a on primary human monocytes, the cells were incubated with STBM-PE for 16 h and cell surface expression was monitored by flow cytometry. More than 95% of the cells expressed the monocyte-specific marker CD14 and its expression remained unchanged with incubation with the microparticles (Figure S1 in Supplementary Material). On the contrary, the expression levels of CD54 were significantly increased [mean increase of median fluorescence intensity (MFI) ± SEM: 641.2 ± 122.5, $p < 0.01$] and the levels of CD11a were significantly decreased (mean decrease of MFI ± SEM: 583.4 ± 77.2, $p < 0.01$) following co-culture with STBM-PE (**Figure 1**). This was very comparable to the changes triggered by STBM-NP, except for CD11a, which was only modestly affected by the latter (**Figure 1B**, lower panels).

In our previous study, we showed that STBM-NP stimulated primary monocytes to secrete soluble mediators of inflammation (19). Therefore, the effect of STBM-PE on the monocytic production of the pro-inflammatory cytokine IL-6 and the chemokine IL-8 was investigated. This analysis showed that STBM-PE stimulated the secretion of IL-6 and IL-8 in a dose-dependent manner, as did STBM-NP. Furthermore, both microparticle populations induced similar concentrations of cytokines at identical doses (**Figure 2**). However, a higher sample number would be required to test for the absence of a difference.

MONOCYTIC ACTIVATION DOES NOT REQUIRE PHAGOCYTOSIS OF THE STBM

As both populations of STBM triggered similar inflammatory responses in primary monocytes, the molecular mechanisms of cell activation were addressed using the more available STBM-NP. To assess whether placental microparticles could interact with human monocytes, they were labeled with the red fluorescent lipophilic dye PKH26 prior incubation with the cells. Flow cytometric analysis revealed that all monocytes became highly positive for PKH26, which indicates that STBM directly interact with the cells (**Figure 3A**). However, this analysis did not permit to localize precisely the microparticles. Therefore, the experiment was repeated with monocytes pre-treated with the green fluorescent

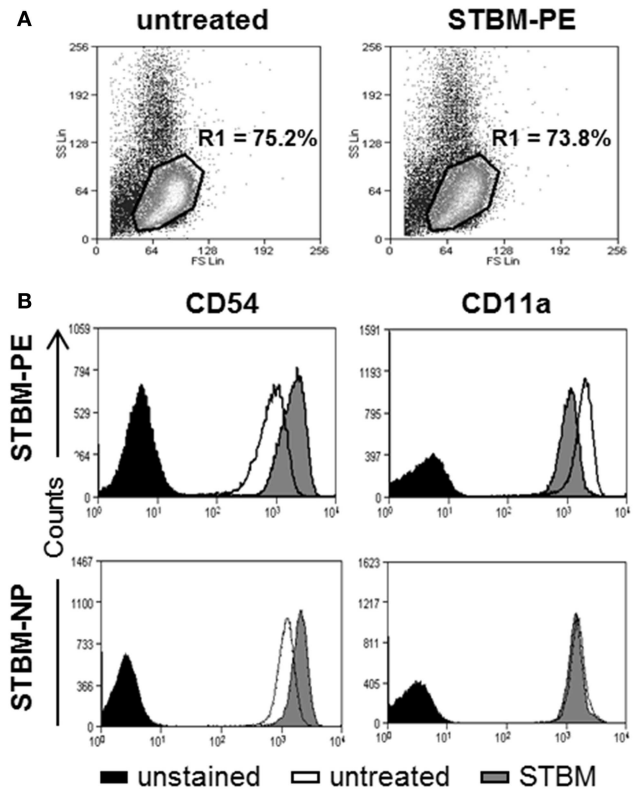

FIGURE 1 | STBM from normal and preeclamptic placentas alter the cell surface expression of CD54 and CD11a on monocytes. Primary human monocytes were left untreated or were co-cultured with 300 μg/ml STBM prepared from normal (STBM-NP) or preeclamptic (STBM-PE) placentas for 16 h. The cell surface expression of CD54 and CD11a was evaluated on CD14-positive cells using flow cytometry. **(A)** Representative forward/side scatter dot plots of untreated or STBM-treated cells. **(B)** Representative histograms of CD54 or CD11a expression on untreated or STBM-treated monocytes. The co-culture experiments were performed two times with five different STBM-NP preparations and three independent STBM-PE preparations.

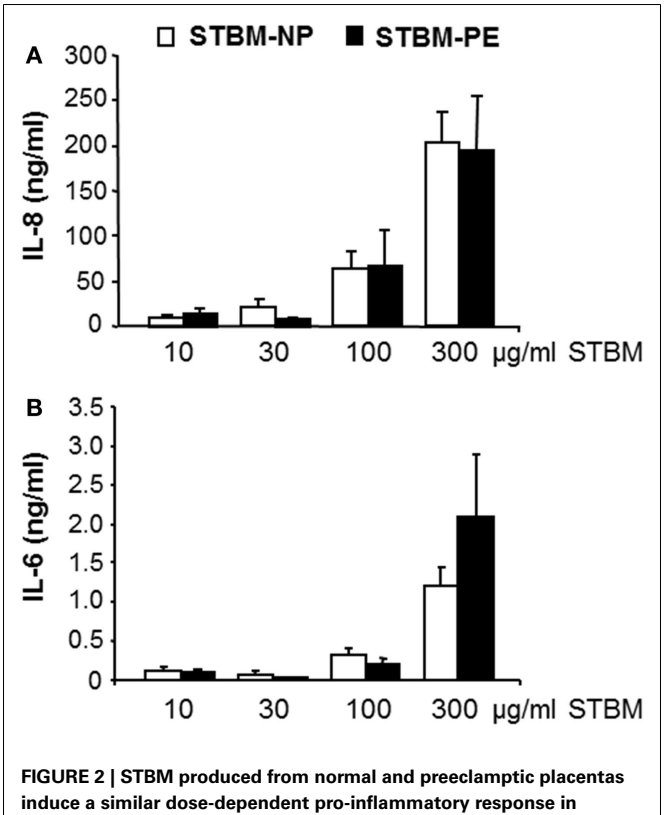

FIGURE 2 | STBM produced from normal and preeclamptic placentas induce a similar dose-dependent pro-inflammatory response in monocytes. Primary monocytes were incubated with increasing concentrations of STBM prepared from normal (STBM-NP) or preeclamptic (STBM-PE) placentas for 16 h. The cellular secretion of IL-8 **(A)** and IL-6 **(B)** was measured by conventional ELISA. Results are illustrated as mean ± SEM of two independent monocyte co-culture experiments with five STBM-NP preparations and three STBM-PE preparations. There is no statistical difference between the responses generated by the two different STBM populations.

intracellular dye CFSE and PKH26-labeled STBM-NP and analyzed by fluorescence microscopy. STBM were regularly found on the rim of the cells, suggesting that they bind to but are not internalized by the cells (**Figure 3B**). In some cases, clamps of STBM formed and attached on the cell surface (**Figure 3B**, lower panel).

To verify that internalization of the placental microparticles was not required to activate the cells, the monocytes were pre-treated with cytochalasin B, a cell-permeable mycotoxin that blocks phagocytosis, prior addition of the microparticles. Neither cytochalasin B nor the drug-carrier DMSO interfered with the STBM-induced secretion of IL-8 and IL-6 (**Figure 3C**). Cell viability after culture was confirmed (Figure S2 in Supplementary Material).

TOLL-LIKE RECEPTORS ARE INVOLVED IN THE STBM-INDUCED ACTIVATION OF MONOCYTES

As STBM interact with the cell surface of monocytes, we checked whether cell membrane receptors of the toll-like family (TLR) may be involved. Accordingly, monocytes were pre-treated with a peptide inhibiting MyD88 homodimerization, a broad TLR blocking agent, prior incubation with LPS or STBM-NP. LPS transmits intracellular activation signals through TLR4. Pre-treatment of monocytes with MyD88 inhibitory peptide partially impaired LPS-induced secretion of IL-6 and IL-8 (**Figure 4**). In a similar way, incubation of monocytes with STBM-NP in the presence of the MyD88 inhibitory peptide lead to significantly reduced secretion of both pro-inflammatory mediators compared to cells cultured with STBM-NP alone (**Figure 4**). Thus, placental microparticles appear to activate monocytes, at least in part, via one or several members of the TLR family.

STBM ACTIVATE MONOCYTES IN AN NF-κB-DEPENDENT MANNER

In order to establish whether the secretion of IL-6 and IL-8 in response to STBM stimulation was due to *de novo* gene transcription, we analyzed mRNA levels by relative real-time PCR. IL-6 and IL-8 mRNA levels were increased 120-fold and 4-fold, respectively, upon STBM-treatment compared to untreated monocytes (**Figure 5A**). The transcription of several other mediators involved in the inflammatory response, such as IL-1α, IL-1β, IL-10, lymphotoxin (LT)-α, and the TNF, was also induced (**Figure 5A**). Besides

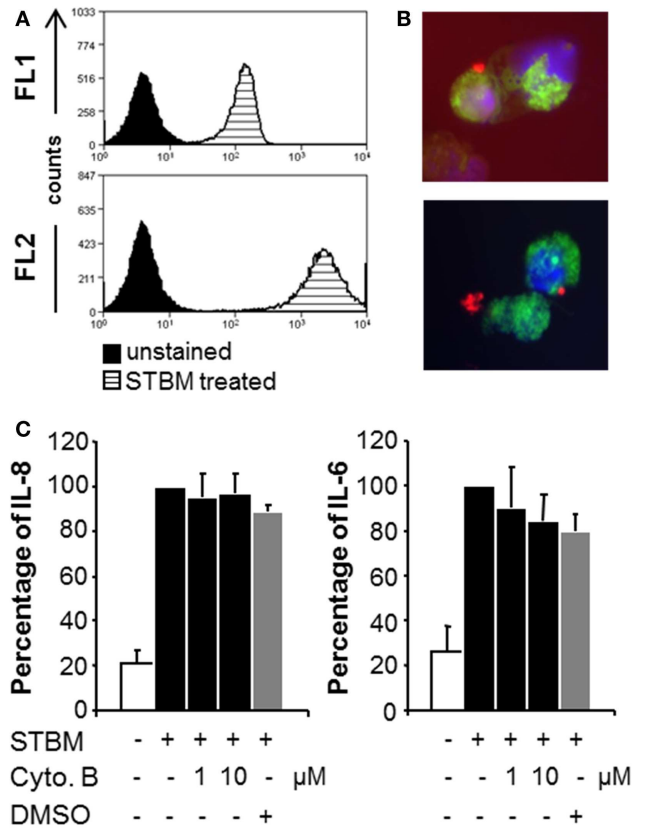

FIGURE 3 | STBM interact with monocytes, but phagocytosis is not required for their stimulatory properties. PKH-26-labeled STBM-NP (300 μg/ml) were incubated with monocytes for 16 h and analyzed by flow cytometry or fluorescence microscopy. **(A)** Representative histograms of CD14-positive monocytes read in the FL1 (CD14) and FL2 (PKH-26) channels. **(B)** Fluorescence microscopy of CSFE-stained monocytes (green) incubated with PKH-labeled STBM (red). DAPI (blue) was used as a nuclear counterstain. **(C)** Monocytes were pre-treated with various concentrations of cytochalasin B in DMSO or with DMSO alone, prior to the addition of 300 μg/ml STBM. The levels of IL-8 and IL-6 secreted by the cells after 16 h of incubation are expressed as a percentage of the amounts produced by cells stimulated with STBM in the absence of the inhibitor, which was set at 100%. Data represent mean ± SEM of three different co-cultures with STBM-NP prepared from three placentas. There was no statistical difference between cytokine secretion in the absence or in the presence of cytochalasin B.

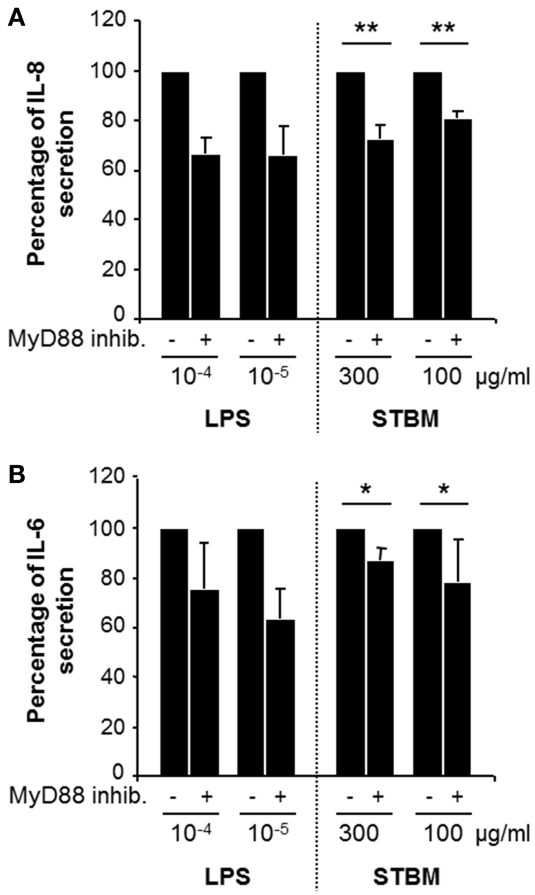

FIGURE 4 | Inhibition of MyD88 homodimerization reduces STBM-induced pro-inflammatory response. Primary monocytes were pre-treated with 1 μM MyD88 inhibitor peptide for 24 h prior incubation with the indicated concentrations of LPS or STBM-NP during 12 h. The concentrations of IL-8 **(A)** and IL-6 **(B)** secreted by the cells were quantified by ELISA and are presented as a percentage of the amounts secreted in absence of pre-treatment with the inhibitor. Data represent mean ± SEM of three monocyte co-culture experiments with STBM-NP prepared from four placentas. $*p < 0.05$; $**p < 0.01$. LPS was used as a positive control.

stimulating the transcription of several targets, STBM-NP also down-regulated the transcription of molecules involved in apoptosis, like caspase-8 ($2^{-\Delta\Delta C_t} = 0.32$), CD27 ($2^{-\Delta\Delta C_t} = 0.43$), and Fas-associated protein with death domain (FADD; $2^{-\Delta\Delta C_t} = 0.5$) (data not shown).

The gene transcription of these pro-inflammatory mediators is controlled in large part by nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (26). Hence, the potential participation of NF-κB in the activated phenotype of STBM-treated monocytes was confirmed using 6AQ and PA, two distinct inhibitors of NF-κB activation. Both inhibitors significantly decreased IL-6 and IL-8 secretion induced by STBM-NP compared to cells co-cultured with the microparticles in the absence of the inhibitors (**Figure 5B**). DMSO, which was used as a carrier for 6AQ, had no effect on IL-6 or IL-8 release. In all instances, cell viability following treatment with the drugs was confirmed (Figure S2 in Supplementary Material). As positive control, LPS, which activates NF-κB function, was used to stimulate monocytes. The LPS-induced release of IL-6 was also partially reduced by the same concentrations of either NF-κB inhibitors (**Figure 5C**).

DISCUSSION

Syncytiotrophoblast microparticles have been attributed potential roles in the systemic maternal inflammatory response during normal pregnancy and in the enhanced inflammatory reaction in preeclampsia (18, 19). In contrast to syncytial knots and larger placental debris, eliciting a local inflammatory response upon phagocytosis by endometrial endothelial cells (27), and

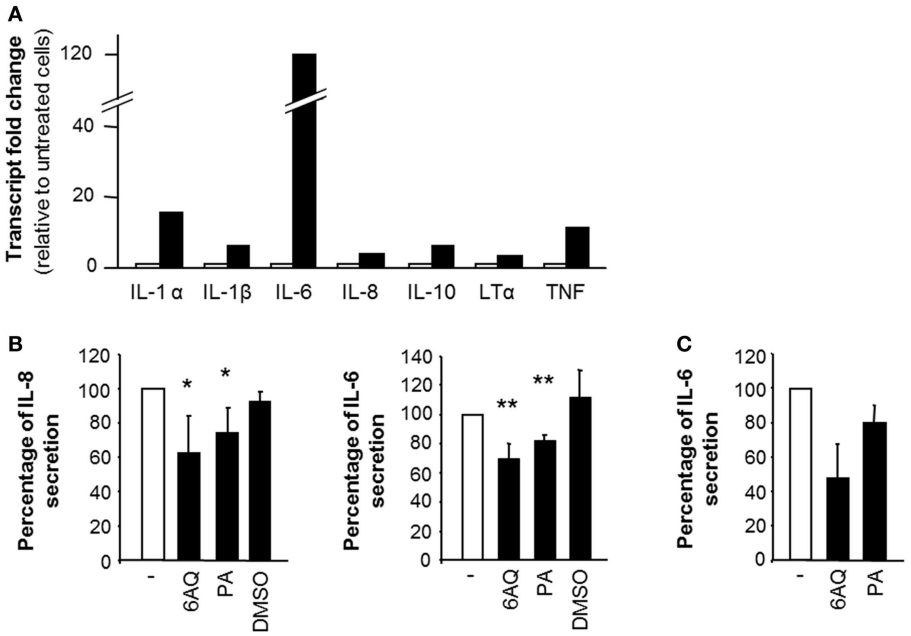

FIGURE 5 | The effect of STBM on monocytes is partly mediated by NF-κB. (A) Monocytes were incubated during 4 h without or with 300 μg/ml STBM-NP prepared from three different placentas. Cellular RNA was extracted, reverse-transcribed, and the pooled cDNA was examined for the expression of several genes. Transcript fold change was calculated with the $2^{-\Delta\Delta C_t}$. **(B,C)** Monocytes were pre-incubated with either 10 μM 6AQ, or 10 μM PA or DMSO alone prior to addition of **(B)** 300 μg/ml STBM-NP or **(C)** 100 pg/ml LPS. After 16 h of incubation, cytokine secretion was measured by ELISA and reported as a percentage of secretion relative to that of monocytes stimulated by STBM-NP in the absence of any drug. Data are presented as mean ± SEM of three independent co-culture experiments with four STBM-NP preparations. $*p < 0.05$; $**p < 0.01$. LPS was used as a positive control.

which are trapped by alveolar macrophages in the lungs (28, 29), STBM reach the maternal peripheral circulation, where they may come into contact with maternal endothelial and immune cells. Here, we showed that STBM derived from placentas collected from women with diagnosed preeclampsia or from women with uneventful pregnancy had a comparable effect on monocytes. STBM-PE, like STBM-NP, increased the cell surface levels of CD54 and induced the secretion of pro-inflammatory factors on monocytes. The cellular response to either STBM population was dose-dependent and equally strong at equivalent concentrations of microparticles. Our data would therefore indicate that the exaggerated inflammatory reaction in patients with preeclampsia may be attributed to the elevated levels of circulating placental microparticles rather than to a differential nature of the particles in preeclampsia compared to normal pregnancy. However, it needs to be mentioned that STBM-PE significantly altered the cellular expression of CD11a, whereas STBM-NP had no effect on this adhesion molecule. Hence, this could indicate that the adhesion properties of the monocytes may change differently upon their interaction with STBM in preeclamptic patients compared to normotensive pregnant women. Others have shown that microvesicles produced from preeclamptic placentas *in vitro* had an exacerbated pro-inflammatory effect on PBMCs when compared to normal term microvesicles (30), giving a hint that the placental debris shed by preeclamptic placentas may possibly be qualitatively different from the placental micro-debris circulating in the blood of normotensive women. Alluding to this hypothesis, a recent study demonstrated that microparticles derived from a trophoblast cell line cultured under hypoxia, as a model for the preeclamptic placenta, triggered a more rapid inflammatory response in PBMC than the particulate material derived from the cell line cultured under normal oxygen conditions (31). It was also recently shown that the monocytic fraction of PBMC was at the origin of the production of IL-6 and IL-8 upon stimulation with STBM obtained through placental perfusion (32). Whether this effect was direct or mediated by the other immune cells present in PBMC was however not determined.

Here, we observed that STBM localized at the boundary of monocytes, suggesting that the microparticles interact with these cells via one or several cell surface molecules. These results are in line with a former study, which documented the physiologic binding of placental microparticles onto circulating monocytes in peripheral blood of normal pregnant women and patients with preeclampsia (18). Similarly, STBM collected from placental perfusion were shown to bind and to be internalized by monocytes *in vitro* (32). Furthermore, we provide evidence against the requirement for STBM internalization by showing that the phagocytosis inhibitor cytochalasin B did not affect the secretion of IL-6 and IL-8 in response to the microparticles.

Therefore, the activation of monocytes involves receptors encompassed on the cellular membrane. In this respect, TLRs

might be potential candidates, as they mediate inflammatory responses in a number of immune cells following stimulation by a large panel of triggers, including host-derived molecules (33). TLRs transduce activation signals through the adaptor protein MyD88 (34). MyD88 is bound to the intracellular domain of the TLRs and, upon receptor stimulation, it homodimerizes and recruits IL-1 receptor-associated kinase, leading to the activation of the transcription factors NF-κB and JNK. We show here that MyD88 homodimerization inhibitory peptide partially blocks the secretion of IL-6 and IL-8, which strongly suggests that one or several members of the TLR family might be involved in the STBM-induced activation of monocytes. Downstream of TLRs, NF-κB is a well-known master switch in intracellular signaling pathways, which regulates the expression of numerous pro-inflammatory genes required to mount a cellular response in immune cells (26). In monocytes, the mRNA levels of a number of NF-κB responsive genes, including IL-6, IL-8, IL-1, LT-α, and TNF, were up-regulated following STBM treatment. The change in mRNA expression was even higher than 100 fold for IL-6. On the contrary, the levels of IL-8 transcripts were only marginally enhanced despite the large amounts of IL-8 that was secreted by the cells. This observation could point to the presence of an intracellular reservoir of pre-stored IL-8 in monocytes, as it is the case in Weibel–Palade bodies in microvascular endothelial cells (35). What may at first hand appear more intriguing is that STBM also induced the expression of the gene coding for the anti-inflammatory cytokine IL-10. This could be part of a negative feedback mechanism to maintain homeostatic control and terminate the inflammatory reaction (36). We also found that STBM decreased transcript levels of the pro-apoptotic molecules FADD, caspase-8, and CD27, which is in agreement with the role of NF-κB in promoting cell survival (37). Both inhibitors of NF-κB function, 6AQ and PA, independently affected cytokine secretion induced by STBM. 6AQ is a cell-permeable quinazoline compound, which inhibits NF-κB transcriptional activation (38), whereas PA is thought to block the calcium-dependent NF-κB signaling (39). It is known that these inhibitors can induce cellular apoptosis, since NF-κB also functions as a cell survival factor. However, the reduced production of IL-6 and IL-8 in the presence of 6AQ or PA was not due to cell death as the viability of the monocytes at the end of the co-culture with the microparticles was confirmed.

Activation of the NF-κB signaling pathway in PBMCs of preeclamptic women remains controversial. On the one hand, an increased activation of NF-κB in PBMC of preeclamptic patients compared to normal pregnant controls was reported (40). On the other hand, evidence for a suppression of the NF-κB activation pathway in preeclampsia was also provided elsewhere (41). However, this suppression might be attributed to the T-cell subset rather than to the monocytes (42).

In conclusion, the current analysis suggests that even though there may be minor qualitative differences between placental microparticles derived from normal or preeclamptic placentas, both can stimulate primary monocytes to produce pro-inflammatory cytokines. This effect appears to be mediated at least in part through TLR and NF-κB, leading to *de novo* gene transcription. Recent compelling investigations have led to the speculation that the size of the placental particulate debris circulating in the maternal blood may also have its importance in preeclampsia (43, 44). Therefore, further investigation will be required to identify the subgroup of placental microparticles and their components, which transmit pro-inflammatory signals to the maternal immune cells in order to complete our understanding of the inflammatory reactions taking place in both normal pregnancy and preeclampsia.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Journal/10.3389/fimmu.2014.00173/abstract

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B cells: the old new players in reproductive immunology

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Reproductive immunology research has long focused on T cell responses to paternal antigens and tolerance mechanisms supporting fetal well-being. The participation of B cells herein was not widely studied. Because of the fascinating immunological uniqueness of pregnancy, it is however to be expected that such pleiotropic cells play a considerable role. In fact, on the one hand B cells contribute toward pregnancy tolerance by secreting the immunomodulatory cytokine IL-10 but on the other hand can seriously harm pregnancy because of their capacity of producing autoantibodies. As for protective B cells, new evidences in mouse models arise suggesting that IL-10 producing B cells, the so-called B10 cells, help in maintaining tolerance toward semi-allogenic fetal antigens. They may be also important to fight danger signals at the fetal-maternal interface as, e.g., in the case of infections with the aim to restore the disrupted fetal tolerance. In human pregnancies, IL-10 producing B cells increase with pregnancy onset but not in the case of spontaneous abortions. *In vitro*, they are able to suppress TNF-α production by T cells from pregnant individuals. Their generation and functionality will be discussed throughout this review article. B cells can be deleterious to pregnancy as well. Aberrant B cell compartment is associated with obstetric pathologies. In particular, the capacity of B2 cells to produce specific autoantibodies or of B-1a B cells to secrete natural autoantibodies that can turn autoreactive will be discussed herein.

Keywords: pregnancy, B cells, autoantibodies, IL-10, Breg, B10 cells

INTRODUCTION

The study of the mechanisms responsible for the paradoxical survival of the conceptus as an intra-uterine semi-allograft within the genetically distinct female host has been an area of substantial scientific devotion. Aspects, particularly those related to the role of the maternal adaptive and innate immune response at a time when the physiological unit of fetus and placenta is framed as well as fertility problems, recurrent miscarriages, premature deliveries, and pre-eclampsia have been widely studied.

B cells are a major component of the immune system thus likely to be involved in maternal fetal immune tolerance. This review will look back in time to the beginnings of B cell discovery, their functional diverse subpopulations and provide the reader with an au courant knowledge regarding their regulatory and pathogenic role in pregnancy. Genuinely B cells were not identified as cells but through their function of secreting antibodies. In the late 1890s, Emil von Behring and Baron Kitasato Shibasaburo described the appearance of protective antibodies in blood in response to introducing foreign antigens into the body. Kitasato and Behring demonstrated the value of antitoxin against diphtheria and tetanus toxins by means of transferring graded injections of blood serum from an animal infected with the disease to a non-immune animal, thus transmitting active humoral immunity and preventing the disease (1). However which cell types were involved in the generation of such antibodies was not discovered for another half century. Murphy and Morton documented lymphocyte infiltration in immunized mice during the process of rejecting inoculated cancer grafts, with either natural or induced immunity. Per contra the destruction of lymphocytes with repeated small doses of x-ray prior to introducing the cancer graft led to a loss of natural or induced resistance toward inoculated cancer growth and the tumor graft grew more readily (2). Following on Jerne postulated that an antigen binds to an antibody by coincidence and upon binding further antibodies to that antigen could be produced. Thus, this theory offered an explanation for the presence of natural antibodies (3); based on this Burnet published the theory of clonal selection in 1957. Herein, he proposed that each lymphocyte carries specific immunoglobulins on its cell surface, reflecting its specificity of the antibody that will be synthesized upon antigen stimulation (4). Nossal and Lederberg confirmed this hypothesis during the following year (5). In 1956, B cells were first identified in chicken by Glick and Chang. Their data demonstrated that the resection of the bursa of Fabricius, called bursectomy, led to a suppressed antibody response and in point of fact B cells did receive their name from this bursa, the place of B cell origin in young birds, and not the term bone marrow (BM) as one would believe because of their origin in humans (6). During the last decades, the key discoveries included the unravelment of the immunoglobulin structure, the antibody–antigen interaction, and the mechanisms involved in the generation of antibody diversity. In particular, the knowledge about the immunoglobulin structure triggered the development of synthetic derived monoclonal antibodies. A breakthrough in the field of specific gene modification in animals came with the

isolation of embryonic stem cells and the discovery of homologous recombination (7, 8). Since then experimental models have undergone significant technological development, beginning with inbred mouse lines toward transgenic mouse models allowing for B cell stock manipulation in the 1980s and gene knockout animals in the 1990s (9).

A DISPLAY OF B CELLS AND B CELL SUBSETS

B lymphocytes are cells in the humoral immunity of the acquired immune system and account for 5–15% of circulating lymphocytes (10). Today, they are classically defined via the presence of endogenous immunoglobulins. A common description is that of a cell population expressing "clonally diverse cell surface immunoglobulin receptors," which recognize specific, antigenic epitopes (11). In adult human subjects, as in all mammals, B lymphocytes are continually formed in the BM from committed pluripotent hematopoietic precursor cells (12). Preceding the BM is populated by hematopoietic stem cells originating from the fetal liver (13). The earliest committed precursor of the B cell lineage is the pro-B cell. Downstream, functionally immature B cells also known as naive B cells (co-expressing IgM and IgD) exit the BM and migrate to the spleen, where they differentiate through transitional stages into B1 cells (albeit not all B1 cells derive from the BM) follicular B cells or marginal zone (MZ) B cells. B1 cells, independently of their origin, are typically subdivided into B-1a and B-1b cells (14).

The principal function of B lymphocytes is the production of antibodies against microbial antigens. Naive B cells not yet exposed to an antigen habitually recirculate secondary lymphoid tissues, chiefly spleen and lymph node follicles in order to encounter antigens. B cell activation, explicitly proliferation and differentiation, is mediated through positive and negative regulation in gene expression upon antigen encounter. Eventually, most B cells differentiate into antibody-secreting plasma cells while a small minority persists as memory cells, the agent of lasting immunity. Crucially, while first exposure to an antigen results in the generation of IgM secreting plasma cells and memory B cells (primary immune response), repeated activation of these memory cells by the same antigen leads to the production of a large quantity of high-affinity, monospecific class-switched IgG antibodies (secondary immune response). The differences of these antibody categories and their role in autoimmunity and pregnancy will be considered.

Beyond the widely recognized role of B lymphocytes in antibody production, B cells can also act as antigen presenting cells (APCs) for the initiation of T cell immune responses, as demonstrated in B cell depleted mice (15). B cells act as APCs by the presence of a transmembrane receptor protein on their surface known as the B cell receptor (BCR). Additionally, B cells can regulate various T cell and DC functions through the secretion of immunomodulatory cytokines (16–18). A novel but less well understood concept describes a phenotypically distinct subset of regulatory B cells to negatively regulate cellular immune responses and inhibiting excessive, tissue specific inflammation (19).

Like other cells, B lymphocytes can be classified into subsets according to their variation in development, anatomical location and ability to migrate, surface marker expression and functional characteristics. Their discovery has been facilitated by means of phenotype recognition using multicolor flow cytometry (20, 21). Two major B cell populations have been described; namely B1 cells and B2 cells.

Follicular B cells and marginal zone B cells (MZ B cells) constitute the B2 cell population. Together, they make up the chief part of splenic B cells but differ among each other in anatomical location, the follicles, and MZ, respectively. Follicular B cells are produced postnatally from BM precursors and colonize the spleen, lymph node follicles, and other peripheral lymphoid tissues. Upon antigen exposure follicular B cells can undergo T cell dependent maturation in form of immunoglobulin class switching, hypersomatic mutation, and differentiation into plasma and memory B cells. Importantly, B2 cells are short-lived and proliferation relies upon antigen stimulation. MZ B cells are innate-like lymphocytes essentially producing natural antibodies in the absence of antigen stimulation and setting up rapid T cell-independent antibody responses against pathogenic antigens. In addition, they are involved in antigen trapping, transport, and presentation (22). In contrast to B2 cells, MZ B cells do not circulate but reside near the marginal sinus of the spleen (23).

A functional distinct B cell population develops earlier in life from hematopoietic stem cells present in the fetal liver and maintain their numbers by self-replenishment (24). As they develop earlier than B2 cells during ontogeny, this population inherited the term B1 cell. With regards to tissue location B1 cells have been found to occupy different areas when comparing human and mouse tissues. In the murine system, B1 cells predominantly localize to pleural and peritoneal spaces whereas in human adults they primarily populate the peripheral blood (13, 25, 26). B1 cells have been readily identified in murine studies and are distinguished by their expression of several surface markers CD45 ($B220^{low}$), IgM^{hi}, $CD23^{-}$, $CD43^{+}$, and IgD^{low} that are not expressed by B2 cells (27, 28). B1 cells can be further subdivided into B-1a and B-1b cells based on distinct phenotypic features within this group. By consensus B-1 cells expressing CD5 are known as B-1a cells and those lacking the expression of CD5 are known as B-1b cells (21, 29, 30). B1 cells are part of the innate immune response and produce the majority of natural antibodies, in particular IgM against a broad spectrum of infections (31–34). These cells do not develop into memory B cells.

The most recently described subset of B2 cells is that of regulatory B cells. This unique population has been found to inhibit excessive inflammatory responses that contribute to the development of autoimmune disease. The main hallmark of regulatory B cells is the production of IL-10, a potent anti-inflammatory cytokine with pleiotropic immunoregulatory activities. Based on their secretory function, they have been labeled B10 cells in the mice and Breg in humans. However, this population exercises their function through more than one mechanism for example the secretion of TGF-β. They represent between 1 and 3% of splenic B cells but much controversy exists regarding surface marker expression. It is fair to say that B10 do have a unique phenotype but within this group are phenotypically distinct subsets. A further regulatory B cell subset with a $CD1d^{hi}CD5^{+}$ phenotype had been identified to secrete IL-10 and control T cell-dependent inflammatory responses (16). However, $CD1d^{hi}$ is expressed by various hemopoietic-derived cells (35).

ANTIBODY PRODUCTION

As stated earlier B2 cells are central players of humoral immunity by giving rise to differentiated antibody-secreting plasma cells. The antibody immune response is highly complex but as a simple outline once secreted, selectively produced antibodies recognize and bind particular external antigens and aid their destruction. For all that, there is a population of antibodies unable to form antigen–antibody complexes due to a structural anomaly in form of an oligosaccharide residue. These so-called asymmetric antibodies compete with their precipitating counterpart by binding the same antigen but are unable to activate effector immune mechanisms, such as complement fixation and phagocytosis. Instead, they have been speculated to function as blocking antibodies and thus may provide protection to the antigen. This blocking property has been demonstrated in previous studies (36).

Chiefly antibodies can be divided into five different classes based on their structural variability, target specificity, and distribution. *Per se* all isotopes are categorized according to their differences in the amino acid sequence in the constant region (Fc) of the heavy chain. As well, they occur in two physical forms: soluble antibodies and membrane-bound antibodies. Membrane-bound immunoglobulins form the B cell antigen receptor complex on B cells. B2 cell derived plasma cells secrete predominantly adaptive antibodies initially in form of IgM and subsequently in form of high-affinity, somatically mutated IgG. Both are dependent upon antigen stimulation. However, en masse IgM secretion is antigen-independent, which brought about the concept of two distinct types of IgM, natural IgM, and antigen-induced IgM respectively (37). Natural IgM is mainly secreted by B1 cells and to a lesser extent by MZ B cells in the complete absence of external antigenic stimulation whereas antigen-induced IgM and IgG are mostly produced by B2 cells (38–43). Antibodies from both cell types have been shown to be necessary and moreover act in concert to provide full immune protection as demonstrated by Baumgarth et al. (44).

In contrast to their adaptive counterparts natural antibodies are defined through their properties of low affinity and polyreactivity. Typically, they are able to recognize cross-reactive epitopes on encapsulated gram-positive bacteria, pathogenic viruses, apoptotic cells, and oxidized low-density lipoproteins and promote their clearance (31, 45). In this way, they provide immediate and broad protection against pathogens within the naive host, making them a crucial component of the humoral innate immune system. Unfortunately, cross reactivity of B1 and MZ B cell derived natural antibodies is not only skewed toward the recognition of pathogenic antigens but also the recognition of self-antigens provoking host cell destruction and ultimately autoimmunity. Thus, it was tempting to speculate that B1 cells may play a central role in the production autoantibodies (42, 46). However, natural antibody production is tightly regulated by the immune system and these natural antibodies rarely enter germinal centers to undergo affinity maturation. Hence, their potential for producing high-affinity antibodies with harmful specificity against their own parts is greatly restricted (45).

Surprisingly, several studies demonstrated that antibodies involved in pathogenic immune deposits within the kidneys are entirely of B2 cell origin (47). On that account, IgG antibodies have been shown to function as dominant mediators for several autoimmune diseases including systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) (48–50). The mechanisms involved in generating autoantibodies are not fully understood. However, through the process of gene segment rearrangement the immune system is capable of generating a virtually unlimited display of antibodies. Despite the establishment of multiple checkpoints which negatively select B cells with self-reactive antigen receptors, by some detrimental mechanism this genetic rearrangement may give rise to autoreactive antibodies; subsequently interacting with self-antigens and contributing toward the clinical picture of autoimmunity.

With reference to the production of natural IgM from B1 cells, there is much debate regarding their protective and destructive contribution toward autoimmune processes. Hayakawa and colleagues have demonstrated in 1999 that murine B1 cells are paradoxically positively selected for the production of autoantibodies (50). Mice deficient in serum IgM not only experienced a diminished response to pathogenic antigens. Moreover, the absence of secreted IgM stimulated the development of IgG autoantibodies (51). This was confirmed by Boes and colleagues in 2000 in normal mice unable to secrete IgM and lupus-prone lymphoproliferative (lpr) mice unable to secrete IgM. Here, lpr mice developed elevated IgG autoantibodies and experienced more severe glomerulonephritis owing to larger numbers of glomerular immune complexes (52). These and subsequent data demonstrate B1 cell-secreted IgM as a critical factor in hampering the development and severity of autoimmunity possible by means of apoptotic cell clearance (53, 54).

B1 cells have been implicated in the pathogenesis of acute inflammation and chronic autoimmune diseases in murine and human studies (55, 56). This was best witnessed in an SLE mice model in which B1 cell depletion reduced the severity of lupus autoimmune pathogenesis in (NZB × NZW) F1 mice (57). Further studies have demonstrated a significant increase of murine B1 cells as well as an increased production of self-reactive antibodies in RA and SLE (28, 58, 59). Like murine B-1a cells, human $CD5^+$ B cells have been reported to produce autoantibodies in form of IgM rheumatoid factor (60). As a number of studies do support whereas others do not support the role of B1 cells involved in the pathogenesis of autoimmune disease, this area remains controversial.

IMMUNE REGULATORY FUNCTION OF B CELLS IN AUTOIMMUNITY, CANCER, AND TRANSPLANTATION

The role of B cells in the pathogenesis of autoimmune diseases extends beyond the production of autoantibodies. Rather B cells are now well-recognized for their positive and negative regulatory functions during immune responses. Newly described so-called regulatory B cells possess the ability of negatively regulating cellular immune responses and inflammation. A variety of cytokines produced by regulatory B cell subsets have been reported, with IL-10 being the most studied. The regulatory immune function was first reported by Janeway and colleagues in 1996 in a B cell deficient mice model of acute experimental autoimmune encephalomyelitis (EAE) (61). Genetically B cell-deficient mice ($IL\text{-}10^{-/-}$) developed a persistent pro-inflammatory immune response and increased severity of EAE in comparison to wild type mice (62). Although

this particular B cell regulatory effect was not IL-10 dependent, various mouse models have reinforced the importance of B cell derived IL-10 in EAE and other human autoimmune disease (62, 63). As such B10 cells have been shown to suppress the progression of intestinal inflammation in inflammatory bowel disease (IBD) and prevent the development of collagen-induced arthritis in murine models (64–66).

Studies of B10 cells and human autoimmune diseases are limited and their relevance in maintaining peripheral tolerance remains unclear. One study demonstrated the presence and moreover significantly increased production of B cell derived IL-10 in untreated RA, SLE, and Sjögren's syndrome patients compared to controls (67). A different study defined a human B cell phenotype with regulatory capacities (67). $CD19^{+}CD24^{hi}CD38^{hi}$ B cells isolated from human peripheral blood and stimulated with CD40 suppressed the differentiation of T_h1 cells. This effect was partially mediated by IL-10. In comparison, the same B cell population isolated from the peripheral blood of SLE patients responded poorly to CD40 stimulation, produced less IL-10 and in this way lost its suppressive capacity.

In both murine and human models, the regulatory effects of B cells are very likely mediated through the anti-inflammatory effects of IL-10 and the ability of B cells to interact with pathogenic T cells to reduce harmful immune responses. IL-10 effects are mediated by multiple mechanisms such as the inhibition of the pro-inflammatory cytokine TNFα production (68, 69). B10 cells suppress T_h1 differentiation and inhibit Ag-specific $CD4^{+}CD25^{-}$ T cell proliferation (70). This key role of B cell derived IL-10 in controlling T cell mediated autoimmunity was supported in several studies (67, 71, 72). A recent murine study identified a different IL-10 independent mechanism through which B cells can regulate autoimmunity. Here, glucocorticoid-induced TNF ligand (GITR ligand) expression by B cells was required to induce the proliferation of Treg in promoting EAE recovery (63).

Although antitumor immunity is not well understood several *in vivo* experiments have shown the regulatory action of B cells in inhibiting immune response against tumors. In B cell knockout (BKO) mice, the depletion of B cells enhanced tumor clearance in Friend murine leukemia virus gag-expressing mouse EL-4 (EL-4 gag) and D5 mouse melanoma whereas tumor progression in wild type mice was uncontrollable (73). Similarly, EL-4 thymomas, MC38 colon carcinomas, and B16 melanomas in $IgM^{-/-}$ B cell-deficient mice exhibited spontaneous tumor regression or significant delayed growth in comparison to wild type mice (74). It has been speculated that IL-10 release from B cells inhibits $CD8^{+}$ T cell memory development and INFγ production from $CD8^{+}$ T and natural killer (NK) cells. Both are important for the tumor immune surveillance. Thus B cells can function as regulatory cells in some tumor settings potentially through the decreased IL-10 production from B cell depleted mice.

GVHD is a pathological condition in which donor T cells from the transplanted tissue initiate an immunologic attack on the recipient's cells. Host APCs particular DCs are crucial for the stimulation of donor T cells and hence the induction of GVHD (75). As previously stated B cells are also able to function as APCs and given the regulatory action of B10 cells in autoimmunity and cancer, their involvement in graft versus host disease has been hypothesized and explored. Rowe and colleagues demonstrated that B cell-deficient μMT mice receiving BM transplantation demonstrated higher mortality rates due to acute GVHD than wild type recipients (76). This seems to be directly linked to the ability of B cells acting as APCs in reducing the proliferation of $CD4^{+}$ T cells as well as the production of pro-inflammatory cytokines within the donor. Moreover, they have demonstrated that the mechanism of B cells in suppressing GVHD is directly related to IL-10 as $IL\text{-}10^{-/-}$ mice developed more severe acute GVHD than recipient mice in which B cells are wild type (76). Finally, although most B cells are eliminated by total body irradiation preceding graft insertion, IL-10 producing B cells appear to be more resistant toward irradiation regimes. A recent cohort study aimed to identify immune parameters that would discriminate tolerant kidney transplant patients from subjects receiving immunosuppression with stable allograft function (77). This study found that tolerant recipients displayed increased total B cell numbers and naive B cells in peripheral serum and had an enhanced expression of three B cell genes in comparison with recipients receiving immunosuppression. These results may also indicate a potential regulatory role for B cells in transplantation tolerance although further studies are needed to identify whether such findings represent a cause or consequence of tolerance.

B CELLS IN PREGNANCY

The concept of immune tolerance and the primary function of the immune system protecting against pathogens becomes a much more complex picture with view toward mammalian pregnancy. During this period of time, the maternal immune system has the double function of tolerating a semi-allogenic fetus, expressing both maternal and paternal antigens, while maintaining the fight against infection. This fine equilibrium between maternal fetal tolerance and immune activation is orchestrated by multiple cellular players. The role of B cells herein is poorly studied, especially when taking into account the many studies dedicated to T cells in pregnancy.

ANTIBODY PRODUCING B CELLS IN NORMAL PREGNANCY AND DURING PREGNANCY COMPLICATIONS

We have previously described a population of IgG-type antibodies that bind to antigens with a relative high-affinity but fail to initiate host mechanisms facilitating the destruction of foreign antigens. Since these asymmetric antibodies seem to provide protection for antigens, it has been speculated that they may play a role in the immunological aspects of protecting the fetus against maternally derived symmetric, antipaternal antibodies at the fetomaternal interface (78). Asymmetric antibodies have been identified in human and other mammalian sera (79). Moreover, their production increased considerably in maternal serum and placental tissue during normal human pregnancy whereas their absence has been associated with pregnancy failure (80–83). One mechanism postulates that these antibodies block placental antigens in order to prevent the immunological attack by maternal NK cells and cytotoxic lymphocytes. Their secretion seems to be partially hormone regulated (84). In contrast to the protective effect proposed for asymmetric antibodies, natural antibodies facilitate pregnancy complications.

Pre-eclampsia (PE) is one of the leading causes of maternal mortality and morbidity worldwide and affects between 6 and 8% of pregnancies (85). It is defined as new onset hypertension presenting after 20 weeks gestation with significant proteinuria and resolves post-delivery. Importantly, this condition not only affects the mother but presents a high risk for the fetus in form of intrauterine death, preterm delivery, and low birth weight. With regards to the pathophysiology both immunological and genetic contributory factors have been proposed in addition to preexisting maternal diseases, i.e., chronic kidney disease and autoimmune conditions such as systemic lupus erythematosis or antiphospholipid syndrome. Pre-eclampsia is an abnormality of placentation, in particular defective remodeling of maternal uterine spiral arteries precipitating high resistance uteroplacental circulation leading to insufficient placental and in this way fetal perfusion. Cells and regulatory molecules have been implicated in the immunological alterations established in the placental microenvironment of patients with pre-eclampsia. One of the main differences in pre-eclampsia is a shift toward T_h1 responses and the production of IFN-gamma of uncertain origin (86). Another mechanism is the blockage of transmembrane receptors for two potent angiogenic substances, vascular endothelial growth factor (VEGF) and placental growth factor (PlGF). Fms-like tyrosine kinase-1 (sFlt-1) has been identified to block these receptors and was found in high concentrations in pre-eclampsia (87–90). This endothelial dysfunction was rescued by administration of exogenous VEGF and PlGF *in vitro* studies. Furthermore heme oxygenase-1 (HO-1), an anti-inflammatory enzyme, able to inhibit sFlt-1 release has been found to be decreased in women who later developed pre-eclampsia. This is supported by data from animal models where it could be shown that HO-1 deficiency is related to uterine growth restriction and development of hypertension at midpregnancy. This seems to be dependent on the number of uterine NK cells. A HO-1 metabolite, carbon monoxide, can rescue this PE-like phenotype when applied continuously during implantation and placentation at low doses (91, 92). A different mechanism suggested autoantibodies against the vascular angiotensin II receptor type 1 (AT_1) to account for disease manifestation. In 1999, Wallukat and colleagues were first to report the presence of circulating autoantibodies (AT1-AA) against the AT_1 receptor in patients with pre-eclampsia, suggesting their involvement in gestational hypertension (93). The current understanding of AT1-AA and their involvement in pre-eclampsia was recently reviewed by Herse and LaMarca (94). As described earlier, $CD19^+CD5^+$B-1a B cells are a major source of natural and polyreactive antibodies. Recent evidence comes from Jensen and colleagues, as they detected a dramatically increased $CD19^+CD5^+$B-1a B cell count in peripheral blood of pre-eclamptic patients in comparison to controls having normal pregnancies. The same cells were further detected in the placenta of pre-eclamptic but not normal pregnancies. This process seems to be driven by higher chorionic gonadotropin (hCG) levels present in the serum and placenta of patients with pre-eclampsia and is supported by the fact that 95% of $CD19^+CD5^+$ cells express the hCG receptor (hCGR) and expand on hCG stimulation *in vitro* cultures. Most importantly, isolated $CD19^+CD5^+$ cells produce autoantibodies against angiotensin II type 1 receptor (95).

Lately, we have learned that pre-eclamptic women exhibit components similar to various chronic inflammatory diseases, such as elevated TNF-alpha, autoantibodies, and autoimmune associated T cells and cytokines (96–99). Recent studies focused predominantly on the role of the agonistic autoantibody to the angiotensin II type 1 receptor (AT1-AA) to account for much of the pathophysiology of pre-eclampsia (100–102). Of those several demonstrated that infusion of purified rat AT1-AA into normal pregnant rats increased blood pressure, the antiangiogenic factor sFlt-1 and sEndoglin (103, 104). To further demonstrate, the important role for B cells and endogenously generated AT1-AA in mediating hypertension in response to placental ischemia, La Marca and colleagues have proposed a model of B cell depletion to suppress endogenously generated AT1-AA. Rituximab, a chimeric murine-human monoclonal antibody against the CD20 antigen located on pre-B, immature, and mature B cells, was used to induce B cell depletion in normal pregnant and reduced uterine perfusion pressure (RUPP) rats (104). RUPP rats treated with rituximab exhibited less blood pressure increases in response to induced placental ischemia (105). This data provides potential insight into adverse pregnancy outcomes in humans and can provide a new approach to the treatment of pregnancy failure. As such a recent case report has described a successful pregnancy after rituximab treatment in a patient with a history of *in vitro* fertilization (IVF) failures and positive anti-cardiolipin antibody (ACA). Following a course of rituximab, the patient's ACA became negative and she successfully conceived with IVF treatment (106). Rituximab destroys B cells that have CD20 on their surfaces, normal, and malignant respectively. CD20 is not expressed on stem cells and most plasma cells, thus B cell regeneration is possible and immunoglobulin synthesis by plasma cells is not affected after treatment with anti-CD20 antibody (107, 108). It has been used to treat diseases, which are characterized by excessive numbers of B cells, overactive B cells, or dysfunctional B cells. This includes several autoimmune diseases, B cell malignancies, and transplant rejection (109).

B10/Breg CELLS IN PREGNANCY

As the fetus is semi-allogeneic to its mother, it is reasonable to assume that the maternal immune response is a key determinant in pregnancy success and failure. In normal pregnancy, immunological adaptations are in place to protect the fetus from the maternal immune system. Failure in the accommodation of such mechanism could lead to sporadic and recurrent pregnancy loss. Recurrent miscarriage is an important complication of human gestation, affecting approximately 1% of the population (110). It is classically defined as a loss of three or more consecutive and clinically recognized pregnancies within the first trimester. Although not fully understood the underlying etiology of recurrent pregnancy is either embryological or maternal driven and can be divided into anatomical, genetic, endocrine, infectious, environmental, thrombophilic, and immunological factors (111). Focusing on the latter one mechanism postulated is the recognition of paternal antigens at the fetoplacental unit by the maternal immune system, resulting in a pro-inflammatory immune response and fetal rejection (112). The concept that B10/Breg cells immunomodulate inflammatory processes and participate in the maintenance of tolerance through IL-10 gave rise to the idea that they may control inflammatory

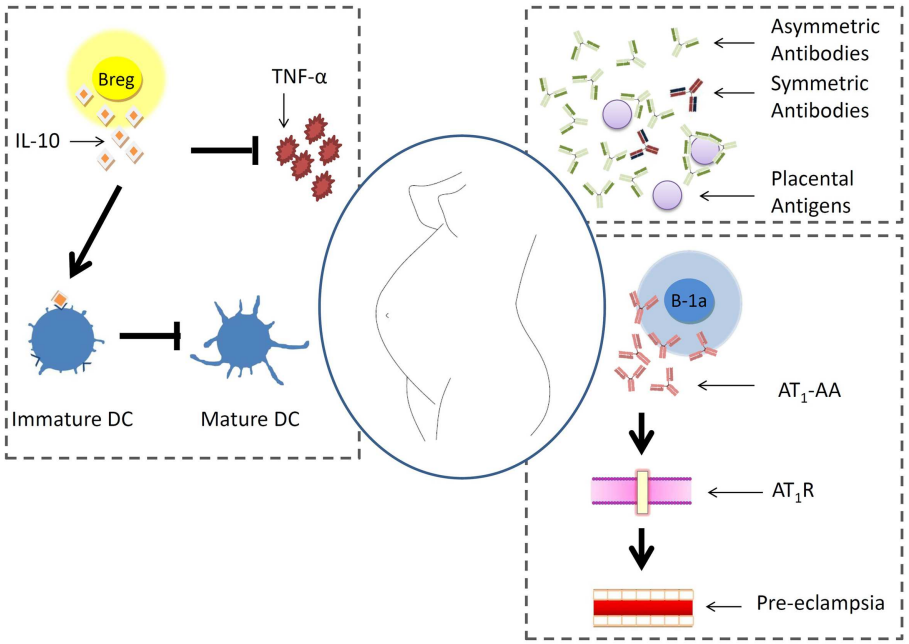

FIGURE 1 | B cell behavior during pregnancy. Pregnancy factors promote the generation of asymmetric antibodies that protect placental antigens from immune reactions. In pre-eclampsia, B-1a B cells can turn autoreactive and secrete antibodies against the angiotensin receptor 1. In patients with autoimmune disorders, it is possible that B cells secrete more autoantibodies and endanger the gestation. A newly described B cell type, the so-called regulatory B cells, produces IL-10 and is proposed to positively influence pregnancy by hindering Th1 immune responses.

processes in pregnancy and are important in the immunological adaptations leading to the survival of the fetus.

While researchers have reported a considerable expression of IL-10 in the decidua and placenta in mice and humans, IL-10 is not crucial for the completion of successful allogeneic pregnancies as demonstrated in breeding experiments of IL-10-null mutant ($Il10^{-/-}$) mice (113–115). However, IL-10 serves an important role in protecting pregnancy from the adverse effects of inflammatory stress during gestation. This became evident in rodent studies using lipopolysaccharide (LPS) to induce pregnancy loss. $Il10^{-/-}$ animals demonstrated high incidences of miscarriage whereas administration of equal doses of LPS did not affect pregnancies in the control group (116). Concomitantly, administration of recombinant IL-10 reversed the harmful effects of injected LPS (117). Of further significance was a report by Chaouat and colleagues utilizing CBA × DBA/2 mice. CBA × DBA/2 mating combinations express high rates of spontaneous fetal resorption accompanied by increased levels of local pro-inflammatory cytokines. This is likely to result from a local defect in the IL-10 production as IL-10 levels in placenta and decidua in pregnancies of CBA/J × DBA/2J mating combination have been shown to be exceptionally low. Exogenous administration of recombinant IL-10 significantly reduced LPS-induced fetal loss in CBA/J × DBA/2J but did not change outcomes of $Il10^{+/+}$ mice (118). Conversely, anti-IL-10 antibodies increased resorption rates in this group (119). Furthermore in the remaining viable fetuses IL-10 deficiency predispose to growth restriction and a progressive decline in fetal weight in the presence of LPS. This was not observed in $Il10^{+/+}$ mice treated with LPS (118).

Several mechanisms have been argued through which IL-10 may accomplish pregnancy preservation from LPS-induced pathologies. IL-10 is well known to inhibit the synthesis of pro-inflammatory cytokines specifically TNF-alpha from monocytes and macrophages *in* autoimmunity (120). Consequent cytokine profile analysis of maternal serum and gestational tissue following LPS injections showed elevated pro-inflammatory cytokine levels in $Il10^{-/-}$ mice compared with tissues from $Il10^{+/+}$ mice. Significantly raised levels were measured for TNF-α, IL6, IL1A, and IL12p40 with TNF-α most dramatically affected by IL-10 deficiency (118, 121). Further evaluation of TNF-α in mediating the adverse effects of IL-10 deficiency in pregnancy using etanercept, a TNF inhibitor, Robertson and colleagues demonstrated a partial reduction in fetal loss in IL-$10^{-/-}$ mice treated with etanercept (118). Another study has reported an important association between fetal resorption in IL-10 deficient mice with a significant rise in uterine NK cell cytotoxicity and placental invasion. Pregnancies in LPS-treated IL-10 deficient mice could be rescued through depleting uNK cells, IL-10 administration, or TNF-alpha reversal. These results suggested an immune mechanism of fetal destruction by which uNK cells mediate inflammation in the absence of IL-10 whereas a regulatory cross-talk between IL-10 and uNK cells contributes toward a positive pregnancy outcome (122).

As discussed earlier, an important source of IL-10 comes from regulatory B cells. In our own studies, we have established that splenic B10 cells increased in frequency during normal murine pregnancy (NP). This B10 cell expansion was not evident in the

non-pregnant and abortion prone (AP) group, both of which demonstrated comparable low levels. Similarly on measuring IL-10, we demonstrated an increase in IL-10 production in NP compared to non-pregnant mice. Again this augmentation was not observed in AP mice. Addressing the participation of B10 cells in establishing pregnancy tolerance, we further identified that the transfer of B10 cells from NP mice into AP animals on day 0 of pregnancy was sufficient to prevent fetal rejection (123). One possible mechanism of IL-10 action is through DCs, as they abundantly express the IL-10 receptor (IL-10R) and can alter the functionality of other immune cells. IL-10 inhibits the maturation process of monocyte derived DCs into efficient IL-12 secreting APCs consequently inhibiting their capacity to present antigens to T cells to activate them and induce the differentiation of naïve T cells to Th1 cells (124). This mechanism may be applicable for pregnancy as suggested by our data. We proved that IL-10 kept DCs in an immature state whereas DC maturation continued in the presence anti IL-10 antibody. Furthermore transferring B10 cells from NP into AP females was associated with decreased numbers of mature DCs and an augmentation of $CD4^+Foxp3^+$Treg. $Foxp3^+$ regulatory T cells play a central role in sustaining maternal fetal immune tolerance and immature DCs are efficient inducers of Tregs in pregnancy (125). Overall the anti-inflammatory properties of B10 cells can provide a new approach to the treatment of spontaneous abortion due to immune mediated fetal rejection.

SUMMARY

B cells are pleiotropic components of the immune system and are at the interface of innate and adaptive immunity. Their role during pregnancy is rather poorly studied. It is however known that pregnancy factors promote the generation of asymmetric antibodies that protect the fetus from immune reactions. In pre-eclampsia, B-1a B cells can turn autoreactive and secrete antibodies against the angiotensin receptor 1. In patients with autoimmune disorders, it is possible that B cells secrete more autoantibodies and endanger the gestation. A newly described B cell type, the so-called regulatory B cells, produces IL-10 and is proposed to positively influence pregnancy by hindering Th1 immune responses. This is resumed in **Figure 1**.

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The unique neonatal NK cells: a critical component required for neonatal autoimmune disease induction by maternal autoantibody

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Human maternal autoantibodies can trigger autoimmune diseases such as congenital heart block (CHB) in the progeny of women with lupus or Sjogren's disease. The pathogenic effect of early autoantibody (autoAb) exposure has been investigated in a murine neonatal autoimmune ovarian disease (nAOD) model triggered by a unique ZP3 antibody. Although immune complexes (IC) are formed in adult and neonatal ovaries, ZP3 antibody triggers severe nAOD only in <7-day-old neonatal mice. Propensity to nAOD is due to the uniquely hyper-responsive neonatal natural killer (NK) cells that lack the inhibitory Ly49C/I receptors. In nAOD, the neonatal NK cells directly mediate ovarian inflammation and oocyte depletion while simultaneously promoting *de novo* pathogenic ovarian-specific T cell responses. Resistance to nAOD in older mice results from the emergence of the Ly49C/I$^+$ NK cells that regulate effector NK cells and from CD25$^+$ regulatory T cell control. In preliminary studies, FcγRIII$^+$ NK cells as well as the ovarian resident FcγRIII$^+$ macrophages and/or dendritic cells were found to be as indispensable players. Activated by ovarian IC, they migrate to lymphoid organs where NK cell priming occurs. Remarkably, the findings in nAOD are very similar to those reported for neonatal responses to a retrovirus and its cognate antibody that lead to long-lasting immunity. Studies on nAOD therefore provide insights into maternal autoAb-mediated neonatal autoimmunity, including CHB, while simultaneously uncovering new properties of the neonatal innate and adaptive responses, lethality of premature infant infection, and novel neonatal antiviral vaccine design.

Keywords: NK cells, Ly49 receptors, neonatal immunology, immune complex, autoimmune ovarian disease, regulatory T cells, congenital heart block, neonatal viral immunity

HUMAN NEONATAL AUTOIMMUNE DISEASE INDUCTION BY MATERNAL AUTOANTIBODY EXEMPLIFIES NEONATAL PROPENSITY TO AUTOIMMUNITY

Increased susceptibility of premature human infants and neonatal mice to infections is well-known (1). What is less appreciated is that neonatal mice are also more susceptible to autoimmune disease. For example, spontaneous autoimmune diseases occur in mice thymectomized between days 1 and 4 but not after day 7 of life (2). Autoimmune diseases induced by tissue antigen or peptide immunization require complete Freund's adjuvant (CFA) in adult but not in neonatal mice (3–6). Indeed, neonatal female mice immunized with an auto-peptide from the gender-specific ovarian zona pellucida 3 (pZP3) antigen, mounted peptide-specific T cell responses, and developed autoimmune ovarian disease (AOD) in 3 weeks. In contrast, the same ZP3 peptide induced tolerance in neonatal male mice as a foreign antigen (4). The opposing responses to self and foreign antigens in the neonates suggest that the neonatal tolerance paradigm (7, 8) should be revisited because it is built mainly on responses to foreign antigens or peptides including alloantigen (9–15). Indeed, in contrast to the neonatal tolerance paradigm, newborn mice responded as efficiently as adult mice to viral, nominal, and autoantigen, when the antigen dose, injection site, and adjuvant type were adjusted (16–18). Finally, neonatal propensity to autoimmunity is supported by the induction of fetal or neonatal self-tissue damage after the transplacental transfer of maternal autoantibody (autoAb) that often does not harm the adult.

Autoimmune disease occurs preferentially in women of reproductive age, and circulating autoAb is a hallmark of autoimmunity. The finding of neonatal autoimmune disease caused by maternal autoAb establishes its pathogenic potential. The impact of maternal autoAb is often transient, and neonates recover as antibody level declines. However, in some cases, maternal autoAb effects persist and induce permanent tissue damage. An example is congenital heart block (CHB) that occurs in the fetuses or infants of women with systemic lupus erythrematosus (SLE) or Sjogren's disease. Although CHB has many possible etiologies (19), a strong candidate is the transplacental transfer of maternal autoAb against the ribonucleoproteins SSA/Ro and SSB/La (20), with SSB/La having a stronger association (21). The maternal autoAb damages the atrioventricular node of the cardiac conduction system by an unknown mechanism. Likely, CHB pathogenesis involves excessive

apoptosis of cardiomyocytes, tissue inflammation and fibrosis, and the interaction of macrophages and fibroblasts (21). The clinical impact of CHB is significant. Although about 2% of neonates from mothers with autoAb to SSA/Ro and SSB/La develop CHB (20), the recurrence rate for an autoAb-positive mother with a previously affected child is 16–18% (22). Importantly, complete or third degree CHB is irreversible with a mortality rate of 12–43% (22). Although first or second degree heart block may reverse with treatment, most children require permanent pacemakers (22).

Maternal autoAb can induce neonatal autoimmune diseases other than CHB. Neonatal myasthenia gravis, associated with severe deformity and difficult deliveries, is caused by maternal autoAb to the fetal form of acetylcholine receptor and a muscle-specific kinase (23, 24). Neonatal Graves' disease involves agonist autoAb targeting the thyroid stimulating hormone receptor (19). Neonatal pemphigus is mediated by autoAb to the desmoglein-1 or 3 antigens (25, 26). Several studies have shown a positive correlation between anti-phospholipid or lupus anticoagulant autoAb, present in patients with SLE or anti-phospholipid syndrome, and a high risk of premature onset of labor, low birth weight and early miscarriages of their progeny (19, 27). In addition, neuronal apoptosis and subsequent abortion are consequences of maternal autoAb to DNA in SLE patients (28); and autism spectrum disorders are linked to maternal autoAb against fetal brain antigens (29, 30). The mechanism of human neonatal autoimmunity is currently uncertain, and we have used the neonatal autoimmune ovarian disease (nAOD) model to address the questions of why neonates are more susceptible to autoimmune disease, and how maternal autoAb induce the tissue damage. Below, we have described some of the characteristics and advantages of nAOD that make it a very useful model.

In addition to its ability to cause tissue injury and disease, antibody has been found to elicit changes in neonatal or very young mice and promote subsequent full-blown autoimmune diseases. An example of the "pre-disease" state is the pre-diabetes that precedes clinical diabetes by months to years (31–34). In NOD mice, a very early cascade of cellular and cytokine responses to local immune complexes (IC) in pancreatic islets was found to cause subsequent development of pathogenic T cell responses and clinical juvenile diabetes (35). In addition, maternal autoAb directed to islet antigens was reported as a requirement for type I diabetes in the NOD mice (36) or to accelerate diabetes onset in a transgenic mouse model (37).

Therefore, maternal autoAb can induce either transient pathology or more persistent tissue damage in the progeny. It may also condition the fetus/infant early in life for a late onset autoimmune disease. In these situations, the design of novel preventive therapies will necessitate a thorough understanding of the pathogenic autoimmune response in the very young individuals, including their innate and adaptive neonatal responses to IC created by autoAb.

NEONATAL AUTOIMMUNE OVARIAN DISEASE MODEL AND ITS UNIQUE FEATURES

The aforementioned findings indicate that newborns are simultaneously more susceptible to infections and to autoimmunity. While "immaturity" of the neonatal immune system might explain the increased sensitivity to pathogens, it is not immediately apparent why newborns are more susceptible to autoimmune disease. A potential explanation is that the maturing neonatal immune system is less stringently regulated relative to the adults. Thus, an overactive neonatal response would induce both autoimmune disease and severe post-infection immunopathology, including sepsis. While other authors support the latter (38), our study focuses on the questions of why newborns are more susceptible to nAOD and how nAOD is induced. We have summarized our results in this review.

Autoimmune ovarian disease is a known cause of primary or secondary premature ovarian failure that can lead to infertility of pubertal and adult women (39, 40). Because ovarian dysfunction is not generally manifested until puberty, it cannot be certain that primary AOD, like type I diabetes, is preceded by a "pre-disease" exemplified by nAOD. While the clinical relevance of nAOD remains unresolved, the murine nAOD model itself has proven to be an excellent platform for investigating the role of the neonatal immune response to autoantigen and the role of autoAb in autoimmune disease pathogenesis.

ZP3 is a major sperm receptor in fertilization (41). The pZP3 (330–342) contains a pathogenic T cell epitope and a native B cell epitope 335–342 (42), and antibodies to this pZP3 B cell epitope inhibit sperm binding to the zona pellucida (41, 43). Adult mice immunized with pZP3 in CFA develop a pathogenic $CD4^+$ T cell response and a non-pathogenic antibody response. The latter was confirmed in adult mice immunized with a chimeric ZP3 peptide that contains the native B cell epitope 335–342 linked to a foreign T cell peptide from the bovine RNAse (43). The mice produce ZP3 antibodies that bind to the ovarian zona pellucida without causing ovarian pathology. However, 70% of the females immunized with the chimeric ZP3 peptide were infertile because of the contraceptive effect of ZP3 antibodies. In the remnant fertile females, the maternal ZP3 autoAb transferred to the neonates induced severe nAOD (**Figure 1**). Remarkably, nAOD developed in the progeny only when the ZP3 antibody exposure was initiated within the first 6 days of life, as documented by feeding antibody-positive milk to normal pups of different ages, and by antibody transfer (44).

Neonatal autoimmune ovarian disease is a unique and versatile model. *First*, ovaries can be safely removed to deplete ovarian autoantigens; and ovaries from donors of different ages and with different genetic modifications can be implanted under the kidney capsule can be used to monitor the effect of putative molecules in nAOD development. This approach has allowed us to show that nAOD susceptibility is not related to the intrinsic differences between adult and neonatal ovaries (44). It has also allowed us to find, unexpectedly, that nAOD induction requires resident ovarian cells that express FcγRIII. *Second*, nAOD induced by maternal immunization with the novel ZP3 chimeric peptide induces ZP3 antibodies without concomitant pathogenic T cell response. In addition to feeding pups with ZP3 antibody-positive mothers, nAOD is also inducible by passive transfer of a monoclonal antibody that recognizes the pZP3 335–342 (45). This allowed us to adjust the antibody dose to body weight between neonatal or adult mice and show that the nAOD resistance of adult mice is not related to insufficient antibody or ovarian IC. *Third*, there has been uncertainties regarding the physiological relevance of the

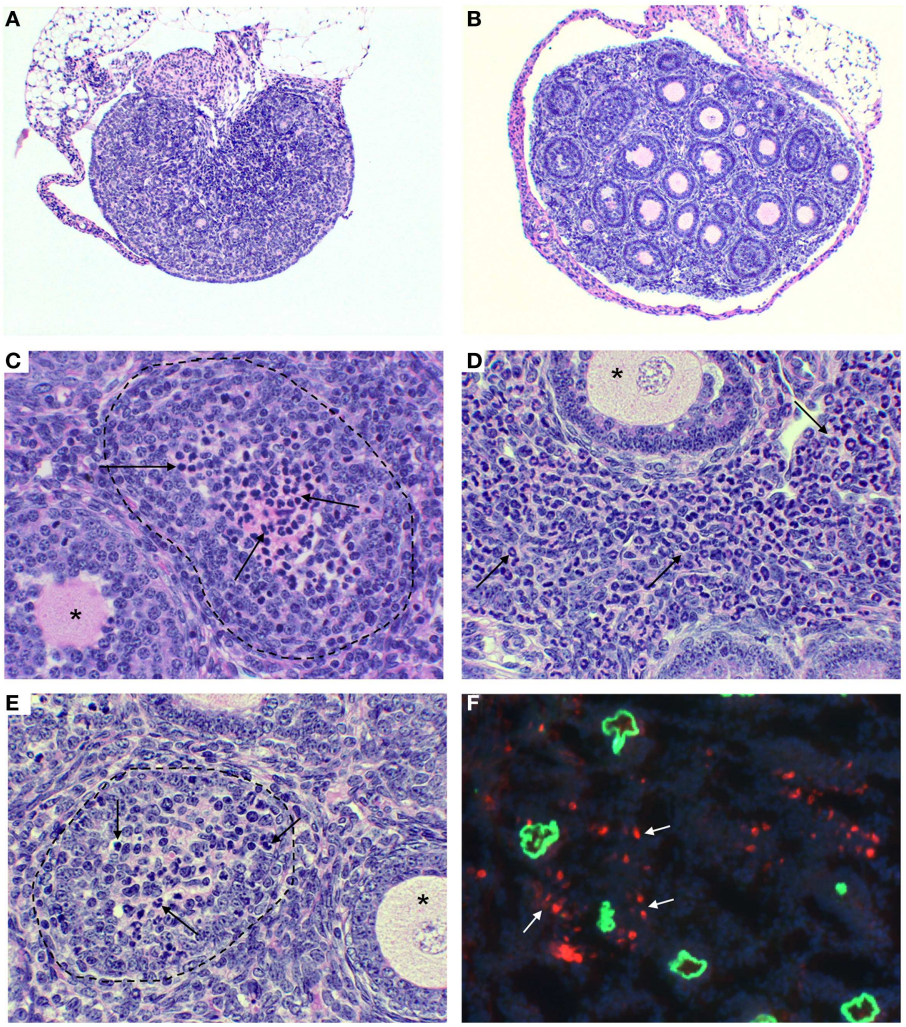

FIGURE 1 | Ovarian immunopathology of TI-nAOD in C57BL/6 Rag1$^{-/-}$ mice injected with pZP3 monoclonal antibody (mAb) in the first week of life. **(A)** Ovarian atrophy with major oocyte depletion and disrupted ovarian architecture. **(B)** Normal ovarian histology in mice injected with ZP3 mAb and NK cell-depleting (NK1.1) mAb. **(C–E)** Mononuclear cell-dominant **(C**, arrows), or granulocyte-dominant **(E**, arrows) infiltrates inside ovarian follicles is a common feature of ovarian pathology in TI-nAOD. The dotted lines **(C,E)** outline ovarian follicles where oocytes are replaced by inflammatory cells while the asterisks **(C–E)** indicate normal ovarian follicles. **(D)**, Inflammatory cells also infiltrate the ovarian interstitium (arrows). In **(F)**, Immunofluorescence detection of mouse IgG (green fluorescence represents zona pellucida-bound ZP3 mAb); and NKG2D^{+} NK cells (red fluorescence) detectable outside and inside the zona pellucida (white arrows). DAPI (nuclear blue staining). Magnification [**(A,B)**: 50×; **(C–F)**: 400×]. Hematolyxin and eosin stain **(A–E)**. [Reproduced from Rival et al. (45), *J. Immunol.* 191, 2865–2869].

ZP3 auto-peptide and its cognate antibody because (1) pZP3 was arbitrarily chosen as a novel auto-peptide with T and B cell epitopes; and (2) AOD induction also requires immunization with adjuvants. However, regulatory T cell (Treg) depletion from normal female mice leads to spontaneous production of autoAb that targets the same ZP3 335–342 epitope and the transfer of the sera from these animals induces nAOD in naïve pups. Thus, the pZP3 that has been studied for over 20 years is, in fact, a physiologically relevant B cell autoepitope. *Fourth*, nAOD develops in wild-type mice, and interestingly, nAOD also develops in mice that lack T and B cells. We call them T cell-dependent nAOD (TD-nAOD) model and T cell-independent nAOD (TI-nAOD) model, respectively. TD-nAOD induction is MHC-restricted, and it provides a useful platform for studying neonatal innate and adaptive responses and their interaction. On the other hand, the study of TI-nAOD in the recombination activation gene (Rag) knock out (KO) mice allows a reductionist approach to critically dissect the neonatal innate response. For example, we can define the novel properties of neonatal natural killer (NK) cells *in vivo* without the complex interaction between the innate and adaptive immune cells.

With the two nAOD models, we have addressed two fundamental questions on neonatal autoimmune disease: (1) Why are neonates more susceptible to autoimmunity; and (2) How do maternal autoAb induce an autoimmune disease in neonatal mice?

WHY ARE NEONATES MORE SUSCEPTIBLE TO nAOD?

The neonatal time window of nAOD induction applies equally to TD-nAOD and TI-nAOD. Therefore, neonatal innate responses are sufficient to confer propensity to nAOD in newborn mice (44, 45). NK cells are components of the innate response that can perform antibody-dependent cellular cytotoxicity (ADCC) through FcγRIII and produce multiple cytokines. Differences in the phenotype and function of neonatal and adult NK cells have been described (46). However, while some reports show a poor neonatal NK cell function compared to adults, others demonstrate equal or enhanced effector functions in neonatal NK cells. Our recent work has convincingly demonstrated that neonatal NK cells and their unique properties are the key explanation for newborn propensity to nAOD (45). Strikingly, the age of the donors of NK cells that restore nAOD in genetically NK cell-deficient recipients that lack the rag and the common gamma chain genes, correlated precisely with the neonatal time window for nAOD induction. Thus, neonatal NK cells are critical for nAOD susceptibility (45). NK cell activation depends on the balance of signaling through stimulatory and inhibitory receptors (47). Self-tolerance by NK cells is achieved by the interaction of major histocompatibility complex class I (MHC I) with the murine Ly49 receptors or the human killer cell Ig-like receptors (KIR). However, expression of these receptors is stochastic. Those NK cells that lack receptors for self-MHC I are potentially autoreactive. However, in a process termed licensing, the NK cells that do not recognize self-MHC I become "anergic," thus ensuring self-tolerance (48). Strikingly, it has been recently shown that the "anergic" NK cells that do not recognize self can become activated during inflammatory conditions and are more efficient in clearing infections or tumor cells than the licensed NK cells (49–51).

The expression of Ly49 receptors is known to be ontogenetically regulated; while ~5% of NK cells express Ly49C/I receptors in the first week of life, the frequency in adult mice is 10 times higher (52–54). This prompted us to investigate the role of Ly49 receptors in nAOD induction, and to address whether the delayed expression of Ly49C/I on NK cells confers adult resistance to nAOD. In a pivotal experiment, we showed that adult Ly49C/I negative NK cells could also induce nAOD only after the Ly49C/I$^+$ NK cell subpopulation has been depleted *in vivo*. Our findings made three important points: (1) neonatal NK cells are documented for the first time to be functional and they are, in fact, hyper-reactive, (2) both neonatal and adult Ly49C/I negative NK cells can induce nAOD, and (3) they raise the interesting possibility that adult Ly49C/I$^+$ NK cells can inhibit the activation of Ly49C/I negative NK cells, as shown by their capacity to block nAOD induction. Our findings are supported by recent literature on NK cells. Ivarsson et al. (55) recently showed that human fetal NK cells are hyper-responsive to cytokine stimulation, and CD16 engagement can overcome their hypo-responsiveness in killing HLA-negative targets. This study provided a clinical correlate to our findings. In addition, it has been shown that Ly49-negative NK cells can acquire full effector functions under inflammatory conditions. We should emphasize that whereas previous work (49, 51) has described intrinsic NK cell regulation, our study on nAOD provides the first evidence of extrinsic NK cell inhibition, where Ly49C/I$^+$ NK cells may inhibit Ly49C/I negative NK cells (**Figure 2**). However, this possibility still requires further investigation. We currently speculate an indirect regulation that depends on the competition between the two NK cell subsets for the interaction with dendritic cells, which are required for NK cell priming (56, 57).

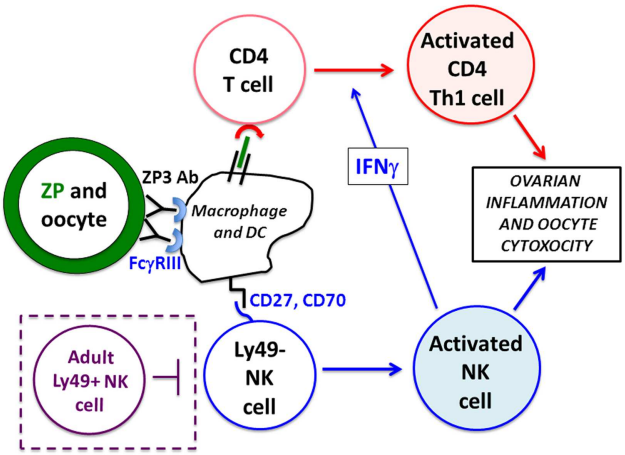

FIGURE 2 | Mechanism of nAOD induction. Ovarian ZP3 immune complexes on the zona pellucida (ZP, green) are formed after maternal autoAb transfer and stimulate FcγRIII$^+$ macrophage/dendritic cells that activate (1) a *de novo* CD4$^+$ T cell response (in red), and (2) Ly49-negative neonatal NK cells (in blue). These NK cells produce IFNγ promoting a Th1 pathogenic CD4$^+$ T cell response to ovarian antigens. Ovarian inflammation and oocyte depletion is, in turn, mediated by the FcγRIII$^+$ neonatal NK cells and activated Th1 effector CD4$^+$ T cells. Mice older than 9 days fail to develop nAOD because of the emergence of Ly49$^+$ NK cells (in purple dotted box) and Treg function (not depicted).

As a mechanism of nAOD resistance in mice older than 7 days, Treg were documented to control susceptibility to nAOD. Thus, when Treg were depleted from 9-day-old pups with CD25 antibody, the neonatal time window was extended and the older mice became fully susceptible to nAOD (44). Therefore, at least two mechanisms acquired by adult mice confer resistance to autoimmune disease: (1) the innate system, by restraining NK cell activation with the acquisition of the inhibitory Ly49 receptors, and (2) the adaptive system, by acquiring Treg function. In contrast, these mechanisms are deficient in the neonatal mouse.

HOW DO THE MATERNAL ZP3 autoAb CAUSE OVARIAN INJURY?

DUAL NK CELL REQUIREMENTS IN nAOD: INDUCTION OF ANTIBODY-DEPENDENT CYTOTOXICITY (ADCC) AND OVARIAN ANTIGEN-SPECIFIC PATHOGENIC CD4 T CELL RESPONSE

Maternal ZP3 autoAb transferred through the milk but not the placenta was critical for nAOD induction (44). Within 24 h, ZP3 IC was detectable in the zona pellucida of the ovary, a process that culminated in significant ovarian inflammation and loss of oocytes over the next 2 weeks [**Figure 1**; Ref. (44)]. The IC can cause immunopathology by: (1) activation of the complement cascade, (2) FcγRIII-dependent ADCC, and (3) T cell activation mediated by FcγRIII and/or complement receptor-bearing antigen presenting cells (APC). Complement C3b and C5b were undetectable in ovaries of mice with nAOD. However, studies with

blocking antibodies and gene KO mice indicated that nAOD is dependent on FcγRIII expression (44, 58). In fact, we found that TD-nAOD pathogenesis requires both FcγRIII-dependent ADCC and a *de novo* T cell response. First, the neonatal NK cell must express FcγRIII to support nAOD (58). Second, the critical $CD4^+$ T cells are required in TD-nAOD because (1) T cell depletion prevents nAOD, (2) $CD4^+$ T cells from mice with nAOD transfer ovarian disease to naïve pups (44). Importantly, NK cells are required in both the inductive phase and the effector phase of the T cell response (58). Likely, the NK cell-derived IFNγ skews the T cell response toward IFNγ-producing Th1 cells (**Figure 2**). Indeed, IFNγ neutralization by antibody in the cell donors blocked the adoptive transfer of nAOD (58) and IFNγ-deficient NK cells failed to induce nAOD. Importantly, requirement of NK cells and FcγRIII expression have also been documented in the adult model of myasthenia gravis (59).

In our ongoing research, we have further clarified the pathogenesis of nAOD by making the following observations. First, in addition to NK cells, ovarian resident $FcγRIII^+$ macrophages and/or dendritic cells are requisites in TD-nAOD. Second, NK cell activation most likely occurs in the lymph nodes where they are primed by the ovarian-derived $FcγRIII^+$ cells. Third, NK cell homing and activation are dependent on IL-15, CD70 and CD27, and CXCR3.

ANTIBODY TO VIRAL ANTIGEN ALSO CO-STIMULATES ACTIVE IMMUNITY TO VIRUS IN NEONATAL BUT NOT ADULT MICE

Maternal anti-microbial antibodies confer transient protection from infection to the progeny. However, compelling studies have shown that co-injection of a small amount of antiviral antibody with a live virus in neonatal mice can evoke effective long-term immunity against the virus. Neonatal infection with the FrCasE murine retrovirus before day 5–6 after birth leads to a virus-specific Treg response that inhibits efficient antiviral immunity (60). This results in fatality from neurodegeneration within 2 months and from erythroleukemia within 4–5 months. However, the Treg response is dramatically curtailed when the virus-infected neonatal mice receive a small dose of epitope-specific antiviral antibody within 2 days after the viral infection (61, 62). Concomitantly, the mice develop a strong antivirus $CD8^+$ T cell response and life-long protection against the virus. As will be described below, the underlying mechanisms behind this successful neonatal antiviral vaccination schema are remarkably similar to the mechanism involved in nAOD induction. Therefore, neonatal exposure to antibody can enhance immunity against both autologous and foreign antigens.

COMMON MECHANISMS ARE SHARED BETWEEN AUTOIMMUNITY AND VIRAL IMMUNITY INDUCTION BY NEONATAL IMMUNE COMPLEXES

It is remarkable that neonatal IC formation by an epitope-specific antibody can lead to both autoimmune disease and antiviral immunity. Even more striking is the fact that the two responses deploy very similar mechanisms. They both require: (1) antibody injection and IC formation within the first week of life, (2) epitope-specific antibody that targets the functional domain of the antigen, (3) NK cells, (4) FcγRIII, (5) *de novo* induction of T cell responses, and (6) IFNγ. In both cases, the responses to IC beyond the neonatal time window were inhibited by Treg. Based on findings from our recent nAOD study, we can now add to this list, the critical influence of NK cell expression of the Ly49 inhibitory receptors.

GENERAL CONCLUSION/SUMMARY

It is generally accepted that the higher susceptibility of newborns to infections is a consequence of the immature neonatal immune system. In support of this concept, many studies have described a weaker response by the neonatal immune system. However, this concept is still controversial, as others have found an equivalent or even stronger neonatal immune responses over the adult response. Moreover, a recent study showed that the poor protection to respiratory syncytial virus infection in newborns is due to the inhibition of the antibody production by the IFNγ produced by neonatal NK cells and T cells (63), suggesting that the neonatal immune system involves a complex cellular and molecular interplay. Experimental data derived from nAOD research have added new insight on the *in vivo* responsiveness of the newborn immune system. They clearly indicated that neonatal mice have the capacity to mount a robust immune response to tissue-associated IC that surpasses an adult response. The primary mechanism is the lower threshold of neonatal NK cell responses to tissue IC; and the capacity of neonatal NK cells to promote a pathogenic neonatal T cell response. It is important to emphasize that recent studies on human NK cells in support of this concept are also emerging (55). Our findings on nAOD are concordant with the observations by Michaud et al. (61) and Nasser et al. (60, 62) on the neonatal response to a foreign antigen in the context of a viral infection in the newborn. In both, the antibody exposure restricted to the first few days of life has been clearly documented to strongly enhance the neonatal immune response against its cognate antigen. The mechanisms shared between these two models should support a novel approach in effective vaccine design for the very young.

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Mast cell-mediated and associated disorders in pregnancy: a risky game with an uncertain outcome?

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During pregnancy, the maternal organism is under the influence of tremendous endocrine as well as immunological changes as an adaptation to the implanted and developing fetus. In most cases, the maternal adaptations to pregnancy ensure both, the protection against harmful pathogens and the tolerance toward the growing semi-allogeneic fetus. However, under certain circumstances the unique hormonal milieu during pregnancy is causative of a shift into an unfavorable direction. Of particular importance are cellular disorders previous to pregnancy that involve cell types known for their susceptibility to hormones. One interesting cell type is the mast cell (MC), one of the key figures in allergic disorders. While physiological numbers of MCs were shown to positively influence pregnancy outcome, at least in mouse models, uncontrolled augmentations in quantity, and/or activation can lead to pregnancy complications. Women that have the desire of getting pregnant and been diagnosed with MC mediated disorders such as urticaria and mastocytosis or chronic inflammatory diseases in which MCs are involved, including atopic dermatitis, asthma, or psoriasis, may benefit from specialized medical assistance to ensure a positive pregnancy outcome. In the present review, we address the course of pregnancy in women affected by MC mediated or associated disorders.

Keywords: mast cells, pregnancy, urticaria, PUPPP, mastocytosis, atopic dermatitis, asthma, psoriasis

INTRODUCTION

Pregnancy represents a unique challenge for the maternal organism. Tremendous endocrine and immunological modifications that occur as an adaptation to the implanted embryo ensure a successful pregnancy outcome. These necessary changes in the hormonal state and the shift toward anti-inflammation can, however, cause a dysregulations in the number and behavior of mast cells (MCs). MCs have been shown to exhibit beneficial function in pregnancy by contributing to implantation, placentation and fetal growth through their release of the glycan-binding protein galectin-1 and, thus, are critically implied in the fetomaternal interface (1). In addition, MCs influence pregnancy by modulating non-immunological responses by contributing to tissue remodeling, angiogenesis, and spiral artery modifications (1). In later pregnancy phases, however, MCs display rather detrimental functions as an excessive release of MC-mediators in parturition are associated with pre-term delivery. MC activation is modulated by hormonal endocrine signals that could lead to altered functional behavior of MCs in various innate and adaptive immune responses (1). In particular, pre-existing MC mediated and associated disorders may affect disease progression and the disease itself may influence pregnancy outcome. Sex hormones are considered to influence the clinical course and severity of chronic allergic and inflammatory diseases, including atopic dermatitis (AD), asthma, and psoriasis (2–5). In fact, estrogen and progesterone have been reported to modulate tissue homeostasis and immunological responses in various conditions.

Here, we review the existent literature on the clinical implications of MC associated disorders in pregnancy, focusing on directly MC mediated disease such as urticaria and mastocytosis, but also summarize the disease impact on pregnancy of common inflammatory disorders in which MC have been reported to critically contribute to the pathogenesis. Hereby, we offer an overview of how disease-specific modifications in MC function and activation could determine the fate of pregnancy.

ROLE OF MAST CELLS AND MAST CELL MEDIATORS IN PREGNANCY

Mast cells reside in the endometrial tissue and uterine MCs exhibit signs of activation during premenstrual stages (6). MCs granules consist of a large array of mediators, including histamine, prostaglandins, leukotrienes, several cytokines, and proteases (7). The release of MC protease such as tryptase has been reported to stimulate the production of matrix metalloproteinases (MMPs) that are involved in the degradation of extracellular matrix components. Increase protease expression is detected during menstruation (8). Histamine, which is produced and released by MCs, has been reported to be involved in blastocyst implantation and placenta development by contributing to and promoting trophoblast invasion, growth, and the expression of adhesion (9, 10) molecules. During pregnancy, the number of MCs increase in the myometrium and equal ratio of tryptase and chymase (MC_{TC}) positive MCs shift toward a tryptase-only (MC_T) phenotype (11). Here, histamine, prostaglandins and MC proteases have

been shown to contribute and modulate myometrical contractility (12, 13). MC proteases may also be involved in post-partum uterine tissue remodeling (14). Given these facts, it is reasonable to suggest that MCs influence pregnancy outcome under physiological conditions and even more relevant when their activation status is modulated by disease. The prevalence of MC mediated and associated disorders, including allergic and non-allergic diseases, is increasing. Severe allergic reaction, i.e., anaphylaxis during pregnancy can indeed result in pre-term labor whereas adequate treatment, including antihistamines and corticosteroids, reportedly inhibited uterine contractions (15). This evidence points toward the importance of disease management of MC-related disorders during pregnancy. Although, the use of systemic treatments should be limited or even generally avoided in pregnancy – especially in the first trimester, pregnant women require best possible treatment. But how to provide best possible treatment by calculated risk profile? The use of second-generation antihistamines (sgAH) for example is widely used for the treatment of allergic disease. In pregnancy, it is recommended to limit the use of sgAH to loratadine (16), and possibly desloratadine, because of best available evidence. Nowadays, several AH are OTC (over-the-counter) products in many countries and it can be assumed that these drugs are frequently used by pregnant women – at least before they knew to be pregnant. However, up-dosing of sgAH as it is recommended in the management of chronic urticaria must be carefully suggested in pregnancy since safety studies have not been performed (17).

URTICARIA IN PREGNANCY

Urticaria is a very common dermatological condition in which an increased activation of MCs and the subsequent release of MC-mediators, mainly histamine among others, lead to the development of wheal-and-flare responses accompanied by an intense pruritus on the skin (18). Urticaria is divided into acute (less than 6 weeks) and chronic (more than 6 weeks) forms as well as into inducible and spontaneous occurring subtypes (17). Urticaria may develop during pregnancy even though it is not considered as a specific pregnancy dermatosis. MCs have also been reported to contribute to the pathogenesis of pregnancy-related dermatoses associated with pruritus that are restricted to pregnancy, e.g., pruritic urticarial papules and plaques of pregnancy (PUPPP). Urticaria can either develop during pregnancy or the symptoms of a pre-existing chronic spontaneous urticaria (CSU) may change in terms of disease activity and severity. Influence of sex hormones on MC functions and the pathogenesis of CSU have long been considered (19). CSU is approximately twice more frequent in women than in men and the disease activity of CSU may be associated or triggered by fluctuation of sex hormone levels, including menstrual cycle, pregnancy, menopause, and hormonal therapies (19). CSU may worsen with pregnancy in some patients but also improve in others (20). Hypersensitivity to sex hormones and their modulating actions on MCs have been implicated in the pathogenesis of CSU and altered hormone serum levels have been described in subgroups of CSU patients (21). Thus, such fluctuations in the hormonal milieu have been suspected to either improve, maintain, or aggravate urticarial lesions during pregnancy (19). PUPPP and other pregnancy-related dermatoses associated with pruritus should be considered as differential diagnosis if wheals and itch newly occur in pregnancy, especially during the third trimester (22). In addition, special treatment considerations should be applied to pregnant and lactating women (17, 23).

PRURITIC URTICARIAL PAPULES AND PLAQUES OF PREGNANCY

Pruritic Urticarial Papules and Plaques of Pregnancy or polymorphic eruption of pregnancy (PEP) is the most common pregnancy-related skin disorder with an incidence of about 1:160–1:200 (24). This disease appears and exists exclusively in pregnant patients. Besides the pruritic urticarial papules and plaques, which represent the key symptoms of PUPPP, more than one-half of the patients later develop polymorphous features including erythema, vesicles as well as targetoid and eczematous lesions (25). Characteristically, the eruptions begin on the abdomen, particularly, within or adjacent to *striae cutis distensae* and occur predominantly in the third trimester in about 83% of the patients (25–27). It is suggested that multiple gestations and an excessive maternal weight gain is associated with the occurrence of PUPPP (25, 28). The fetal weight and sex does not seem to be related to the onset of PUPPP (25). Cortisol serum levels have been found to be significantly reduced in PUPPP patients whereas estradiol concentrations were comparable with unaffected women (27). It is tempting to speculate that MCs are involved in the onset of PUPPP although no studies are existing showing a direct link between MCs and this disease. It is suggested that the activation of the skin immune system characterized by increased numbers of dentritic cells and activated T cells in lesional skin contribute to the pathology of PUPPP (29). Skin infiltrates of macrophages (30) and eosinophils (25, 27) have been described in affected tissue. Although, a direct implication of MCs has, as of yet, not been reported in PUPPP there are several lines of evidence that clearly suggest a role for MCs. First, as in urticaria, antihistamines are the first line option in the treatment of PUPPP and are effective in most patients. MCs are considered as the main source of histamine in the skin (31). Second, even though PUPPP and urticaria are different diseases there are several similarities in terms of the clinical symptoms including pruritic erythema and urticarial lesions. Third, autologous whole blood injections have been reported as an effective treatment option in PUPPP as it is in auto-reactive CSU (32, 33). Therefore, even if still speculative it is not unlikely that the release of MC-mediators critically contribute to the pathogenesis of PUPPP.

MASTOCYTOSIS AND PREGNANCY

Mastocytosis represents a group of related disorders, each characterized by a pathological accumulation of MCs in one or more organs ranging from indolent to very rare aggressive forms (34). Mastocytosis is classified as a rare disease with an estimated prevalence of around 1 per 10,000 and dividing cutaneous from systemic forms (35). About 80% of mastocytosis patients carry an Asp816Val mutation in the catalytic domain of the c-Kit receptor downstream tyrosine kinase in peripheral blood mononuclear cells (36). This point mutation mediates an increased proliferative rate of MCs (37). An addition explanation for the increased numbers of MCs in tissues from mastocytosis patients might be the enhanced chemotaxis of CD117 positive cells. It is speculated

that MC progenitor cells bearing the D816V mutation preferentially migrate to SCF produced by stroma cells, endothelial cells, fibroblasts, and keratinocytes in the skin (38). It could be shown that MCs present in the lesions express SCF suggesting a potential autocrine or paracrine growth and differentiation loop for MCs and lymphoid progenitors (39, 40). Differentiation of the mutant progenitor cells into mature MCs occurs locally based on the specific microenvironment. Thus, enhanced MC migration combined with aberrant proliferation contribute to the extensive MC hyperplasia observed in affected tissues (38). Beside the elevated serum tryptase levels in mastocytosis patients (41), the coexpression of CD25 antigen in bone marrow MCs turned out as diagnostic marker in mastocytosis (42, 43). In bone marrow biopsies from mastocytosis patients, MCs are surrounded by lymphoid aggregates, which consist of a mixture of B and T cells (39, 44). Similar to MCs, these B and T cells carry the D816V mutation (44).

Between 20% and one third of pregnant women with mastocytosis reported a worsening of the diseased-related symptoms (45, 46). Around 30% experienced a clinical improvement during the first trimester. In the other half of the affected population, MC-mediator related symptoms remained unchanged (46). Interestingly, worsening of symptoms was observed during the first or third trimester (46) when Th1-mediated pro-inflammatory conditions dominate (47–49). Although, women diagnosed with mastocytosis are often required to continue the intake of medications including antihistamines during pregnancy the doses are often decreased because of fetal safety concerns (45). The reduction in medication as well as an irregular medication intake could contribute to worsening of mastocytosis symptoms as well. Undiagnosed and not appropriately treated mastocytosis can be associated with severe pregnancy complications including fetal demise (50).

Parturients suffering from mastocytosis that do not undergo a natural birth represent a particular challenge for anesthesiologists. During the process of labor, life-threatening complications may occur, particularly due to the risk of anaphylactoid reactions triggered by anesthesia. Medications such as glucocorticoids, antihistamines, and epinephrine should be available during the critical phases of labor and the early post-partum period (51).

In general, studies describing the impact of mastocytosis in pregnancy and vice versa are limited. Thus, one can only speculate that the unique pregnancy-associated micromilieu composed of hormones and myriads of mediators contribute to variations in the disease pattern.

ATOPIC DERMATITIS IN PREGNANCY

Atopic dermatitis is a complex chronic inflammatory condition in which MCs have been shown to contribute critically to the pathogenesis and the induction of inflammation and pruritus. MC number and degranulation is increased in atopic lesions (52). It is suggested that the invasion and degranulation of MCs within peripheral nerve bundles may provoke and aggravate itchiness of AD (53). MC-derived mediators might participate in epidermal hyperplasia seen in lichenified lesions in AD (54).

Atopic dermatitis is one of the most prevalent dermatoses during pregnancy (55, 56) and pregnancy may alter the clinical course and severity of AD. More than half of pregnant women with pre-existing AD were reported to experience worsening of their disease during the second or third trimester (57, 58) when a constant Th2 response is maintained. During pregnancy an immunologic homeostasis tolerating the fetus is of crucial importance. To prevent fetal rejection, maternal T cell mediated immunity is modulated. AD is widely accepted as a Th2-dominated disorder. Therefore, alteration in the Th1/Th2 balance is suggested to promote AD severity, which is often observed in pregnancy (59). MCs found in AD lesions are a major source of IL-4 and store higher amounts of IL-4 compared to MCs in normal skin (60). Exogenous IL-4 has been shown to be important for the differentiation of T helper cells into Th2 cells (61). Thus, one can speculate that MCs participate in the Th1/Th2 switch and therefore disease severity (**Figure 1**). Moreover, IL-4 induces the proliferation of fibroblasts (62) and atopic fibroblasts contribute to the pathogenesis of AD by initiating strong proliferation and differentiation defects in keratinocytes (63).

Variations in sex hormone concentrations seem to be related with the severity of AD that is supported by the finding that ca. one third of women reported a premenstrual deterioration in the symptoms (57, 58). Sex hormones may also directly influence AD symptoms by their effects on MCs, mediator release, and IgE production (64) (**Figure 1**). No reported evidence point toward direct influence of AD on infertility or increased rates of miscarriage, birth defects, or prematurity (65). However, AD patients that are more prone to suffer from bacterial or viral super-infection may be at a higher risk for birth complications including premature delivery, intrauterine growth restriction, or miscarriage (66). The application of large doses of triamcinolone acetonide for treatment of AD should be avoided as it was related with intra uterine growth retardations (67). Therefore, specific considerations about the treatment strategies in AD during pregnancy will apply to control the impact of AD progression and complications that may occur on pregnancy.

ASTHMA AND PREGNANCY

In 2004, the number of people affected by asthma worldwide was estimated as 300 million with a further increase up to 400 million asthmatics by 2025. The rate of asthma increases as communities adopt western lifestyles and become urbanized (68). It is assumed that environmental estrogens participate in the development of asthma as they induce MC degranulation via the estrogen receptor-α. These pollutants show estrogen-like activities, tend to degrade slowly, have a long biological half-life, and bioaccumulate and bioconcentrate in the food chain (69).

Asthma is characterized by recurrent episodes of airway obstruction, which reverse either spontaneously or after use of medication. It is usually associated with bronchial hyperresponsiveness and evidence of chronic airway inflammation (70). The earliest text where the term "asthma" was mentioned in a medical context is in the Corpus Hippocraticum. Several centuries later during the second half of the first century A.D. Aretaeus the Cappadocian was the first one who dealt with asthma as an autonomous clinical disease and not as a symptom (71). One characteristic of asthma is the exaggerated narrowing of the airways that is caused by contraction and shortening of airway smooth muscle (ASM) cells. However, the cause of the induced

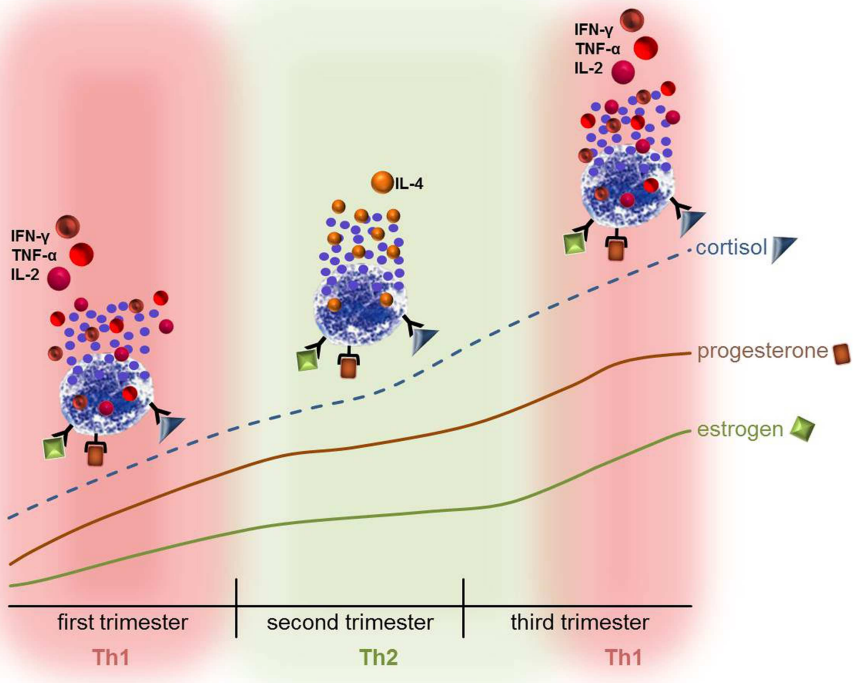

FIGURE 1 | The hormonal stimulation of mast cells during pregnancy might contribute to the Th1/Th2 switch that takes place in pregnancy. The ever-going increase in estrogen, progesterone, and cortisol could directly influence the activation status and behavior of MCs and lead to the release of either pro- or anti-inflammatory mediators thus contributing to the Th1 or Th2-based micromilieu. Hence, disorders that are mediated by MCs or in which MCs are involved may turn into an unfavorable direction. It is however also possible that the symptoms ameliorate due to the hormone-modulated behavior of MCs.

displacement behavior of the ASM is still a matter of discussion. It is proposed that following processes could contribute to the SMC response: (1) changes in ASM structure and/or behavior; (2) structural and/or mechanical alterations in the non-contractile structures of the airway wall, and (3) variations in the relationship of the airway wall to the surrounding lung parenchyma (72). The airway remodeling observed during the course of asthma includes the increase in the smooth muscle cells surrounding the airway wall, a deposition of extracellular matrix components under the epithelial basement membrane that causes a thickened appearance, a breach in the integrity of the airway epithelium and an increase of mucus-producing goblet cells in the epithelium or submucosal glands (73).

Various immune cell types including macrophages, eosinophils, and MCs participate in the process of airway remodeling; accordingly they were found in high numbers in bronchoalveolar lavage and in bronchial biopsies from asthmatic patients (74). Asthma is i.a. characterized by an infiltration of MCs in the bronchial epithelium (75), mucous glands, and smooth muscle (76, 77). It is hypothesized that the ASM itself induce the migration of MCs and their progenitors via the release of the chemoattractants CXCL9, CXCL10, CXCL11, stem cell factor (SCF), and transforming growth factor (TGF)-β (78–80). ASM would also induce MC proliferation and survival (81). Once resident in the ASM bundle, MCs adhere to it via cell adhesion molecule 1 (CADM1) (82). The release of MC-mediators such as histamine, prostaglandin D_2, and leukotriene C_4 upon activation induces typical asthmatic symptoms including bronchoconstriction, mucus secretion, and mucosal edema (80). It is often assumed that the activation of MC during asthma is allergen-dependent via the high affinity IgE receptor FcεRIα. Mucosal MCs present in bronchial tissue from asthmatic patients exhibit features of chronic activation (83).

About 1% of pregnant women are diagnosed with active asthma (84). The inflammatory response induced in asthmatic airways contributes to the pathophysiology of this disease (85). This in turn could interfere with the necessary variations in the cytokine milieu mandatory for pregnancy to occur and be maintained. The first trimester and also the early phases of the second trimester of pregnancy require a strong inflammatory response in order to ensure uterine tissue remodeling and clearance of cellular debris (47–49). During the course of pregnancy, the concentrations of the steroid hormones estradiol (E_2) and progesterone (P_4) raise more than fivefold followed by a further fivefold increase by term (86). The immunological shift toward Th1 responses at the beginning of pregnancy is mediated by enhancing pro-inflammatory cytokine production by monocytes and macrophages (87), dendritic cells (88) as well as MCs (89) (**Figure 1**). The ever-going increase in estrogen, progesterone, and cortisol concentrations at

midpregnancy supports a shift toward Th2 responses, which are necessary for maintaining pregnancy (90). As asthma is a classical Th2-driven disease its course may be negatively influenced by the establishment of an anti-inflammatory milieu as it is observed in the second and early third trimester (91). Indeed, during pregnancy asthma worsens in 35% of the women with an increase in asthma symptoms starting at the third trimester (92) when a constant Th2 milieu was established. MCs express the receptors for E_2 and P_4 (93, 94) and degranulate upon treatment with theses hormones (94). Based on these findings, one might speculate that the rising levels of estrogens and progesterone during pregnancy stimulate MCs to degranulate (**Figure 1**) whereby they could negatively influence the course of asthma. This could be an explanation of why one third of women present a worsening of asthma symptoms at midpregnancy (92). However, 28% of the women experienced an improvement of asthma symptoms and further 33% showed no changes (92). Women who reported changes in their asthmatic symptoms during pregnancy reverted post-partum toward their pre-pregnancy asthma course (92).

An elegant study by Perlow et al. revealed that pregnant women requiring long term administration of oral steroids are at an increased risk of pre-term labor and delivery as well as to develop gestational diabetes (95). Moreover, steroid-dependent (95, 96) but also non-steroid medicated (95) asthmatic mothers delivered more often low birth weight neonates with less than 2500 g than mothers without asthma. Intriguingly, the boost in asthma symptoms during pregnancy seems to correlate with the gender of the fetus. While women who delivered boys reported an improvement in their asthma during their pregnancy, mothers of girls, however, had increased asthma severity during gestation (97, 98). Women who were pregnant with a female fetus needed significantly more inhaled glucocorticoids in late pregnancy. For this particular group, it is proposed that an upregulation of inflammation is associated with asthma as gestation progressed (98). The mechanism behind is not entirely understood. In asthmatic mothers pregnant with female fetuses, the placental activity of the enzyme 11-hydroxysteroid dehydrogenase type 2 (11-HSD2) that metabolizes cortisol to inactive cortisone is reduced (98). This would increase the intracellular cortisol concentration in relation to variations in cytokine production in female placentas compared to male placenta explants (99). It was reported that the inflammatory response of placental trophoblast cells from male pregnancies after LPS stimulation is boosted probably because of the enhanced toll-like receptor (TLR)-4 expression in these cells (100). These fetal gender-specific differences should be taken into account during pregnancy in asthmatic women.

PSORIASIS AND PREGNANCY

About 25 million people in Europe and North America are affected by psoriasis that counts to the most prevalent immune-mediated skin disease in adults (101). Psoriasis is considered to be a genetically programed, organ-specific (skin, or skin and joints) inflammatory disease (102) characterized by red, scaly, and raised plaques (101). Vascular dilation, bridged fenestrations, and gaps in endothelium, edematous areas in the cytoplasm of endotheliocytes, myocytes, and pericytes, basement membrane zone thickening and cell extravasation are reported as microvascular changes that occur in psoriatic lesions and represent signs of increased vascular permeability (103). Compared to asthma, psoriasis is considered to be a Th1-driven disease characterized by the infiltration of several T cell subsets, neutrophils, dendritic cells, natural killer T cells, and MCs (102, 104). All of them contribute to the inflammatory microenvironment that is composed of increased levels of interferon (IFN)-γ, tumor necrosis factor (TNF)-α, IL-2, and IL-12 (105) as well as IL-23 and IL-17A. MCs have been demonstrated to be key modulators of T-cell mediated responses and essentially involved in neutrophil recruitment (106). Recently, the important role of IL-17 producing Th17 cells in the pathogenesis of psoriasis has been reported (107). Neutrophils and MCs are other significant potential sources of IL-17A in psoriasis (108). In the superficial dermis of psoriatic skin, MC density is increased (104). It has been shown that alterations in psoriatic tissue appear to be initiated by degranulating MCs (109) and MC degranulation is among the earliest events in relapsing psoriasis lesions (109). Degranulated MCs have been found in close proximity to blood vessels in the area of psoriatic lesions (103). One can speculate that the survival of MCs in the tissue is regulated by their most important growth factor, the SCF that is intensely expressed in psoriatic tissue while its receptor KIT is upregulated in the surface of MCs (110).

Mast cells are typically classified into either MC_T that contain only tryptase or MC_{TC} that contain both proteases tryptase and chymase. Both subtypes in human are suggested to be equivalent to the murine MC phenotypes. While MC_T seem to be related to immunological processes, MC_{TC} appear to be linked to non-immunological responses including tissue remodeling and angiogenesis. The tryptase is the quantitatively dominant protease present in all MC phenotypes (111). In psoriatic skin, tryptase-positive cells are increased in number (112, 113). However, the determination of serum tryptase levels is not an appropriate tool to assess the severity of psoriasis as no correlation could be found between serum tryptase and psoriasis severity in patients (114). Tryptase levels in normal subjects are undetectable (<1 ng/ml) whereas in systemic MC disorders such as mastocytosis and anaphylaxis elevated tryptase levels can be detected (41).

It was reported that approximately 50% of patients develop psoriasis before the age of 25 (115). There are several studies showing that the natural course of psoriasis in women is modulated by menstrual cycle, pregnancy, and menopause (5, 115, 116). All of these events during the reproductive cycle are under hormonal regulation. During pregnancy, more than 50% of the women reported an improvement of psoriasis at ca. the 30th week of pregnancy (midpregnancy) while more than 20% observed a worsening (116). At this time point, the shift from Th2 to Th1 immunity occurs mainly mediated by increased levels of estrogen, progesterone, and cortisol (90). It is assumed that during pregnancy and when hormone levels are increased, psoriatic symptoms improve. During puberty, post-partum and menopause when hormone levels decrease the disease severity seems to peak (117). Several Th1-characterized diseases including psoriasis (116), multiple sclerosis (118), and rheumatoid arthritis (119, 120) have been shown to improve during pregnancy. Psoriatic body surface areas (BSA) decreased significantly from 10 to 20 weeks' gestation. Thereby, increased levels of estrogen relative to progesterone correlate with

the improvement of psoriasis while progesterone concentrations alone did not correlate with changes in psoriatic symptoms. Interestingly, post-partum more than 60% of the patients reported worsening of symptoms while only 8.7% observed an improvement. But the authors found out that the "post-partum flare" was a return to the patients' baseline, rather than a real worsening (116).

It was reported that pregnant psoriatic women are at increased risk to adverse pregnancy outcomes including spontaneous and recurrent abortion, gestational hypertension, ectopic pregnancy, and pre-term rupture of membranes (121, 122). In fact, study results are controversial. Some showed an increased risk of adverse pregnancy outcomes, others did not. Psoriasis is definitely no contraindication for a pregnancy but a well-controlled disease and the monitoring of comorbidities, such metabolic syndrome, during pregnancy is of advantage. In contrast, generalized pustular psoriasis of pregnancy (GPPP), a special subtype of psoriasis occurring during pregnancy might be harmful for mother and child (123). In such cases, women should be kept on medical care by dermatologists. Several medications are available whose suitability and unsuitability for the treatment of psoriasis during pregnancy are reviewed by Lam et al. (124). However, safety data are limited because most of the data are based on case reports, which lack the comparison group of untreated patients (124). Women who need a disease-specific treatment should keep in mind that leaving a disease untreated during pregnancy may carry a greater risk to both the mother and fetus than any teratogenic risk of the drug to the fetus (124). Systemic treatment should be avoided when possible, but if necessary the best treatment option should be chosen on a case to case basis for optimized treatment during pregnancy.

RESUME

In general, there is no contraindication to pregnancy when MC-related pathologies are under appropriate medical control. Women who were diagnosed with MC mediated or associated disorders and especially those whose disease is active, should be carefully advised by medical specialists to avoid severe pregnancy complications and to monitor disease progression. The unique modifications of the maternal endocrine and immune system can influence the number and behavior of MCs. Further studies addressing the molecular mechanisms behind the impact of pregnancy on MC mediated and associated disorders are needed in order to optimize pregnancy course and outcome.

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Placental Protein 13 (PP13) – a placental immunoregulatory galectin protecting pregnancy

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Galectins are glycan-binding proteins that regulate innate and adaptive immune responses, and some confer maternal-fetal immune tolerance in eutherian mammals. A chromosome 19 cluster of galectins has emerged in anthropoid primates, species with deep placentation and long gestation. Three of the five human cluster galectins are solely expressed in the placenta, where they may confer additional immunoregulatory functions to enable deep placentation. One of these is galectin-13, also known as Placental Protein 13 (PP13). It has a "jelly-roll" fold, carbohydrate-recognition domain and sugar-binding preference resembling other mammalian galectins. PP13 is predominantly expressed by the syncytiotrophoblast and released from the placenta into the maternal circulation. Its ability to induce apoptosis of activated T cells *in vitro*, and to divert and kill T cells as well as macrophages in the maternal decidua *in situ*, suggests important immune functions. Indeed, mutations in the promoter and an exon of *LGALS13* presumably leading to altered or non-functional protein expression are associated with a higher frequency of preeclampsia and other obstetrical syndromes, which involve immune dysregulation. Moreover, decreased placental expression of PP13 and its low concentrations in first trimester maternal sera are associated with elevated risk of preeclampsia. Indeed, PP13 turned to be a good early biomarker to assess maternal risk for the subsequent development of pregnancy complications caused by impaired placentation. Due to the ischemic placental stress in preterm preeclampsia, there is increased trophoblastic shedding of PP13 immunopositive microvesicles starting in the second trimester, which leads to high maternal blood PP13 concentrations. Our meta-analysis suggests that this phenomenon may enable the potential use of PP13 in directing patient management near to or at the time of delivery. Recent findings on the beneficial effects of PP13 on decreasing blood pressure due to vasodilatation in pregnant animals suggest its therapeutic potential in preeclampsia.

Keywords: actin cytoskeleton, biomarker, danger signal, evolution, extracellular vesicles, glycans, lectins, maternal-fetal interface

PREFACE

Many authors of this review have collaborated with Dr. Hans Bohn, the discoverer of Placental Protein 13 (PP13), who passed away on January 25, 2014. We dedicate this manuscript to his memory. His scientific legacy and enormous contribution to placental protein research have strongly influenced placentology and inspired our studies on PP13 (1).

Hans Bohn was born in Munich on October 18, 1928. He graduated in 1954 and completed his doctoral thesis in 1956 in chemistry at the University of Würzburg. A research fellowship starting in 1963 in the Protein Research Laboratory at the University of Pittsburgh was critical in directing his interest in protein research. After two years, Dr. Bohn returned to Germany to work on proteins in Behringwerke in Marburg/Lahn (**Figure 1A**). He was the first to isolate factor XIII from human placenta for the treatment of patients with factor XIII deficiency and wounds after injury or surgery, and a side-fraction of this experiment yielded human placental lactogen. This experience strongly influenced him to

focus his research on the systematic isolation and characterization of placental, endometrial and pregnancy serum proteins. These studies have greatly supported our knowledge on pregnancy-related proteins and their application in the diagnosis of pregnancy complications (1).

Dr. Hans Bohn processed large amounts of human placental, amniotic fluid and serum specimens and utilized combinations of classical fractionation techniques to isolate more than 50 proteins, which he named sequentially. He characterized these proteins for their physico-chemical characteristics, and then developed specific rabbit antisera against them for further protein purification and for the development of immunoassays to determine these proteins' diagnostic significance. In collaboration with scientists around the world, Dr. Bohn also determined the amino acid sequence as well as biological functions of many of these. Among the proteins he isolated were Placental Protein (PP) 4 (annexin-V), PP5 (tissue factor pathway inhibitor-2, TFPI-2), PP10 (plasminogen activator inhibitor-2, PAI-2), PP12 (insulin-like growth factor binding protein-1, IGFBP-1) and PP13 (galectin-13), which were subsequently identified to be important regulators of the fundamental processes in pregnancy (1).

Dr. Bohn's collaboration with Professor Gábor N. Than (University of Pécs, Pécs, Hungary) had a fundamental impact on the cloning, sequencing, structural, and molecular biological characterization of a large number of PPs including PP13, and their pioneering collaborative research significantly improved our understanding on the biological role and diagnostic significance of these proteins in pregnancy complications and malignancies. Beyond these scientific discoveries, their friendship and close collaboration strongly inspired a new generation of scientists. The existing knowledge and advancements in the field were summarized in their book entitled *Advances in Pregnancy-Related Protein Research*, co-written with Dr. Dénes G. Szabó in 1993 (2) (**Figure 1B**).

For Dr. Bohn's founding research and discovery of PP14 (glycodelin), he shared the prestigious Abbott Award in 1997. His remarkable contributions to placental and pregnancy-related protein research were published in 198 research articles. Dr. Bohn continued to contribute to placental protein research after his retirement, and closely followed the studies implementing novel molecular and cellular biological techniques on the proteins he isolated, leading to further discoveries and improvements in clinical diagnostics and patient care (1).

Dr. Hans Bohn was an exceptional scientist, an enthusiastic catalyzer of collaborations and friendships who inspired many peers and followers. He was a wonderful, kind and charismatic person, a silent giant, who will be greatly missed.

THE DISCOVERY AND MOLECULAR CHARACTERIZATION OF PP13

ISOLATION, PURIFICATION AND PHYSICO-CHEMICAL CHARACTERIZATION OF PP13

Dr. Bohn's scientific vision combined with his thorough work using state-of-the-art methods of the 70's and 80's yielded the discovery of 26 soluble placental tissue proteins, among which PP13 was purified, physico-chemically characterized and described in 1983 (3). Dr. Bohn homogenized term placental tissues and

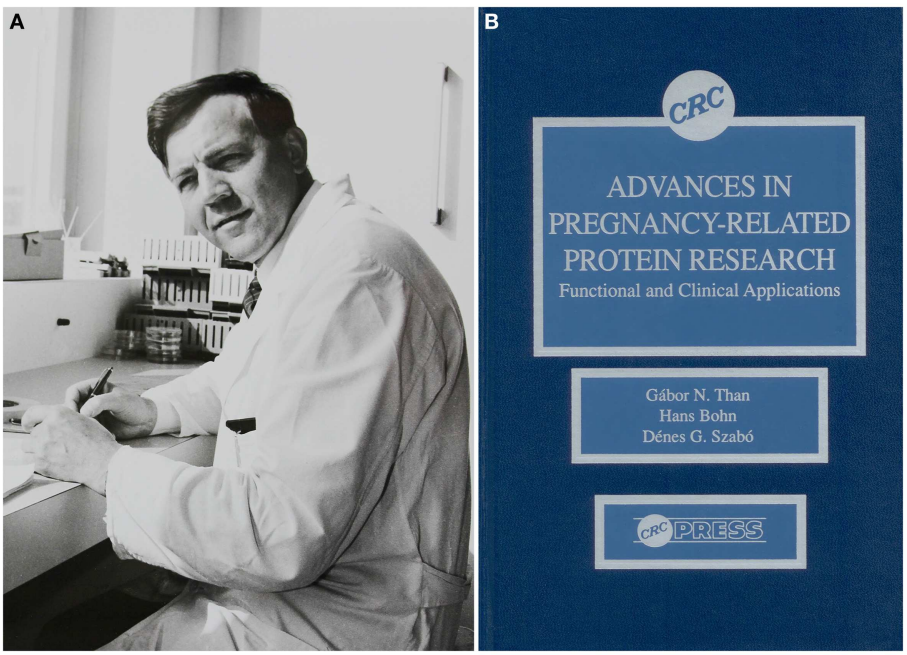


FIGURE 1 | Dr. Hans Bohn and his scientific legacy in pregnancy-related protein research. **(A)** Dr. Hans Bohn is depicted in his Protein Laboratory at Behringwerke (Marburg/Lahn, Germany), where he discovered and isolated more than 50 placental, endometrial and pregnancy serum proteins including Placental Protein 13 (PP13), and developed specific antisera and immunosassays for them between 1965 and 1989. The photo was obtained as a courtesy from Gabriele Bohn. **(B)** A book co-written by Hans Bohn, Gábor N. Than and Dénes G. Szabó published in the USA summarized the existing knowledge in the research field in 1993.

fractionated the protein extracts by a step-wise process that included Rivanol and ammonium sulphate fractionation, gel-filtration, ethanol precipitation, and immunoabsorption techniques. The resulting PP13 protein was >99% pure, and SDS-polyacrylamide gel electrophoresis found it to be composed of two identical ~16 kDa subunits held together by disulphide bonds. The carbohydrate content of PP13 was found negligible, a feature that later became important in understanding its functions (2, 3). Utilizing the purified PP13-specific rabbit antiserum, an electroimmunoassay and an Ouchterlony's gel-diffusion test found an average amount of 3.7 mg PP13 in human term placentas and detected PP13 solely in the placenta among fetal and adult tissues. The sensitivity of a radioimmunoassay (0.8 ng/ml) that utilized this rabbit antiserum was insufficient to detect PP13 in maternal and fetal serum or in amniotic fluid (2–4). Indeed, this is in accord with the 0.1–0.4 ng/ml concentration range of PP13 in maternal blood as was discovered with sandwich ELISA techniques using mouse monoclonal antibodies a decade later (5).

CLONING, SEQUENCING AND INITIAL MOLECULAR BIOLOGICAL ANALYSIS OF PP13

Professor Gábor N. Than's team in Hungary isolated the full-length cDNA (GenBank Acc. No.: AF117383) encoding PP13 from a human placental cDNA expression library using Dr. Bohn's rabbit anti-PP13 antiserum. Sequence analysis revealed a 578 bp insert with a 417 bp open reading frame encoding a 139 amino-acid protein (6). The predicted molecular mass and amino-acid composition of the cloned protein corresponded with Dr. Bohn's estimate of the purified PP13 protein. A BLAST search of nucleotide and protein sequences showed PP13 to be homologous to members of the beta-galactoside binding galectin family, and computer analysis detected 8 out of 16 invariant residues in galectins conserved in PP13, suggesting its place in the galectin family (6). The highest sequence similarity of PP13 was found with the eosinophil Charcot–Leyden Crystal (CLC) protein, which forms crystals at sites of eosinophil-associated inflammation (7), a phenomenon similar to that found with PP13 immunostainings on first trimester decidual tissue sections (8). Interestingly, using a functional assay and highly sensitive NMR measurements, the native and recombinant PP13 was observed to have weak lysophopholipase activity (6, 9) similar to CLC protein. This lysophopholipase activity was also inferred from the release of free fatty acids from cultured primary trophoblasts exposed to PP13 (5). However, it was later revealed that this enzymatic activity of CLC protein is caused by an associated lysophospholipase (10), and further research is required to understand whether the lysophospholipase activity of PP13 is intrinsic or indeed related to an associated protein.

DETAILED STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF PP13 AS A GALECTIN

The homology of PP13 to members of the galectin family inspired further structural and functional investigations. Homology modelling based on the "jelly-roll" fold of galectins observed by X-ray crystallography revealed the 3D model of PP13, which was deposited into the Protein Data Bank (Acc. No.: 1F87) (11). This fold consists of five- and six-stranded β-sheets linked by two α-helices characteristic for "prototype" galectins (**Figure 2A**). Out of the eight consensus residues in the galectin carbohydrate-recognition domains (CRDs), four identical and

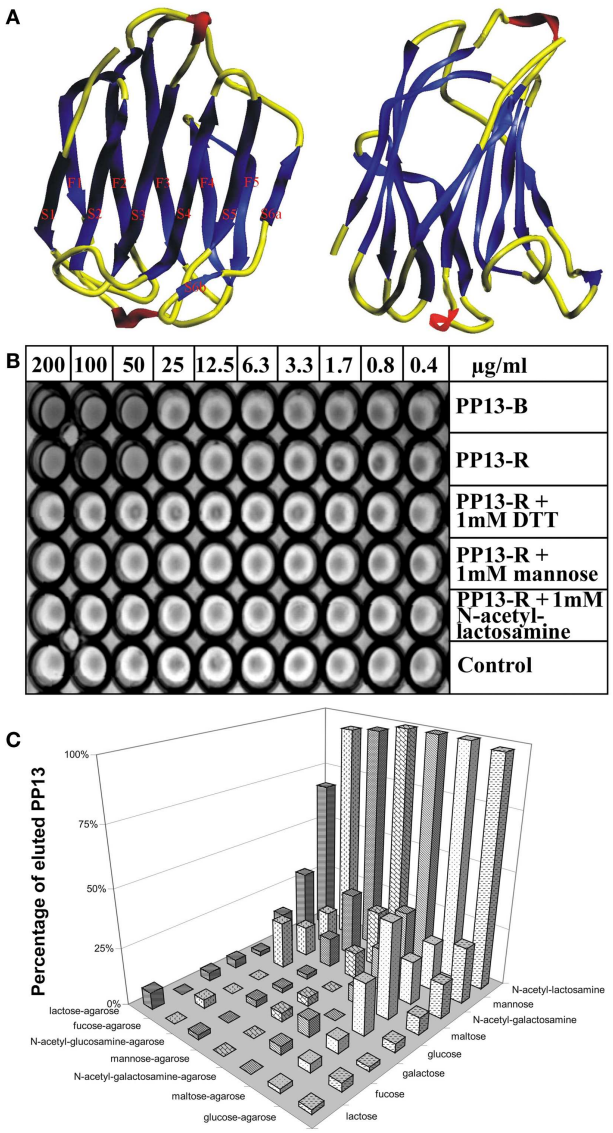


FIGURE 2 | The structural and functional basis for renaming of PP13 as galectin-13. **(A)** The figure depicts the "jelly-roll" fold of PP13, which consists of five- and six-stranded β-sheets linked by two α-helices. **(B)** *In vitro* assays revealed haemagglutinating activity of placenta-purified (PP13-B) and recombinant (PP13-R) PP13. In non-reducing conditions, both PP13-B and PP13-R agglutinated human erythrocytes at ≥50 µg/ml protein concentrations. The haemagglutinating ability of PP13-R was inhibited by dithiothreitol or sugars at ≥1 mM concentrations. **(C)** The strength of PP13-R binding to sugar-coupled agarose beads increased from lactose-agarose to glucose-agarose (left to right). PP13 bound to sugar-coupled agarose beads was competitively eluted by sugars (1 M) listed back to front. PP13 had the best eluting capacity (sugar affinity) for *N*-acetyl-lactosamine, mannose and *N*-acetyl-galactosamine. Figure **(A)** was published in Ref. (11), and Figures **(B,C)** in Ref. (9). Kind permission for the reuse of figures was obtained from Oxford University Press **(A)** and John Wiley and Sons **(B,C)**.

three conservatively substituted residues were found in PP13. Computational docking simulations showed that the PP13 CRD may bind sugars, e.g. *N*-acetyl-lactosamine and lactose, similar to most galectins (**Figure 2A**). Since these lines of evidence demonstrated that PP13 is a novel galectin, it was designated as galectin-13 (11).

Galectins constitute a subgroup among the superfamily of lectins, carbohydrate-binding proteins that are important in the regulation of cellular interactions with cells, the extracellular matrix and pathogens. They bind to glycans residing on glycoproteins, glycolipids and other glycoconjugates that constitute a complex array coined the "glycome", which stores orders of magnitude larger biological information than nucleic acids and proteins store. For example, the numbers of 4,096 hexanucleotides and 64 million hexapeptides are far surpassed by the 1.44×10^{15} isomer quantity of hexasaccharides (12). Galectins can bind to a diverse set of glycoconjugates, and therefore, they have pleiotropic functions in a variety of key biological processes including signal transduction, cell differentiation, apoptosis, or cell adhesion. Moreover, galectins are positioned at the cross-roads of adaptive and innate immune functions as they are key determinants of acute and chronic inflammation, immune tolerance and host-pathogen interactions (12–18).

Triggered by the recognition that these galectin functions are important determinants of healthy pregnancies (19), the detailed molecular characterization of PP13 allowed greater insight into its function in the placenta during pregnancy. Similar to other galectins, PP13 also hemagglutinated human erythrocytes *in vitro* (**Figure 2B**). Furthermore, sugar-binding assays showed the affinity of PP13 for carbohydrates widely expressed in the human placenta (**Figure 2C**), particularly for *N*-acetyl-lactosamine, mannose and *N*-acetyl-galactosamine (9) as already predicted by molecular modelling (11). Assay performance in reducing conditions decreased the hemagglutinating (**Figure 2B**) and sugar binding activity of PP13, suggesting that homodimerization of PP13 subunits by disulphide bridges are important for these functions (9).

Through placental immunostaining, PP13 was found in the cytoplasm and brush border membrane of the syncytiotrophoblast. Using affinity chromatography and mass spectrometry, annexin II and beta/gamma actin were identified as ligands of PP13, a finding that was also supported by high colocalization of PP13 with annexin II in the syncytiotrophoblast brush border membrane. These results suggested the galectin-like externalization of PP13 to the cell surface by extracellular vesicles containing actin and annexin II (9). It has to be elucidated whether, similar to other galectins (20), PP13 may bind to glycoconjugates on cell surfaces and form "galectin-glycan lattices" that are important in cellular interactions and signaling.

THE INTERACTIONS OF PP13 WITH ABO BLOOD GROUP ANTIGENS

As an indirect sign of PP13 binding to glycoconjugates on cell surfaces, placental immunostainings showed PP13 positivity of maternal and fetal erythrocytes, confirming the *in vivo* erythrocyte-binding of PP13 (21). These results were consistent with the tendency of PP13, similar to other galectins, to bind beta-galactosides that are present at terminal positions on ABO blood-group antigens (9, 11, 22, 23). Flow cytometry measurements further demonstrated the binding of PP13 but not its CRD-truncated variant to erythrocytes, proving that PP13 binding is mediated by its CRD (**Figure 3A**). PP13 binding was similar in intensity to blood group A and O erythrocytes, while PP13 had the weakest binding intensity to blood group B and the strongest binding intensity to blood group AB erythrocytes (**Figure 3A**). Similar to other galectins (24, 25), PP13 binding to various ABO blood group erythrocytes changed dynamically with increasing PP13 concentrations (**Figure 3C**).

Computational studies have indicated that the structural basis for ABO blood group antigen binding includes the following: (1) three out of four residues in the core galectin CRD involved in disaccharide-binding are conserved in PP13 (11, 23); (2) PP13 has a similar "B-site" involved in ABO antigen binding as human galectins that exhibit ABO blood group antigen binding (21, 25); and (3) PP13 accommodates blood group H trisaccharide in its CRD similar to a fungal galectin's CRD (21) (**Figure 3B**).

It is interesting to note that ABO blood group antigens are oligosaccharides attached to cell-surface glycoconjugates on epithelia, endothelia and erythrocytes, which might have been evolutionarily advantageous in conferring resistance against certain pathogens (26). The gene encoding for the enzymes that catalyze the transfer of these oligosaccharides to cell-surface glycoconjugates emerged in primates (22, 27). If the evolution of the ABO blood group system and genes encoding for PP13 and closely related galectins was somehow associated, that would suggest a potential functional relevance of PP13 binding to ABO blood group antigens.

THE EVOLUTION AND HUMAN DISEASE-RELATED POLYMORPHISMS OF *LGALS13*

EVOLUTIONARY ANALYSES OF GENES ENCODING FOR PP13 AND CLOSELY RELATED GALECTINS

An evolutionary study presented compelling evidence that a cluster of galectin genes, including *LGALS13* that encodes PP13, emerged on chromosome 19 in anthropoid primates, which differ from other primates by having larger brains and longer gestations (23). The analysis of this galectin cluster in the available genome assemblies revealed frequent gene duplication, inversion and deletion events characteristic of repeat-mediated "birth-and-death" evolution, a process that leads to novel phenotypes in species adapting to their changing environment (28). Detailed analysis showed that transposable long interspersed nuclear elements (LINEs) were positioned at the majority of boundaries of large inversions and gene duplication units, suggesting that LINEs had mediated the rearrangements within this cluster. Genes in this cluster have four-exon structures as other "prototype" galectin genes. Of the two major clades in the cluster, one contains genes with predominant placental expression including *LGALS13* and related pseudogenes. Of note, *LGALS13* was only found in Old World monkeys and apes. Sequence analyses of 24 newly determined sequences and 69 annotated sequences in 10 anthropoid species indicated functional diversification among PP13 and related galectins during evolution as can be inferred from the amino acid replacements in their CRDs (23).

Sequence comparison showed a strong conservation of more than half of the residues of PP13 and cluster galectins

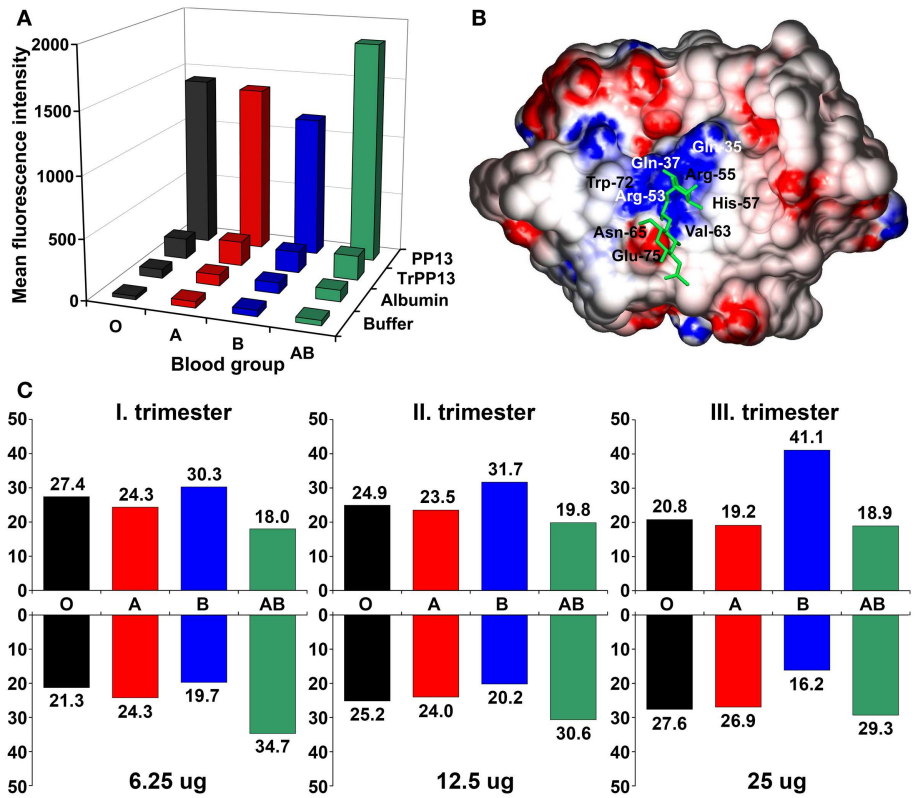


FIGURE 3 | The differential binding of PP13 to ABO blood group antigens *in vitro* and *in vivo*. **(A)** Flow cytometry showed that recombinant PP13 binding to erythrocytes was specific and mediated by its carbohydrate-recognition domain (CRD) since recombinant truncated PP13 (TrPP13) bound negligibly similar to bovine serum albumin (BSA). PP13 had the strongest affinity to blood group AB erythrocytes and weakest affinity to blood group B erythrocytes. **(B)** Surface representation of PP13 complexed with blood group H trisaccharide (green). Blue and red colors indicate positive and negative electrostatic potentials on the molecular surface, respectively. The binding groove of the CRD contains a central positive channel flanked by negative regions. **(C)** The relative binding of PP13 (lower panel) to different ABO blood group erythrocytes dynamically changed and inversely mirrored the relative serum PP13 concentrations (upper panel) in women with different ABO blood groups with advancing gestation from the first to third trimesters. All figures were published in Ref. (21). Kind permission for the reuse of figures was obtained from the Public Library of Science.

predominantly located in the protein cores that determine their overall structure, whereas residues on their surface, especially in the loop regions, have undergone rapid evolution (23) (**Figure 4A**). From the eight conserved residues in the galectin CRD, four (positions: 53, 65, 72, and 75) that are key in the overall sugar binding form a pocket in one side of the CRD and were under purifying selection in PP13 and cluster galectins. The other four residues (positions: 55, 57, 63, and 77) on the opposite side of the CRD had more variability among cluster galectins, with frequent replacements in several lineages following gene duplications including K>T77 in PP13 (**Figure 4B**). As these latter four residues are crucial for galactose or glucose binding, the structural differences might have resulted in differing functions between PP13 and other cluster galectins. Indeed, functional experiments with human recombinant PP13 and five other galectins proved the different sugar-binding profiles of these investigated proteins (23).

A large number of pseudogenes in the studied species were found in the cluster (23). These emerged by the deletion of exons, mutations of the exon–intron boundaries, and by the introduction of one or more in-frame premature stop codons. As a striking observation, 18 out of the identified 38 pseudogene variants contained the "163C>T" DNA variant leading to the introduction of a premature stop codon at the site encoding residue 55, which may result in truncated proteins of 54 amino acids that lack the entire CRD. In fact, functional experiments proved that this "163C>T" DNA variant results in the expression of a truncated PP13 that cannot bind carbohydrates (23). The question why nature utilized the same process to silence so many galectin genes in certain lineages, including *LGALS13* in baboon, Bornean orangutan and Sumatran orangutan, remains unanswered.

DNA VARIANTS IN HUMAN *LGALS13*

Somewhat related to evolutionary selection, a total of 933 *LGALS13* DNA variants have been already identified in the genomes of 1,092 individuals from 14 populations by the "1000 Genomes Project"[1]. These included mostly upstream ($n = 277$), downstream ($n = 261$) or intron ($n = 240$) variants. Besides these, non-coding transcript ($n = 98$) or exon ($n = 56$) variants and missense variants ($n = 56$) were frequently detected. Nevertheless, the

[1]www.1000genomes.org

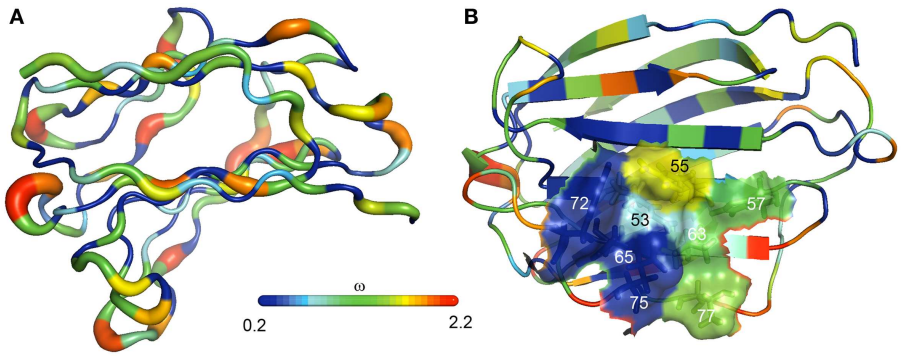


FIGURE 4 | The evolution of PP13 and closely related galectin genes in the chromosome 19 cluster. **(A)** Evolutionary changes leading to structural diversification in chromosome 19 cluster galectins are depicted on the molecular backbone of PP13 (left). The width and color of the ribbon varies in proportion with site-specific ω values (d_N/d_S; $\omega < 1$, purifying selection; $\omega > 1$, positive selection) for chromosome 19 cluster galectins. ω, indicated by the color spectrum on the bar, is the smallest along β-strands and highest in loop regions. **(B)** The same color coding shows that four residues in the PP13 CRD (residues: 53, 65, 72, 75) have been conserved in chromosome 19 cluster galectins, while the other four residues on the opposite side of the CRD (residues: 55, 57, 63, 77) show more evolutionary changes among these galectins.

"1000 Genomes Project" has not provided information regarding the association of *LGALS13* DNA variants with disease susceptibility. In search of *LGALS13* DNA polymorphisms by targeted genotyping studies, the association of certain *LGALS13* DNA variants with severe complications of pregnancy has been identified. These studies utilized whole blood DNA samples obtained from pregnant women and their neonates in a South African cohort of the Black and Coloured population, and the following DNA variants were revealed for *LGALS13*: (1) variants in Exon 3 including single nucleotide polymorphisms (SNPs) and a single nucleotide deletion, which latter causes a frame-shift in the open reading frame, leading to the formation of a premature stop codon and a truncated protein (221delT); (2) SNPs in Introns 2 and 3 including an intron boundary polymorphism that is associated with alternative splicing and the deletion of Exon 2; and (3) an SNP in the promoter region (29–34).

Of interest, in a prospective cohort of 450 low-risk primigravid women of Black and Coloured origin, carrying the naturally occurring "221delT" mutation conferred a 2.27-fold relative risk for preterm labor (34). The frequency of heterozygous carriers of this mutation was higher in the group of women with preterm preeclampsia (5.7%) than in controls (2.4%), and no individuals were found to be homozygous. In another study conducted on the same population, there was a significant association for this mutation and preeclampsia, particularly among Coloured women (33). These results suggest that the placental expression of a functionally impaired, truncated PP13 may put women at increased risk for severe pregnancy complications. However, so far no polypeptide derived from the "221delT" DNA polymorphism could be identified in placental or body fluid samples, most likely due to the rapid degradation or insufficient immunodetection of such a protein because of the anticipated major misfolding (35).

The "-98A/C" promoter polymorphism was also associated with the risk of preeclampsia (31, 33, 34). In a prospective cohort of low-risk pregnant women, controls were in the Hardy-Weinberg equilibrium, while cases deviated from that, and the heterozygous A/C genotype appeared to be protective against preeclampsia (31). Another study comprising the same population found a significant difference between "-98A/C" genotype distributions in patients with placental abruption and controls among Coloured women (33). These results suggest that the "-98A/C" promoter polymorphism may negatively affect *LGALS13* expression and PP13 functions.

THE EXPRESSION PATTERN OF PP13 IN HUMANS

WIDE-SCALE EXPRESSION PROFILING OF PP13 IN HUMAN TISSUES

Besides the studies on *LGALS13* DNA variants, the investigations on the expression patterns of PP13 have revealed interesting insights. The study describing the cloning of PP13 also presented compelling evidence for the predominant placental expression of PP13 in the human body (6). In fact, the expression profiling of human adult and fetal, normal and tumorous tissues by Western blot (26 tissues) and Northern blot (16 tissues) detected a 16kDa PP13 immunopositive band in extracts of human term placentas, and unique placental PP13 mRNA expression, respectively. These findings were supported by GenBank evidence of only placental expressed sequence tags for *LGALS13*. Later, the wide-scale expression profiling of *LGALS13* and related chromosome 19 cluster galectin genes using qRT-PCR on a human 48-tissue cDNA panel confirmed the predominant placental expression of *LGALS13* (**Figure 5A**) (23).

PLACENTAL EXPRESSION PROFILING OF PP13 IN NORMAL PREGNANCIES

In human villous placental tissues at term, immunohistochemistry and immunofluorescence consistently found predominant PP13 positivity of the syncytiotrophoblast and villous capillary endothelium but not the cytotrophoblasts (8, 9, 21, 23, 36, 37). *In situ* hybridization (23) detected PP13 mRNA expression in the same placental cells, further confirming the specificity of the immunostainings (**Figure 5B**). The same PP13 expression pattern was found in the placentas of Old World monkeys, suggesting the conservation of PP13 expression during evolution (**Figure 5C**). Moreover, *in situ* hybridization revealed *LGALS13* expression in the amnion

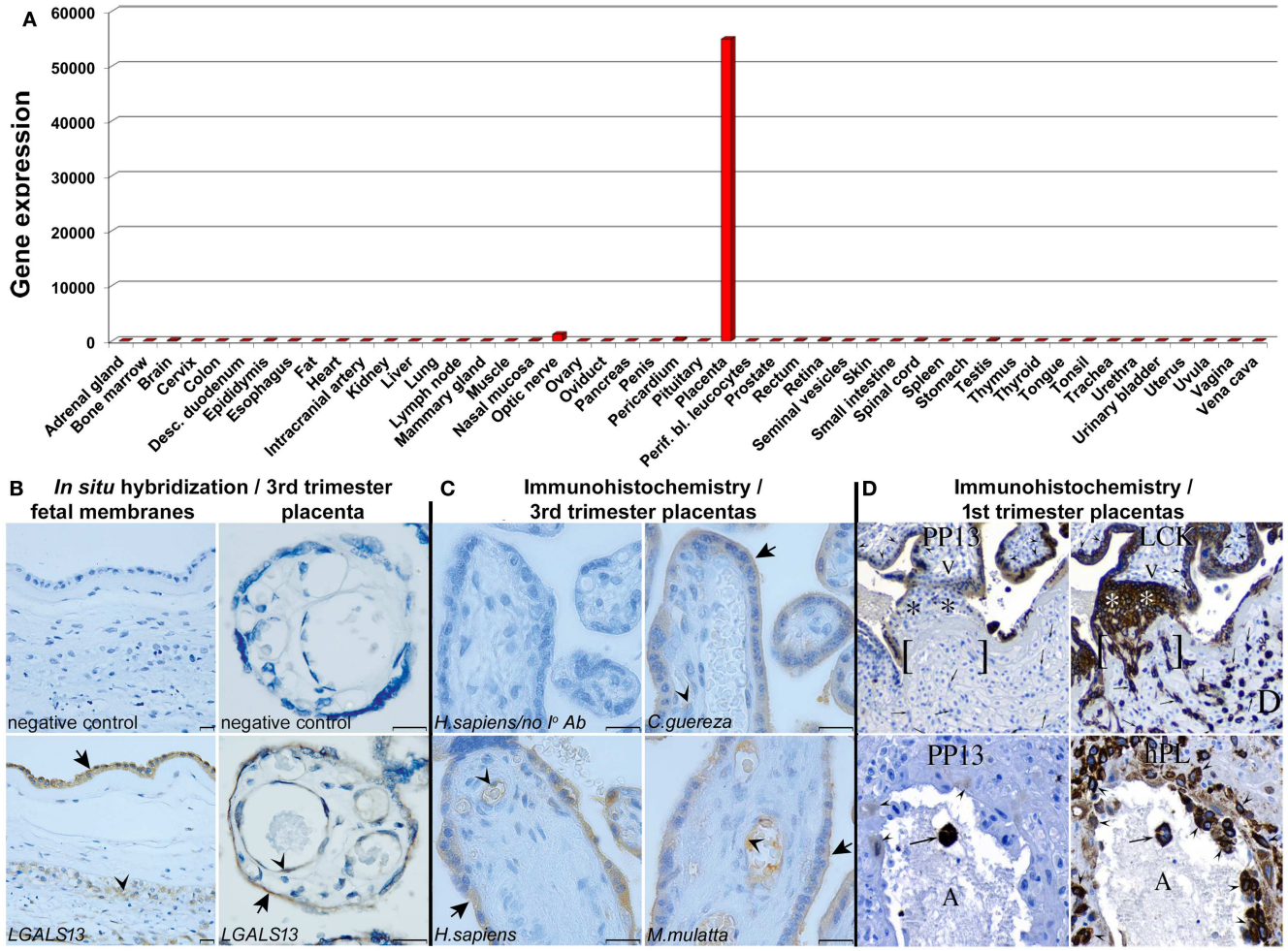


FIGURE 5 | PP13 expression profiling. **(A)** qRT-PCR on a human 48-tissue cDNA panel uncovered that *LGALS13* is predominantly expressed by the placenta. The y axis shows gene expression levels [$2^{(-\Delta Ct)}$]. **(B)** *In situ* hybridization revealed *LGALS13* expression in the amnion (arrow) and chorionic trophoblasts (arrowhead) in the fetal membranes in normal term pregnancy (left). In normal term placenta, *LGAL13* is predominantly expressed by the syncytiotrophoblast (arrow) and endothelium (arrowhead) (right). Scale bars: 20 μm. **(C)** PP13 immunostaining is conserved in the syncytiotrophoblast, its apical membrane (arrows), and the endothelia (arrowheads) of human and anthropoid primate placentas. (Scale bars: 20 μm.) **(D)** (Upper panel) Serial sections of a 15 week junctional complex stained for PP13 and low molecular weight cytokeratin (LCK). LCK immunostained epithelial cells, including cytotrophoblasts (arrowheads), anchoring trophoblasts (*), early infiltrating trophoblasts [ ], and invasive trophoblasts (arrows) in the decidua (D). Mesenchymal villous core cells (V) and decidual cells were negative. PP13 immunostaining was found in the syncytiotrophoblast. The cytotrophoblasts (arrowheads), anchoring trophoblasts (*), early infiltrating trophoblasts [ ], and the invasive trophoblasts (arrows) in the decidua (D) were negative. (Lower panel) Serial sections of 8 week maternal spiral arterioles immunostained for PP13 and human placental lactogen (hPL). All the decidual invasive, intravascular and endovascular trophoblasts (arrowheads), and a single luminal (A) syncytiotrophoblast (arrow) were stained for hPL. The monoclonal anti-PP13 antibody did not stain decidual invasive trophoblasts, lightly stained endovascular trophoblasts (arrowheads), and it intensely stained luminal syncytiotrophoblasts (arrow). Figure **(A)** represents data published in Ref. (23). Figures **(B,C)** were published in Ref. (23). Figure **(D)** was published in Ref. (8). Kind permission for the reuse of the figures was obtained from the National Academy of Sciences of the United States of America **(A-C)** and SAGE US **(D)**.

and chorionic trophoblasts in the fetal membranes. These findings showed PP13 expression predominantly in locations where maternal-fetal immune interactions occur.

In the first trimester, PP13 was immunolocalized to the syncytiotrophoblast and multinucleated luminal trophoblasts within converted decidual spiral arterioles (8). Villous cytotrophoblasts and invasive extravillous trophoblasts in the anchoring trophoblastic columns were immunonegative (**Figure 5D**). The syncytiotrophoblastic staining intensity declined with gestational age, being the strongest between 6 to 8 weeks of gestation. This study also confirmed previous findings in term placental tissues on predominant, diffuse cytoplasmic and also nuclear immunopositivity of the syncytiotrophoblast.

EXPRESSION PROFILING OF PP13 DURING VILLOUS TROPHOBLAST DIFFERENTIATION AND FUSION

Based on this immunohistochemical evidence, it was hypothesized that PP13 expression is related to the biochemical and

morphological differentiation and syncytialization of the villous trophoblast (36). These processes are primarily governed by cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA), which regulate the resetting of the transcriptional program during the shift from cytotrophoblast into the syncytiotrophoblast (38–40). The resulting unique transcriptome of the syncytiotrophoblast (41) controls the production of placental hormones, immune proteins and other proteins predominantly expressed by the placenta, which support pregnancy. Besides the exchange of feto-maternal gas, nutrients and waste, and the hormonal regulation of fetal development, the syncytiotrophoblast is also active in generating immune tolerance between the mother and her semi-allogeneic fetus (2, 42, 43).

In vitro assays with trophoblast-like BeWo cells demonstrated that indeed *LGALS13* expression is related to trophoblast fusion and syncytium formation induced by the cAMP-analogue Forskolin, and that a PKA inhibitor could block BeWo cell syncytialization and *LGALS13* expression (44). A recent study confirmed these findings in BeWo cells, and demonstrated the syncytialization and differentiation-related *LGALS13* expression in primary villous trophoblasts (45). The evolutionary and functional investigations of the trophoblastic regulatory mechanisms of *LGALS13* expression showed that promoter evolution and the insertion of an anthropoid-specific LINE element into the 5′ untranslated region (UTR) of an ancestral gene introduced binding sites for several transcription factors (e.g. ESRRG) key in villous trophoblastic gene expression, leading to the gain of placental expression of *LGALS13* and related chromosome 19 cluster galectin genes (43, 45). Glial cell missing-1 (GCM1), the transcription factor that governs villous trophoblast fusion and syncytialization (46), was shown to facilitate the expression of ESRRG and other key villous trophoblastic transcription factors, and thus, to indirectly promote the placental expression of *LGALS13* and cluster galectin genes. In addition, DNA methylation was also observed to regulate developmental expression of *LGALS13* and cluster galectin genes (45).

PLACENTAL ASPECTS OF PREECLAMPSIA

It is important that the impairment of villous trophoblast syncytialization characterized by the decreased trophoblastic expression of GCM1 and syncytin-1, a fusogenic protein regulated by GCM1, has been observed in preeclampsia (47, 48), an obstetrical syndrome originating from impaired early placentation (49, 50). Preeclampsia is diagnosed by new-onset hypertension and proteinuria after 20 weeks of gestation, and it is a major cause of maternal, fetal and neonatal morbidity and mortality (51). Moreover, this syndrome consists of various subtypes defined by gestational age (e.g.: early-onset: <34th weeks; preterm: <37 weeks; and term: ≥37 weeks) (52, 53). Early-onset and preterm preeclampsia are severe subtypes of the disease that require premature delivery and are more often associated with intrauterine growth restriction (IUGR), hemolysis, elevated liver enzymes, and low platelet count (HELLP) syndrome, and fetal death, while term preeclampsia may be severe or mild in its clinical presentation (51–55). Although the molecular pathways of preeclampsia are incompletely understood, it appears to be associated with impaired placentation as the only definite therapy of preeclampsia is still the delivery of the fetus and the removal of the placenta (50, 51, 53). It is also evident that heterogeneous causes can trigger early placental pathologic events, and that these are followed by the onset of the terminal pathway of preeclampsia in a later stage, leading to the subsequent clinical onset of the symptoms (50, 51, 56).

Several studies providing histopathologic or transcriptomic evidence have suggested that the placental pathogenesis of preeclampsia may differ in its subtypes as more pronounced differences could be observed in early-onset than late-onset preeclampsia when compared to gestational age-matched controls (57–61). In line with these findings, the extent of histopathologic changes in the placental bed was most extensive in early-onset preeclampsia, especially in cases associated with IUGR. These abnormal findings were consistent with impaired trophoblast invasion into the uterine tissues and the consequent abnormal remodelling of the maternal spiral arterioles, placental pathologic events that occur in the first trimester (62, 63).

Previously it was thought that impaired early placentation is associated with placental hypoxia (64); however, it has recently become evident that the resulting fluctuation in uterine blood supply leads to placental ischemic injury, causing oxidative stress, pro-inflammatory conditions, and apoptosis (65–67). In response, the placenta expresses and releases increased amounts of anti-angiogenic factors, pro-inflammatory cytokines and aponecrotic syncytiotrophoblast microvesicles. The latter might induce maternal anti-angiogenic and exaggerated systemic pro-inflammatory states, hypertension and proteinuria (50, 51, 53, 66, 68–70).

DECREASED PLACENTAL PP13 EXPRESSION IN PREECLAMPSIA

In this context, *LGALS13* expression was found to be down-regulated in villous placental tissues in preeclampsia. This was first described for preterm preeclampsia compared to gestational age-matched controls at the time of disease, and this phenomenon was suggested to be associated with problems in trophoblast syncytialization (36) (**Figures 6A,B**). Since then, other studies confirmed these findings in the third trimester, including one that investigated placental *LGALS13* expression at the time of disease (71) and another that looked for syncytiotrophoblastic *LGALS13* expression in laser captured specimens in the first trimester (72). The latter study detected decreased *LGALS13* expression in the syncytiotrophoblast from chorionic villus samples obtained at 11 weeks of gestation in women who subsequently developed preeclampsia compared to controls. Such first trimester down-regulation of placental *LGALS13* expression may be one of the earliest pathological indications for the subsequent development of preeclampsia.

A recent study has revealed the possible molecular mechanisms leading to decreased placental *LGALS13* expression in women with severe preterm preeclampsia. It was found that in this subform of preeclampsia there is a decreased placental expression of *GCM1* and *ESRRG*, genes encoding transcription factors that regulate trophoblastic *LGALS13* expression (45). Moreover, functional experiments showed that the knock-down of *GCM1* in BeWo cells led to the down-regulation of ESRRG and other transcription factors that regulate *LGALS13* expression. Accordingly, it was concluded that there is a decreased GCM1-mediated trophoblast fusion and trophoblastic gene expression in severe preterm preeclampsia that leads to the down-regulation of *LGALS13*. Furthermore,

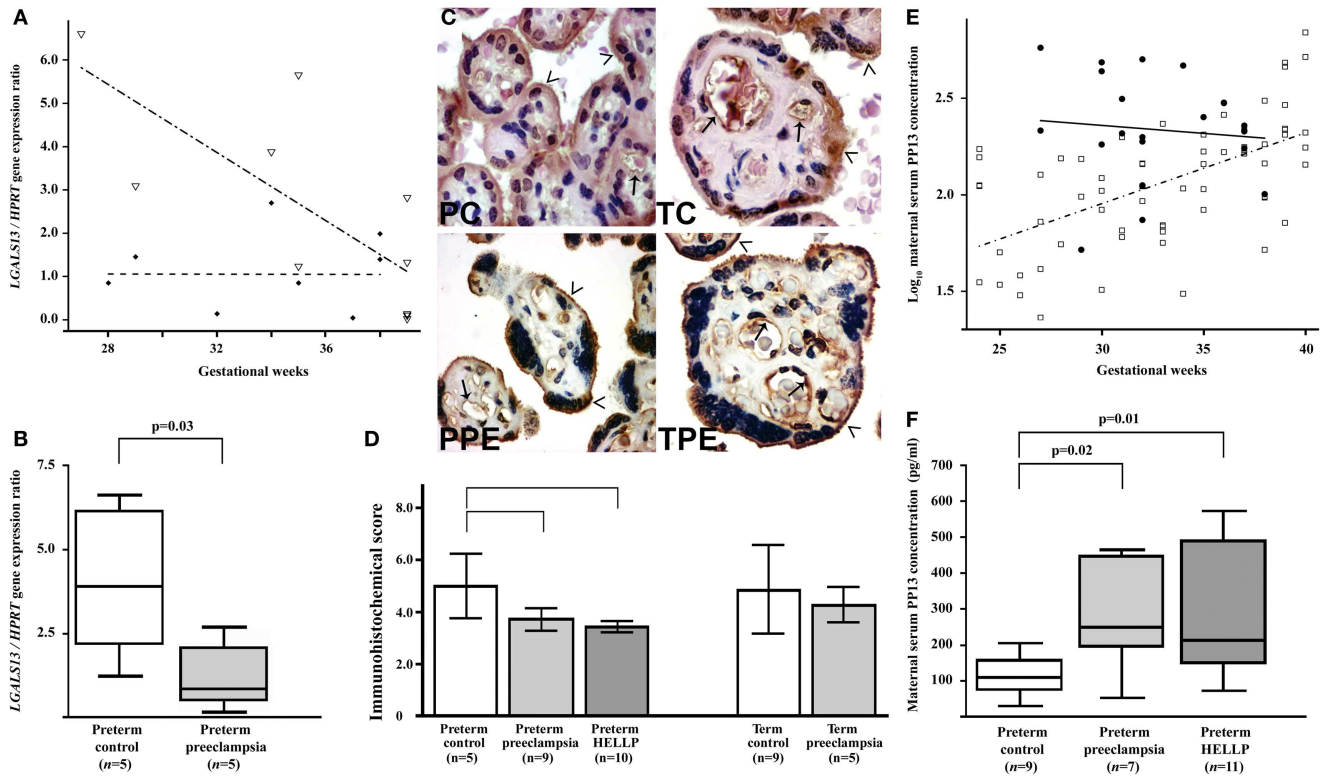


FIGURE 6 | Decreased placental *LGALS13* expression and increased trophoblastic PP13 shedding in preterm preeclampsia. **(A)** Relative *LGALS13* expression decreases with advancing gestational age in controls (open triangles), while it is constantly low in patients with preeclampsia (diamonds). **(B)** Relative *LGALS13* expression is lower in women with preterm preeclampsia than in controls. **(C)** Syncytiotrophoblastic PP13 immunostaining is decreased in preterm preeclampsia compared to controls. PC: preterm control, 35 GW (weeks of gestation); TC: term control, GW38; PPE: preterm preeclampsia, GW29; TPE: term preeclampsia, GW37. The endothelium (arrows) is also PP13 immunopositive in all sections. The microvillous membrane (open arrowheads) stains moderately for PP13 in controls, while it is strongly PP13 immunopositive in preeclampsia. 500× (left) or 700× (right) magnification. **(D)** The immunohistochemical score of the syncytiotrophoblast is higher in preterm controls than in preterm preeclampsia, with or without HELLP syndrome, while it is not different between cases and controls at term. **(E)** Maternal serum $\log_{10}$ PP13 concentrations increase as a function of gestational age in control women (open rectangle), while these do not correlate with gestational age in patients with preeclampsia (filled circle). The regression line for $\log_{10}$ PP13 concentrations is significantly different in the two groups. **(F)** Median maternal serum PP13 concentrations are higher in women with preterm preeclampsia, with or without HELLP syndrome, than in controls. All the figures were published in Ref. (36). Kind permission for the reuse of figures was obtained from Springer Science+Business Media.

the differential methylation of *LGALS13* was also found in the villous trophoblast in preterm preeclampsia, which may interfere with *LGALS13* expression, suggesting that potential additional disease-mechanisms may account for the trophoblastic pathology in preterm preeclampsia (45).

ALTERED PLACENTAL LOCALIZATION AND INCREASED SHEDDING OF PP13 IN PREECLAMPSIA

In accord with gene expression data, immunostainings revealed that cytoplasmic PP13 positivity of the syncytiotrophoblast was weaker in preeclampsia compared to controls, particularly in preterm cases. Similar changes were also observed at the time of disease in preterm HELLP syndrome (36) (**Figures 6C,D**). Paradoxically, PP13 immunostaining of the syncytiotrophoblast microvillous membrane was stronger in preeclampsia and HELLP syndrome compared to controls (**Figures 6C**, **7A**). Syncytial cytoplasmic protrusions and membrane microvesicles shed from the syncytiotrophoblast stained strongly for PP13 in preeclampsia (**Figure 7A**). It was suggested that the increased release of PP13 positive microvesicles from the syncytiotrophoblast may lead to elevated maternal serum PP13 concentrations in preterm preeclampsia and HELLP syndrome before or at the time when the clinical symptoms of preeclampsia appear (**Figures 6E,F**) (36, 37).

The subcellular redistribution of PP13 in the syncytiotrophoblast was further observed by confocal imaging of placental samples from patients with preeclampsia and HELLP syndrome compared to gestational age-matched controls (73). In all study groups, PP13 highly colocalized with placental alkaline phosphatase, a glycophosphatidylinositol-anchored lipid raft-resident protein. However, there was also a high degree of colocalization of PP13 with CD71, a non-raft plasma membrane protein, which decreased in preterm preeclampsia and HELLP syndrome. In contrast, the colocalization of PP13 with cytoskeletal actin, a protein earlier found to bind to PP13 with high affinity (9), was increased in all patient groups compared to controls. These results indicated that the translocation of PP13 to the juxta-membrane region of

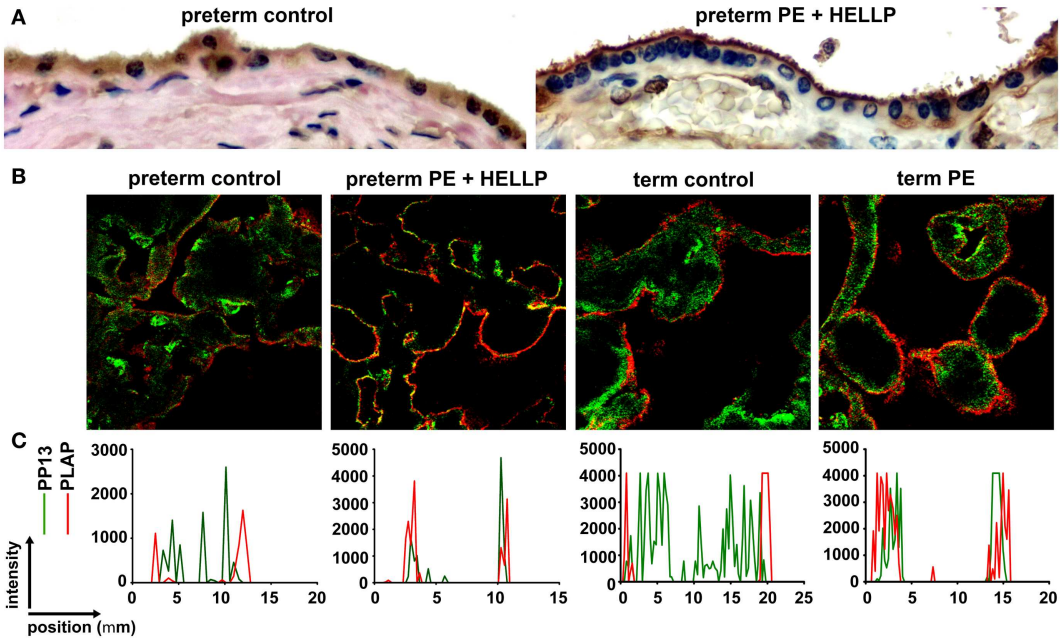


FIGURE 7 | Subcellular relocalization of PP13 in preeclampsia and HELLP syndrome. **(A)** Representative images show uniformly moderate cytoplasmic and brush border membrane PP13 immunostaining of the syncytiotrophoblast in a preterm control placenta (left), while its weak cytoplasmic and strong membrane immunostaining in a placenta from a woman with preterm preeclampsia (PE) associated with HELLP syndrome (right). Cytoplasm protrusions, membrane blebs and shed membrane microvesicles immunostained intensely for PP13 (right). 800 x magnification. **(B)** Representative confocal images show the subcellular relocalization of PP13 (green) near to placental alkaline phosphatase (PLAP) immunopositive (red) lipid rafts in the juxtamembrane regions of the syncytiotrophoblast in term preeclampsia and preterm preeclampsia associated with HELLP syndrome compared to controls. **(C)** Line scan intensity distributions of PP13 and PLAP in representative confocal images shown in subfigure **(B)**. Figure **(A)** was published in Ref. (36). Figures **(B,C)** were published in Ref. (73). Kind permission for the reuse of the figures was obtained from Springer Science+Business Media **(A)** and Elsevier **(B,C)**.

the syncytiotrophoblast in preeclampsia and HELLP syndrome is associated with actin (**Figures 7B,C**) (73). Supporting these observations in the placenta, subsequent *in vitro* experiments revealed that Latrunculin B, a selective blocker of actin polymerization, decreased PP13 release from BeWo cells and led to its intracellular accumulation (**Figure 8A**) (73).

This result may also explain how PP13 is released from the syncytiotrophoblast since the actin cytoskeleton and associated motor proteins drive intracellular and plasma membrane trafficking amongst a wide variety of cellular processes (74, 75). In this regard, galectins predominantly utilize unconventional trafficking routes, either vesicular or direct translocational, avoiding the endoplasmic reticulum (ER) and Golgi apparatus, since they are synthetized on free ribosomes and lack an *N*-terminal signal sequence for the translocation to the ER/Golgi system (76–78). However, other vesicular transport mechanisms for PP13 cannot be excluded, such as the "kiss and run" exocytosis, which was described for many hormones and neurotransmitters and was proved to be an actin- and calcium-dependent process (79, 80).

The role of actin cytoskeleton in the release of extracellular vesicles (EV; e.g. microvesicles/microparticles and exosomes), which also carry various galectins, has also been demonstrated (81–83). In addition, annexin II, another protein that specifically bound to PP13 (9), has also been found in various types of EVs along with actin (84, 85). Similar to galectin-9, which was shown to be associated with many different types of EV fractions (86), PP13 may also translocate with different EVs through the syncytiotrophoblast membrane. This type of release is supported by evidence on the PP13 release from BeWo cells mediated by exosomes (73) and the observed PP13 immunopositivity of microvillous membrane microvesicles shed from the syncytiotrophoblast (36).

THE ROLE OF CALCIUM AND ISCHEMIA IN TROPHOBLASTIC PP13 RELEASE

Recent *in vitro* experiments with BeWo cells transfected with *LGALS13* to enable high PP13 expression (73) also showed an increased PP13 release from calcium ionophore-treated cells, evidenced by decreased cellular PP13 content and elevated amounts of PP13 in cell culture supernatants (**Figure 8B**). This finding is in accord with a previous report showing that galectin-3 is secreted by exosomes from monocytes upon calcium ionophore treatment (87). Calcium serves as a ubiquitous second messenger responsible for controlling numerous cellular processes including exosome secretion (88). Since calcium regulates the actin cytoskeleton at multiple levels including the organization of actin monomers into actin polymers and the super-organization of actin polymers into a filamentous network (89), it is not surprising that stimuli resulting in the elevation of intracellular calcium concentration can induce microvesiculation and membrane shedding of exposed cells (90, 91). As a mechanism, the dynamics of actin assembly and

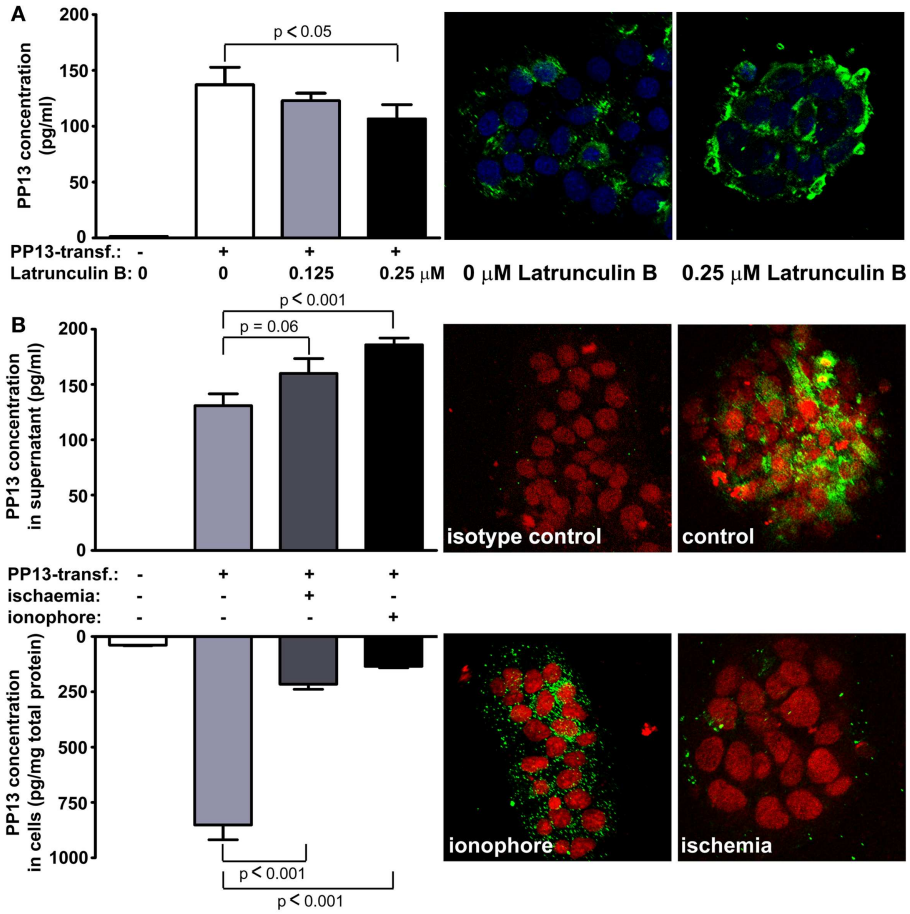


FIGURE 8 | Blocking of actin polymerization inhibits while calcium and ischemia promotes trophoblastic PP13 release. **(A)** PP13 content of BeWo cell culture supernatants of non-transfected controls, as well as *LGALS13*-transfected, untreated or Latrunculin B-treated cells were measured by ELISA (left). *LGALS13*-transfected, untreated or Latrunculin B-treated cells were stained with anti-PP13 (green) and nuclei were counterstained with DRAQ5 (blue) followed by confocal microscopic analysis (right; 40x magnifications). The disruption of the actin cytoskeleton with Latrunculin B treatment decreased PP13 release from BeWo cells. **(B)** *LGALS13*-transfected BeWo cells were treated with calcium ionophore to increase intracellular calcium level, or kept under ischemic stress to mimic placental milieu in preterm preeclampsia. (Left) PP13 content of BeWo cells and cell culture supernatants of non-transfected controls, as well as *LGALS13*-transfected, untreated, calcium ionophore-treated or ischemia exposed cells were measured by ELISA. (Left) Representative confocal images of *LGALS13*-transfected control, calcium ionophore-treated or ischemia exposed BeWo cells immunostained with monoclonal anti-PP13 antibody (green) and counterstained with DRAQ5 (red) nuclei dye. Either ionophore treatment or ischemia induced the release of PP13 from BeWo cells. Figures were published in Ref. (73). Kind permission for the reuse and modification of the figures was obtained from Elsevier.

disassembly is regulated by certain actin-binding proteins such as annexin II in a calcium-dependent manner (92–96).

The release of PP13 from BeWo cells appears to be similar to the *in vivo* release when comparing the effect of calcium ionophores and ischemic stress (**Figure 8B**) (73). Ischemic stress of the placenta is a major component of the pathophysiology of preterm preeclampsia (97). In accord, higher PP13 release was observed in placental villous tissue explants obtained from women with preeclampsia compared to gestational age-matched controls in the third trimester (98). A possible explanation is that ischemic stress causes elevation in intracellular calcium levels, which leads to actin depolymerization supported by findings of separate studies (99, 100). All of these results indicate that different kind of actin- and calcium-dependent release mechanisms exist side by side for PP13, and most probably the dominant sort depends on the cell type and also on the nature of the received stimuli.

As a functional aspect of the increased PP13 release from the placenta in preeclampsia, ischemic and other stress conditions pose danger to the organism, which is signaled to the immune system by endogenous danger signals called "alarmins" (101, 102). Indeed, "danger signals" in the placenta have also been proposed to create an abnormal placental cytokine milieu and link the activation of the innate immune system and preeclampsia (8, 103–105). In this context, some galectins with cytokine-like properties (106, 107) may act as alarmins, since they are increasingly secreted from inflamed or damaged tissues, and they may elicit effector responses from innate and adaptive immune cells (19, 102, 108). Although direct evidence for the role of PP13 as an alarmin has not yet been

established, these findings suggest that PP13 may function in such way in the placenta in preeclampsia (19, 73).

IN VITRO AND *IN VIVO* FUNCTIONAL STUDIES ON PP13

IN VITRO PARACRINE EFFECTS OF PP13 ON HUMAN IMMUNE CELLS

PP13 released from the trophoblast into the extracellular space may have various functions similar to other galectins, which may exert their pleiotropic extracellular functions in an autocrine and paracrine manner. Since PP13 is secreted by the trophoblast to the maternal circulation from where it gets into the decidual extracellular matrix (8), PP13 may affect various types of circulating and tissue-resident maternal leukocytes throughout pregnancy. Thus far only a couple of functional experiments were carried out, focusing on the examination of potential extracellular effects of PP13. As several members of the galectin family regulate adaptive immune responses by the induction of apoptosis of activated T lymphocytes (19, 109–111), the apoptosis-inducing effects of PP13 and other chromosome 19 cluster galectins were investigated on activated T cells freshly isolated from healthy donors (23). Among the studied recombinant galectins, PP13 had the strongest apoptosis-inducing effect (**Figures 9A,B**), stronger than galectin-1, a protein that has central role in maintaining maternal-fetal immune tolerance in eutherian mammals (16, 19, 112). A subsequent study also investigated the effect of PP13 on the secretion of cytokines and chemokines from mononuclear cells isolated from peripheral blood of pregnant women (8). The treatment with placenta-purified PP13 slightly increased the secretion of interleukin (IL)-1α and IL-6 into the culture medium. These *in vitro* experimental evidences suggest various effects of PP13 on immune cells, which may also be largely dependent on the type, activation and differentiation status of the affected cells, the microvesicle-bound or free nature and concentration of PP13, and the redox status of the environment, similarly to other galectins (19).

As with galectins that bind ABO blood group antigens, the paracrine effects of PP13 may also be affected by this phenomenon (21). In this context, large cohort studies showed that preeclampsia is more frequent among patients with AB blood group compared to those with non-AB blood groups (113, 114). Thus, recently it has been hypothesized that the higher susceptibility to preeclampsia among AB blood group women may be related to the decreased bioavailability and paracrine effects of PP13 on maternal immune cells (21). As a similar phenomenon, the ABO blood group antigens linked to the protein backbone of coagulation factor VIII and von Willebrand factor significantly affect the bioavailability of these blood clotting factors and coagulation (115–118). These findings altogether further underline the important immunoregulatory functions that PP13 may have in early pregnancy and warrant further investigation of the effect of ABO blood group system on PP13 bioavailability and functions. In summary of all of the above, PP13 may have a complex role in the regulation

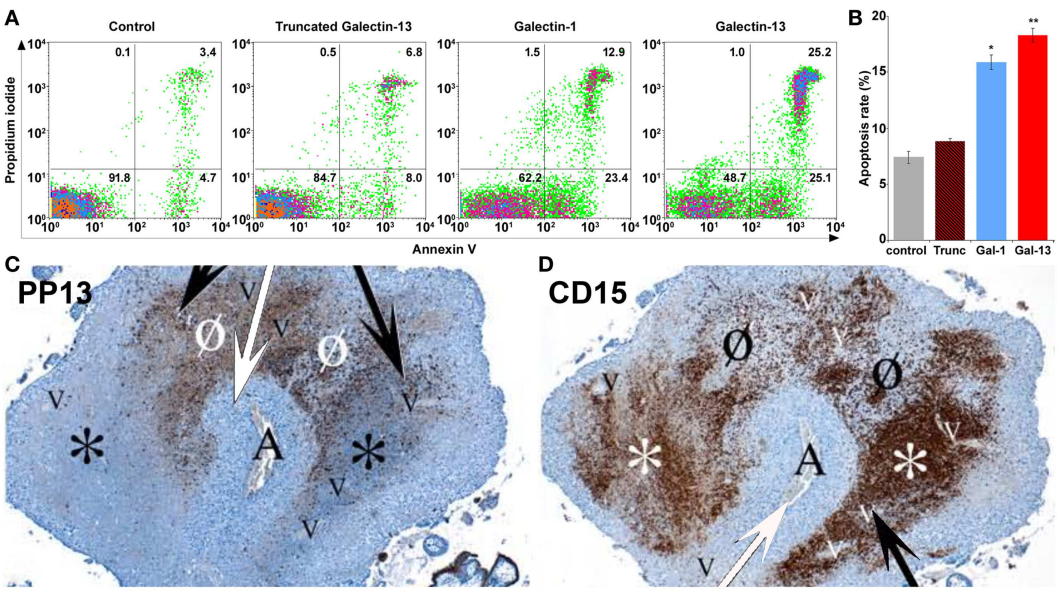


FIGURE 9 | Extracellular PP13 induces apoptosis *in vitro* and *in vivo*. **(A)** The *in vitro* apoptosis-inducing effect of PP13 on activated CD3+ T cells was comparable or stronger than that of galectin-1, whereas truncated PP13 did not have such effect when proteins were applied in 8μM concentration. Numbers in quadrants indicate the percentage of CD3+ T cells. **(B)** The *in vitro* apoptosis-inducing effect of PP13 on activated CD3+ T cells was stronger than that of galectin-1. Apoptosis rate was calculated as the percentage of Annexin V and propidium iodide double-positive cells. Gal: galectin; Trunc: truncated galectin-13; $*P < 0.05$; $**P < 0.01$. **(C,D)** Serial sections of decidua basalis samples in the first trimester. A spiral arteriole ("A" and white arrows) is surrounded by decidual veins ("V" and black arrows). **(C)** PP13 immunostainings revealed areas of intense PP13 depositions consistent with early and active zones of necrosis ("ZONE") formation (Ø), and areas with weak PP13 immunoreactivity consistent with end-stage "ZONEs" (*). **(D)** CD15 immunostainings revealed neutrophil accumulation showing an inverse pattern with PP13 depositions. The least intense staining was observed in early "ZONEs" (Ø), while the most intense staining in end-stage "ZONEs" (*). Figures and data **(A,B)** were published in Ref. (23). Figures **(C,D)** were published in Ref. (8). Kind permission for the reuse and modification of figures was obtained from the National Academy of Sciences of the United States of America **(A,B)** and SAGE US **(C,D)**.

of adaptive and innate immune functions at the maternal-fetal interface depending on the changing environment.

THE *IN VIVO* PARACRINE EFFECTS OF PP13 ON HUMAN IMMUNE CELLS

This latter study also described an interesting finding in placental tissue specimens obtained from elective terminations of pregnancies between 6 to 15 weeks of gestation (8). In addition to the PP13 immunopositivity of the syncytiotrophoblast, crystal-like PP13 deposits in the decidual extracellular matrix and phagocytosed PP13 immunopositive material in immune cells were also documented. These deposits were always adjacent to decidual veins but not to arteries, and they were coincident with unique zones of necrotic and apoptotic immune cells ("ZONEs") (**Figures 9C,D**) (8), a phenomenon that may be consistent with previous findings on zones of decidual necrosis in the first trimester (119). In addition, immunostainings demonstrated the expression of IL-1α and IL-6 within and around macrophages in these "ZONEs", suggesting the potential pro-inflammatory effect of PP13 (8). The highest number of "ZONEs" appeared to be between 7 to 8 weeks of gestation, and their occurrence declined by the end of the first trimester in parallel with the completion of the spiral artery remodelling and the establishment of the blood circulation to the placenta. This study also revealed that spiral artery transformation by invasive trophoblasts and "ZONEs" were rarely seen in specimens obtained from women with low maternal serum PP13 concentration compared to those with normal PP13 values. In summary, these findings prompted the authors to suggest that syncytiotrophoblast-secreted PP13 reaches the decidual veins, crosses their wall, deposits into the extracellular matrix and forms perivenous crystal-like aggregates near the veins. The lysophospholipase activity associated with PP13 was implicated in this process, but no evidence yet exists to prove it. These PP13 deposits were suggested to serve as "diversion sites" to attract, activate and kill maternal immune cells, drawing these away from sites where the semi-allogeneic fetal trophoblasts invade and remodel maternal spiral arteries. It was also hypothesized that decreased PP13 expression may lead to deficient "ZONE" formation, decreased trophoblast invasion, and the subsequent failure of spiral artery transformation (8). Functional and causal evidence for these *in situ* observations needs to be provided in the future.

PP13 AND UNIQUE ASPECTS OF DEEP PLACENTATION IN ANTHROPOID PRIMATES

These *in vivo* findings are important from an evolutionary point of view since PP13 evolved in Old World monkeys and apes (23), species that have endovascular trophoblast invasion and spiral artery remodelling different from lower primates (120–123). In fact, a growing body of evidence suggests that PP13 may belong to primate-specific molecules (e.g. human leukocyte antigen C, killer-cell immunoglobulin-like receptors), which are involved in the regulation of immune mechanisms related to invasive placentation (124). The findings on PP13 and "ZONE" formation may be mostly related to the pro-apoptotic effect of PP13, similar to the effect of galectin-1 on activated decidual T cells, which is critical in the down-regulation of maternal adaptive immune responses at the maternal-fetal interface in early pregnancy (111). However, the pro-inflammatory action of extracellular PP13 may also fit with early placentation events.

In fact, the early pregnancy decidua is infiltrated by a large number of leukocytes, mainly natural killer (NK) cells (70%), macrophages (20–25%), and T cells (10%) (125–127). These leukocytes, especially decidual natural killer (dNK) cells, macrophages and T regulatory cells, are indispensable for the success of pregnancy since they produce a large variety of chemokines, cytokines, matrix metalloproteinases and angiogenic molecules that regulate maternal-fetal interactions, trophoblast invasion and spiral artery remodelling (127–130). On one hand, these immune cells are involved in the establishment of a delicate immune tolerance between the mother and the fetus, and on the other hand they promote local pro-inflammatory responses that facilitate implantation, trophoblast invasion and placentation events (126, 127). These complex immune interactions between the mother and the fetus are conveyed by cell membrane- and vesicle-bound as well as soluble molecules. Among the best studied molecular mechanisms are the effect of progesterone-induced blocking factor (PIBF) on the shift towards Th2 over Th1 cytokine production (131), the anti-inflammatory role of decidual macrophages (132), the immunosuppressive effects of decidual galectin-1 (16), trophoblastic indoleamine 2,3-dyoxignease (IDO), FAS/FAS ligand and galectin-1 (133, 134), and the roles of human leukocyte antigen (HLA)-C and HLA-G in protecting fetal cells from NK- and cytotoxic lymphocyte (CTL)-mediated cytolysis (135, 136). It is a question for future studies how the actions of PP13 are related to this complex, dynamically changing cellular and molecular network during placentation.

As the result of these complex interactions at the maternal-fetal interface, aggregates of extravillous endovascular trophoblasts plug the openings of uterine spiral arteries; therefore, they inhibit intervillous circulation at the beginning of gestation (49, 120, 128). This is suggested to be a protective mechanism to keep the developing embryo in a relatively low oxygen environment, minimizing oxidative stress that would lead to developmental defects during organogenesis (137). Of importance, the observed "ZONE" formation peaks when placental circulation is not yet established (8), and the low flow of endometrial gland secretions around the placenta allows the increased transport of PP13 from decidual veins into the decidua. This is also the period when extravillous trophoblast invasion starts into the decidua (128, 137). Remarkably, after the start of the placental intervillous circulation at around 8–10 weeks of gestation (49, 128, 137, 138) PP13 deposits and "ZONE" formation rapidly declines and diminishes by the time intervillous circulation is fully established at about 10–14 weeks of gestation (8). This suggests that PP13 transport is reduced to the decidual extracellular matrix due to the continuously increasing blood flow in spite of the increasing total production of PP13 by the placenta. Importantly, if trophoblastic plug formation is incomplete, placental circulation starts earlier, which leads to the oxidative stress of the placenta, the subsequent development of preeclampsia, and early pregnancy loss in more severe cases (49, 137, 138). In this context, the earlier start of placental blood flow would theoretically restrict PP13 transport into the decidua and "ZONE" formation, providing another mechanism to hamper normal placentation.

THE *IN VITRO* AUTOCRINE EFFECTS OF PP13 ON HUMAN TROPHOBLAST

An *in vitro* study showed the autocrine effect of PP13 measured by its ability to depolarize the membrane of primary trophoblasts isolated from normal and preeclamptic placentas (5). For these experiments, either the patch-clamp technique or a voltage-sensitive fluorescence dye was used, and PP13 was transiently added to the cells. PP13-induced trophoblastic membrane depolarization was increased with extracellular calcium concentrations according to the Nernst equation, and it was blocked in the presence of EGTA, a calcium chelator (5). Furthermore, a two-minute exposure of cells to PP13 resulted in linoleic acid release and subsequent prostacyclin liberation in a calcium-dependent manner. Galectin-1 did not elicit a similar response, indicating the specific effect of PP13. It is interesting that, in contrast, galectin-1 has various effects on trophoblasts including the regulation of hCG and progesterone production (139), proliferation (140), and syncytium formation (141). Based on these results, it would be interesting to further investigate additional autocrine signaling effects of PP13 on the trophoblast at various stages of syncytialization.

THE *IN VIVO* PARACRINE EFFECTS OF PP13 IN PREGNANT ANIMALS

Besides *in vitro* experiments, the *in vivo* effects of PP13 in an animal model have also been investigated. Initially, non-pregnant rats were exposed to a single bolus of intravenous PP13 injection followed by immediate hypotension and heart rate increase resulting from generalized vasodilatation (142). PP13 was also administered to pregnant rats subcutaneously via osmotic pumps that slowly released PP13 over a period of five days starting from day 15 of pregnancy. In these animals, the hypotension and increased heart rate lasted through the five days of PP13 administration. Furthermore, isolated uterine and mesenteric arteries responded with dilatation to *in vitro* PP13 treatment as measured by angiography (142). In subsequent studies, the effect of PP13 on uterine vasculature was investigated between days 8 and 15 of pregnancy during a prolonged intraperitoneal exposure through a slow release from osmotic pumps (35, 143). Again, PP13 treatment led to a general hypotension that lasted throughout the treatment period, and then blood pressures returned to normal. PP13 treatment affected uterine vasculature with the main effect elicited on uterine veins. These veins had an increased diameter on day 15, while their size returned to normal by day 20. Interestingly, PP13-treated rats delivered slightly larger pups and placentas than saline-treated controls, possibly because of the increased uterine blood flow in these animals (143). These findings may be related to the *in vitro* prostacyclin liberalization ability of PP13 (5).

PP13 is a primate-specific protein, and thus, certain differences exist between the set of potential "receptors" to which PP13 may bind in rats and primates. Moreover, various differences exist between primate and rodent gestations regarding the length of gestation, uterine anatomy, placentation, litter size, immune regulation, and other aspects. Therefore, the most appropriate context for the *in vivo* investigations of PP13 effects would be in a pregnant primate model; however, there are ethical limitations for such studies. While there could be major differences between the effects of PP13 in rats compared to humans due to the reasons described above, the effect of PP13 on hypotension and vasodilatation are novel and have not previously been described in regard to any other galectins. In the future, these *in vivo* effects of PP13 need to be further investigated in humans, presumably on placental bed arteries in hysterectomy specimens and also on placental derived *in vitro* decidual models in order to evaluate the potential therapeutic use of PP13 to prevent preeclampsia, along with many additional considerations.

FUNCTIONAL CONSIDERATIONS REGARDING THE PP13 CRD

In vitro experiments on activated T cells also included their treatment with a truncated, 54-residue PP13 variant that lacks the entire CRD. This truncated protein was expressed from a mutated cDNA that contains the "163C>T" DNA variant frequently observed in cluster galectin pseudogenes in primates (23). Compared to the strong apoptosis inducing effect of PP13, this truncated PP13 had no effect on T cell apoptosis, confirming the crucial role of the CRD in this function (23). In addition, *in vivo* experiments included the administration of a different truncated PP13 (35), which contains the first 73 amino acids of PP13 similar to the "221delT" native mutant (34). Although this truncated PP13 variant contains 6 out of 8 amino acids from the CRD, its *in vivo* functional properties were different from PP13 since it caused hypotension in pregnant animals throughout the period of its active release between days 8 to 15 of pregnancy, while it did not increase the birth weight of the pups. Since an increased misfolding of this truncated protein was observed during the isolation from bacteria and the monoclonal anti-PP13 antibodies could not recognize it, it was concluded that its functional properties are different from those of the full length PP13 because of the misfolded structure (35). Further studies are warranted with these truncated PP13 variants to reveal their structural characteristics and effects.

THE EVALUATION OF PP13 IN THE DIAGNOSIS OF PREECLAMPSIA

LOW CIRCULATING PP13 mRNA IN MATERNAL BLOOD IN PREECLAMPSIA

The discovery of fetal DNA and RNA in maternal blood stimulated the experimental assessment of free and cellular PP13 mRNA species in pregnant women's blood in the first half of pregnancy. In accord with the previously discussed placental *LGALS13* expression data in preeclampsia, recent studies showed a lower PP13 mRNA content in the maternal blood in the first half of pregnancy in preeclampsia compared to matched controls (144, 145). The source of these PP13 mRNA species in maternal blood is only the placenta since no other human tissue expresses PP13 (23), and PP13 mRNA is not detectable in the blood of non-pregnant controls (146). These findings combined with those from placental studies have indicated that pathophysiological changes in PP13 expression appear very early in pregnancy. However, the predictive value of PP13 mRNA species in maternal blood is currently limited, which is most likely associated with varying and low amounts of trophoblastic mRNA reaching the maternal circulation. It is possible that advanced RNA processing techniques and sensitive detection methods like deep sequencing may enable a more robust PP13 mRNA detection in maternal blood for a better performance in preeclampsia prediction in early pregnancy. This aim is currently being supported by the European Union FP7 funded "ASPRE" project.

FIRST TRIMESTER MATERNAL BLOOD PP13 FOR PREDICTING THE RISK OF THE DEVELOPMENT OF PREECLAMPSIA

The evaluation of PP13 as a protein biomarker for the first trimester prediction of preeclampsia was analyzed with a recent meta-analysis based on studies performed with two immunoassay platforms (147). This meta-analysis explored 68 studies and included 19 into the final analysis, which were published between 2006 and 2013 (21, 37, 148–164). The analysis pooled the results from only singleton pregnancies of low or high risk women or all-comer cohorts, which were included in prospective or nested case-control studies, or fully prospective studies. A total of 16,153 pregnant women were tested for PP13 in the first trimester (between gestational weeks 6 and 14), among whom 1,197 developed subsequently preeclampsia. Out of these cases there were 19% who developed early-onset preeclampsia (<34 weeks) and 45% who developed preterm preeclampsia (<37 weeks).

Ten studies used the ELISA platform developed in Israel (21, 37, 148–152, 154, 160, 164), one study used the ELISA platform recently developed in China (161), and the remaining studies used the DELFIA platform. In all studies, PP13 blood concentrations were converted into gestational week-specific multiples of the medians (MoMs) (165), and then were further adjusted to maternal weight in two studies (156, 164) or to body mass index (BMI). In 10 studies, the PP13 MoMs were further adjusted to smoking, ethnicity, maternal age and parity. Interestingly, one study also adjusted PP13 MoMs to conception by *in vitro* fertilization (IVF) (164), and another study, which yielded the highest sensitivity and specificity, further adjusted PP13 MoMs to ABO blood groups (21).

All studies in the meta-analysis utilized algorithms that calculated the receiver operating characteristics (ROC) curves to detect the sensitivity and specificity, and logistic regression analysis to predict the risk of preeclampsia (147). When all cases of preeclampsia were included in the meta-analysis, the mean detection rate (DR) for predicting preeclampsia was 47% (95% confidence interval, CI: 43–65) at a 10% false positive rate (FPR). The DR of PP13 for preterm preeclampsia was higher, 66% (95% CI: 48–78), and for early-onset preeclampsia it was 83% (95% CI: 25–100). The assessment of likelihood ratios (LRs) for all cases of preeclampsia revealed a positive LR [sensitivity/(1-specificity)] of 5.82, a negative LR [(1-sensitivity)/specificity] of 0.46 and an overall LR (positive LR/negative LR) of 26.35, while the positive, negative and overall LRs for preterm preeclampsia were 6.94, 0.34, and 40.07, respectively.

The median PP13 MoMs and 95% CIs varied considerably between the different studies. Comparison of the DELFIA and ELISA studies showed that the DR for all preeclampsia cases at 10% FPR was 78.75% (95% CI: 68.44–88.22) with the ELISA platform and 40.29% (95% CI: 0–61.19) with the DELFIA platform. The positive, negative and overall LRs were 8.25, 0.19 and 53.26 with the ELISA platform and 5.03, 0.55 and 13.29 with the DELFIA platform, respectively. It has also been demonstrated that the ELISA assay of the same samples provides better segregation of PP13 values between preeclampsia cases and controls than the DELFIA assay (166). Among the eight DELFIA assay based studies, good preeclampsia prediction was achieved in two (156, 157), no prediction was achieved in three (155, 159, 162), while varying, moderate prediction was achieved in the rest of the studies. The DELFIA platform differs from the ELISA platform since it includes the capture and detection antibodies in an inverted order, and it utilizes Europium amplification compared to the use of the biotin-extravidin-horse radish peroxidase amplification in the ELISA. These differences may account for some of the differences detected in assay performances (166). However, recent results may suggest that batch differences in the Fab domain of one of the antibodies also play a role in this phenomenon, which is now under examination with the new generation of PP13 kits developed by the "ASPRE" project.

In view of the differential binding of PP13 to cell surfaces containing ABO blood group antigens, and its varying bioavailability in maternal blood depending on the ABO blood type, the adjustment of PP13 MoMs to ABO blood groups further improved their predictive value for preeclampsia as well as for IUGR and the two combined (21). For example, Caucasian and Hispanic women with blood group AB had the lowest, and those with blood group B had the highest first trimester maternal serum PP13 MoMs, while individuals with blood group A or O had intermediate MoMs (21). After adjustment of PP13 MoMs to ABO blood groups, the overall LR for predicting IUGR increased from 2.2 to 5.32, the overall LR for predicting preeclampsia increased from 6.9 to 18.1, and the overall LR for predicting preeclampsia associated with IUGR increased from 5.6 to 27.9.

Earlier studies have shown increased accuracy for the prediction of severe cases of preeclampsia over the mild ones (152, 154, 157). Based on these findings, the large differences in prediction accuracy demonstrated in the meta-analysis can probably be attributed to the differences in the severity of the included cases. This phenomenon as well as the observation on the reduced first trimester PP13 MoMs after IVF (164) are under further examination by the "ASPRE" project, which targets the longitudinal, multi-center examination of 33,000 maternal blood specimens.

PERFORMANCE OF FIRST TRIMESTER PP13 AS PART OF A MULTIPLE MARKER PANEL

A growing body of evidence suggests that the prediction of preeclampsia can be improved using multi-parametric approaches, combining data derived from multiple biomarkers (153, 165). Initially, PP13 was evaluated as a single marker with MoMs adjusted to various pregnancy features as detailed above. It was then evaluated over a background risk calculated according to preeclampsia in a previous pregnancy, medical history of gestational diabetes, kidney and cardiovascular diseases, maternal age, ethnicity, BMI and conception by assisted reproduction techniques. This analysis showed that the sensitivity of PP13 for predicting all cases of preeclampsia increased from 52 to 59% at 10% FPR after combining with background risk factors (167). Subsequently, PP13 and background risk factors were also combined with the mean arterial pressure (MAP), which further increased the detection rate to 93% for all cases of preeclampsia at 10% FPR (167). Combining PP13 with placental growth factor (PlGF) (156) or with additional biochemical markers [i.e. pregnancy associated plasma protein A (PAPP-A), PlGF and ADAM metallopeptidase domain 12 (ADAM12)] were also accompanied by an increased

DR for preeclampsia in spite of the varying predictive values of the individual biomarkers (165). In seven studies, the risk prediction was based on combining PP13 and uterine artery Doppler pulsatility index (PI), which also showed increased prediction accuracy (148, 150, 154, 157, 165, 168, 169). Comprehensive risk algorithms were further developed based on a combined multi-marker analysis that took into consideration the background risk (as detailed above), MAP, Doppler PI, and a panel of serum biomarkers. This approach yielded much higher predictive value and accuracy than individual markers (157), especially for early-onset (<34 weeks) and preterm (<37 weeks) preeclampsia. This is consistent with the results of several other studies that used combined biomarker panels and various types of risk prediction algorithms to obtain better risk prediction (170–172). It was therefore concluded that the introduction of a broad biomarker panel for the evaluation of preeclampsia and other maternal and fetal pregnancy disorders could present a change in deploying antenatal care as formulated by the inverted pyramid model of perinatal evaluation in pregnancy (173). In agreement with these, the combination of PP13, Doppler PI, MAP (or maternal artery stiffness) increased the DR of preeclampsia to 93% for early-onset preeclampsia and to 86% for all preeclampsia cases at 10% FPR (174). This preeclampsia prediction accuracy satisfies the World Health Organization (WHO) requirements for the clinical introduction of a disorder predicting procedure in terms of clinical usefulness in disease management and disorder prevention (175, 176).

LONGITUDINAL ASSESSMENT OF PP13 IN MATERNAL BLOOD

A repeated measure of a marker level was identified as a better method to get a more accurate prediction of the risk to develop pregnancy disorders, initially for Down syndrome (177) and also for preeclampsia (178, 179), or for IUGR and preeclampsia combined (180). A large study on PP13, which utilized repeated measures in the first, second and third trimesters, provided increased prediction accuracy compared to the first trimester test alone, either when a contingent or a combined model was used (151). This was further confirmed in a smaller study using repeated measures of PP13 every 2–4 weeks (37, 147).

The significance of the repeated measure of PP13 is also high since gestational age-related changes in maternal blood PP13 concentrations are very different between normal pregnancy and those with preeclampsia. While in patients who develop preeclampsia PP13 concentrations are lower in the first trimester than in normal pregnant women, the use of repeated measures of PP13 in longitudinal or cross-sectional studies showed that PP13 concentrations sharply increase in preeclampsia patients between the first and third trimesters compared to the moderate change that can be observed in women with normal pregnancy. The most prominent increase is seen when preeclampsia enters into the clinico-pathological stage. For example, between the first and third trimesters, maternal PP13 MoMs were detected to increase by ~1.5 to 3-fold in normal pregnancy compared to the 3.5 to 7.7-fold increase in preeclampsia, and occasionally even more (21, 36, 37, 98, 151, 181). Interestingly, the slope of increase was different among individuals, and it seemed to be related to patient characteristics like obesity, ethnicity, maternal age, parity, and particularly the severity of the disease.

Of importance, when taking into account the effect of ABO blood groups on the longitudinal changes in PP13 across the three trimesters, the regression slope of PP13 concentrations and MoMs were steeper in blood group B than in blood groups A and O, but not in blood group AB. The characteristic changes during gestation in serum PP13 concentrations in women with different ABO blood groups inversely mirrored the relative binding of PP13 to various ABO blood group erythrocytes, suggesting a dynamic change in PP13 sequestration on erythrocyte surfaces depending on gestational age and actual PP13 concentrations (**Figure 3C**) (21).

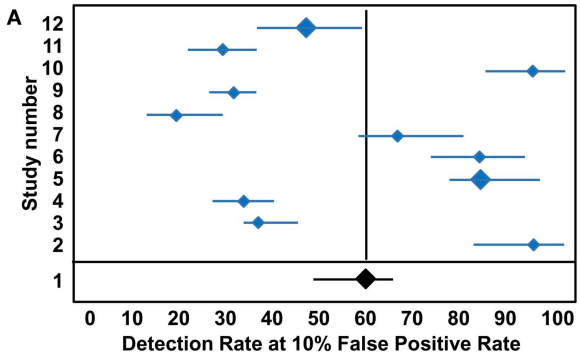
As described before, there is an increased shedding of PP13-rich syncytiotrophoblastic microvesicles from placental villi when women enter into the clinico-pathological stage of preterm preeclampsia (36, 37). Importantly, it was proposed that these microvesicles release their PP13 content, leading to the increased maternal blood PP13 concentrations in these cases (36, 37, 73). Since the extent of microvesicle shedding is related to the extent of placental ischemic stress and it is significantly more pronounced in severe cases of preeclampsia, particularly with preterm than with term onset (182), the longitudinal slope of changes in PP13 could be used as an additional parameter to predict case severity (36, 151). This phenomenon explains why so much difference was found in early-onset or preterm preeclampsia in PP13 compared to gestational-age matched controls in the third trimester.

META-ANALYSIS FOR THE PREDICTION OF THE RISK FOR PREECLAMPSIA WITH THIRD TRIMESTER PP13

METHODS AND INCLUDED STUDIES

Because of the increased PP13 concentrations in preeclampsia in the third trimester, it was hypothesized that PP13 testing can be further utilized for the prediction and diagnosis of preeclampsia during this period. To address this question, we have conducted a meta-analysis on third trimester datasets and found studies that utilized the PP13 ELISA but not DELFIA platform. The PP13 ELISA utilizes a pair of anti-PP13 mouse monoclonal antibodies (27-2-3 and 215-28-3 MAbs) that were selected based on their high (10^{-9} M) affinity to native and recombinant PP13 (5, 71). As a result, the detection limit of the ELISA was 3–8 pg/ml, the linear detection range was between 12.5–400 pg/ml, and the kit-to-kit, operator-to-operator and batch-to-batch variations were between 3–12% (5).

From the 71 studies published on PP13, the current meta-analysis identified eight clinical studies published between August 2008 and March 2014 that contained third trimester maternal blood PP13 data. These had an international scope involving Israel (71, 98, 151), Hungary (36), Austria (37, 181) and the USA (21). These studies were either performed as part of longitudinal clinical trials or cross-sectional studies that focused on the ELISA-based evaluation of PP13 between 26 and 40 weeks of gestation. In all studies, the ROC curves were based on PP13 adjusted to gestational week specific MoMs, which were further adjusted to BMI, smoking, ethnicity, maternal age as well as parity. In one study, PP13 values were further adjusted to the ABO blood groups (21). In a very recent study the adjustment was further made to conception by IVF (167).



B

Study number	Reference	Publication year	Study design	Population	GA at test	PE patients	All patients	Weight in analysis	DR (95% CI)	Overall LR
12	Gonen et al.	2008	Prospective cohort	All-comers	24-28	20	1,198	0.435	48.0 (38-57)	8.31
11	Huppertz et al.	2008	Prospective cohort	All-comers	31-35	4	67	0.024	30.8 (24-36)	4.02
10	Huppertz et al.	2008	Prospective cohort	All-comers	36-40	4	65	0.024	94.6 (85-100)	156.60
9	Than et al.	2008	Case-control	All-comers	28-32	11	57	0.021	33.0 (28-36)	4.43
8	Than et al.	2008	Case-control	All-comers	33-38	9	57	0.021	21.0 (15-29)	2.39
7	Grimpel et al.	2011	Case-control	All-comers	28-32	11	78	0.028	67.0 (59-79)	18.27
6	Sammar et al.	2011	Case-control	All-comers	30-34	12	75	0.027	83.7 (74-92)	46.21
5	Than et al.	2011	Prospective cohort	All-comers	24-28	15	815	0.296	84.0 (78-95)	47.25
4	Huppertz et al.	2013	Prospective cohort	All-comers	28-31	30	154	0.056	35.0 (28-40)	4.85
3	Huppertz et al.	2013	Prospective cohort	All-comers	32-36	34	150	0.054	38.0 (35-45)	5.52
2	Meiri et al.	2014	Prospective cohort	All-comers	24-28	3	38	0.014	95.0 (83-100)	171.00
1	Total		Meta-analysis		24-40	193	2,754	1.00	59.4 (49.7-64.5)	26.24

FIGURE 10 | Meta-analysis of PP13 in predicting preeclampsia in the third trimester. **(A)** A Forest plot analysis was performed including 11 studies based on unaffected and all preeclampsia cases. The detection rate (DR) at 10% False Positive Rate (FPR) of all cases of preeclampsia is shown in case-control and prospective cohort studies using all-comers. The DR was extracted from Receiver Operation Characteristics (ROC) curves based on the adjusted multiple of the medians (MoMs) of PP13. The final analysis took into consideration the total study size and the size of the preeclampsia group. Number 1 on the study list reflects the results of the meta-analysis depicted with a dark filled diamond compared to individual studies depicted with blue diamonds. The relative weight of a certain study in the analysis is reflected by the relative size of the diamonds. **(B)** The table lists all studies used to perform the Forest plot for the meta-analysis. Weight represents the relative impact of the study in the meta-analysis. DR for 10% FPR is shown along with the 95% confidence interval (95% CI). The Likelihood ratio (LR) was calculated for positive LR [sensitivity/(1-specificity)], negative LR [(1-sensitivity)/specificity] and overall LR (positive LR/negative LR). For the meta-analysis, the values were adjusted to the relative weight of each study in the meta-analysis. The numbers on the left side of the table correspond to the graph numbers in **(A)**. PE, preeclampsia; GA, gestational age in weeks.

The meta-analysis was performed by a Forest plot method (183). There were three occasions in which the determination of PP13 in maternal blood was extracted from studies performed in separate time points, which were included as separate studies (36, 181, 184). Accordingly, the dataset for the analysis was based on 11 cohorts. The DR at 10% FPR was extracted from the published ROC curves or by communicating with the authors and obtaining complementary data. The 95% CI of the DR was extracted or calculated from ROC curves of the published study or by using the web-calculator[2]. The analysis pooled clinical results from all singleton pregnancy studies, irrespective whether these were prospective cohort studies or case-control studies that enrolled low- or high-risk patients or all-comers. The pooled dataset included all preeclampsia cases and then a sub-analysis was performed for preterm and early-onset preeclampsia cases. Preeclampsia associated with IUGR and/or HELLP syndrome was compared to all preeclampsia cases, but there were too few cases with these additional complications to enable a true meta-analysis.

Regarding the detection level, the meta-analysis has also evaluated the overall LR of developing preeclampsia by dividing the positive LR with the negative LR as described earlier. The overall LR calculation took into consideration the relative weight of each of the cohorts in terms of study size and the number of women with preeclampsia.

RESULTS OF THE META-ANALYSIS

In total, 2,750 third trimester pregnant women were tested. 193 women subsequently developed preeclampsia out of whom 30.7% had preterm preeclampsia and 7.6% had early-onset preeclampsia.

[2]http://www.causascientia.org/math_stat/ProportionCI.html

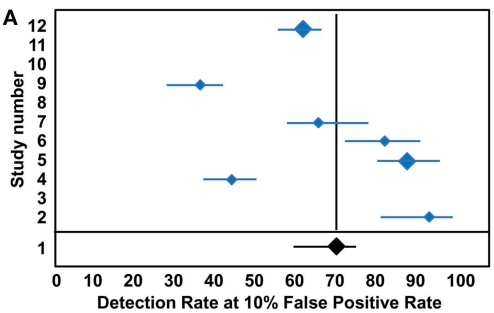
All but one study enrolled all-comers, and only one study enrolled high-risk patients. In this latter study the correlation between having prior risk of preeclampsia based on major risk factors and a high level of PP13 in the third trimester was low ($R = 0.13$), indicating that the two are independent evaluators (167).

In all studies, maternal blood PP13 MoMs were higher in women who subsequently developed preeclampsia compared to unaffected women although the variation between individual data-points within a study or between studies was large. Therefore, the sensitivity of using PP13 as a biomarker for predicting the risk of the subsequent development of preeclampsia had a broad range (14–100%). The mean DR at 10% FPR for all preeclampsia cases was 59.4% (95% CI: 49.7–64.5) (**Figures 10A,B**). The DR of PP13 for preterm preeclampsia (which included all early-onset preeclampsia cases) was 71.7% (95% CI: 60.3–75.3) (**Figures 11A,B**). Since there were few studies with data from patients with early-onset preeclampsia, this separate analysis had insufficient power for statistical analysis.

The time of detection ranged between 28–32 to 36–40 weeks, and the evaluation of the DR per gestational week yielded a regression line of $Y = 1.3986X + 100.58$, where X was the gestational week and the regression coefficient (R) was 0.2. These results have indicated that the variations are indeed independent of the gestational week at testing. When evaluated according to the correlation with MAP or urine protein, the DR appeared to be related to the severity of the cases in a given study, with regression coefficient values of 0.61 and 0.73, respectively. This means that the higher the hypertension and proteinuria, the higher the third trimester PP13 MoMs in maternal blood, and the better the prediction. A combined algorithm of PP13, MAP and proteinuria, which was available for nine out of the 11 studies, yielded a 95% DR for preterm preeclampsia and 85% for all preeclampsia at 5% FPR, showing the value of combining all parameters (data not shown). In conclusion, the meta-analysis indicates that higher third trimester maternal blood PP13 among women who will subsequently develop preeclampsia reaches the clinical diagnostic level.

The positive LR for all cases of preeclampsia in the meta-analysis was 5.94 and the negative LR was 0.45, providing an overall LR of 26.24 (**Figure 10B**). The positive LR for preterm preeclampsia in the meta-analysis was 7.17 and the negative LR was 0.31, providing an overall LR of 37.99 (**Figure 11B**). These LRs are lower compared to the overall LRs of first trimester PP13, but these can still be considered respected LRs by the criteria of the WHO (176).

A



B

Study number	Reference	Publication year	Study design	Population	GA at test	PE patients	All patients	Weight in analysis	DR (95% CI)	Overall LR
12	Gonen et el.	2008	Prospective cohort	All-comers	24-28	5	1183	0.503	63.1 (57-67)	15.32
9	Than et al.	2008	Case-control	All-comers	28-32	8	54	0.023	37.0 (29-42)	5.29
7	Grimpel et al.	2011	Case-control	All-comers	28-32	11	78	0.033	67.0 (59-79)	18.27
6	Sammar et al.	2011	Case-control	All-comers	30-34	12	75	0.032	83.7 (74-92)	46.21
5	Than et al.	2011	Prospective cohort	All-comers	24-28	5	805	0.343	89.0 (82-97)	72.82
4	Huppertz et al.	2013	Prospective cohort	All-comers	28-31	14	117	0.050	45.0 (38-50)	7.36
2	Meiri et al.	2014	Prospective cohort	All-comers	24-28	3	38	0.016	95.0 (83-100)	171.00
1	Total		Meta-analysis		24-32	58	2,350	1.00	71.7 (60.3-75.3)	37.99

FIGURE 11 | Meta-analysis of PP13 in predicting preterm preeclampsia in the third trimester. **(A)** Forest plot analysis was performed including seven studies based on unaffected and preterm preeclampsia cases. The detection rate (DR) at 10% False Positive Rate (FPR) of cases of preterm preeclampsia is shown in case-control and prospective cohort studies using all-comers. The DR was extracted from Receiver Operation Characteristics (ROC) curves based on the adjusted multiple of the medians (MoMs) of PP13. The final analysis took into consideration the total study size and the size of the preeclampsia group. Number 1 on the study list reflects the results of the meta-analysis depicted with a dark filled diamond compared to individual studies depicted with blue diamonds. The relative weight of a certain study in the analysis is reflected by the relative size of the diamonds. **(B)** The table lists studies used to perform the Forest plot for the meta-analysis of cases of preterm preeclampsia <37 weeks), including early onset preeclampsia (<34 weeks). Weight represents the relative impact of the study in the meta-analysis. DR for 10% FPR is shown along with the 95% confidence interval (95% CI). The Likelihood ratio (LR) was calculated for positive LR [sensitivity/(1-specificity)], negative LR [(1-sensitivity)/specificity] and overall LR (positive LR/negative LR). For the meta-analysis, the values were adjusted to the relative weight of each study in the meta-analysis. The numbers on the left side correspond to the graph numbers in **(A)**. PE, preeclampsia; GA, gestational age in weeks.

Reduced blood concentrations of PIGF in the third trimester have been suggested for predicting the symptoms of preeclampsia within 14 days of the test. This fast and quantitative TRIAGE test, measuring the decreased PlGF concentrations in maternal blood, also predicts the anticipated disease severity (185, 186). Of interest, the combination of anti-angiogenic factors and PlGF (or their ratio) increase the prediction rate of severe late-onset preeclampsia in the third trimester (179). Combining low PIGF with high PP13 maternal blood concentrations may generate an even better test. Thus, it is essential to investigate the PP13/PIGF ratio as a better diagnostic tool for preeclampsia in the third trimester. This will be further explored by the "ASPRE" project, in which at least 1,500 high-risk patients out of the 33,000 enrolled pregnant women will be tested longitudinally in the first, second and third trimesters of pregnancy.

SUMMARY AND CONCLUSIONS

Galectins are glycan-binding proteins that regulate innate and adaptive immune responses, and some galectins confer maternal-fetal immune tolerance in eutherian mammals. A chromosome 19 cluster of galectin genes has emerged in anthropoid primates, species with deep placentation and long gestation, in which this galectin network may confer additional immunoregulatory functions to enable deep placentation. These cluster galectins, including PP13, have a conserved structure, CRD and sugar-binding preference resembling other mammalian galectins. PP13 is solely expressed by the human placenta, predominantly by the syncytiotrophoblast, from where it is released into the maternal blood. PP13 expression and release from the individual placental villi is highest in the first trimester when maternal immune cell infiltration into the decidua is at its peak (**Figure 12**) to promote successful placentation including embryo implantation, trophoblast invasion, repair of the uterine epithelium and removal of cellular debris. Of interest, PP13 released from the villi is deposited around uterine veins and contributes to the formation of "ZONEs" of apoptotic and necrotic immune cells, which peak parallel with the start of spiral artery remodelling in the first trimester. Because PP13 is capable of inducing the apoptosis of activated T cells and the cytokine production of macrophages, it was postulated that these PP13 deposits in the decidual extracellular matrix may

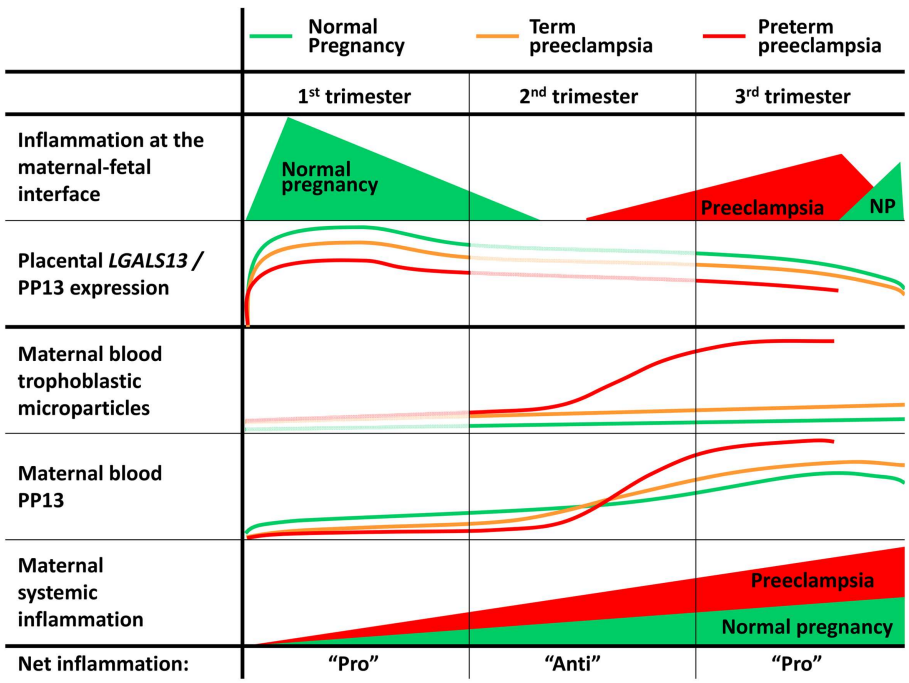


FIGURE 12 | PP13 expression is related to inflammatory changes at the maternal-fetal interface and in maternal circulation. This summary figure consolidates the results of numerous studies related to various facets of PP13 in normal pregnancies and in preeclampsia. The placental expression of *LGALS13* and PP13 is strong in the first trimester, and the secreted protein can be detected in maternal blood from gestational weeks 5 to 6 in normal pregnancies. From the decidual veins PP13 gets into the decidua, where it is deposited extracellularly or phagocytosed, coincident with maternal immune cell infiltration and the formation of the "Zones of Necrosis" (ZONEs) adjacent to the decidual veins. Although maternal serum PP13 concentrations do not change, the relative placental expression and decidual deposition of PP13 declines until the end of the first trimester in parallel with the decrease in the number of ZONEs. In the second and third trimesters, maternal serum PP13 concentrations rise due to the growing number of villi and trophoblast volume in the placenta, paralleling the escalation in maternal systemic inflammation. In preeclampsia, especially in early-onset cases, there are lower placental expression and maternal serum concentrations of PP13 in the first trimester, coincident with impaired trophoblast invasion and spiral artery remodelling. Starting from the second trimester, ischemic placental stress and pro-inflammatory changes at the maternal-fetal interface are also reflected by the increased shedding of aponecrotic microvesicles, which carry a considerable amount of PP13, elevating maternal blood PP13 concentrations. PP13 expression in the first trimester is associated with inflammation at the maternal-fetal interface. Similarly, maternal blood PP13 concentrations in the second and third trimesters parallel maternal systemic inflammation. As a consequence, PP13 has a good diagnostic value for the prediction and diagnosis of preeclampsia in the first and third trimesters. NP: normal pregnancy.

attract maternal immune cells away from the sites of maternal spiral artery formation to the decidual veins, and may promote a tolerogenic environment that facilitates trophoblast invasion and placentation. How important the roles of PP13 are during early placentation may be well reflected by observations showing decreased placental expression and maternal serum concentrations of PP13 in the first trimester in preeclampsia (**Figure 12**), a syndrome originating from severely impaired trophoblast invasion and placentation. Moreover, mutations in the promoter and in the exons of *LGALS13* presumably leading to altered, misfolded or non-functional protein expression are associated with a higher frequency of preeclampsia and also other obstetrical syndromes which involve immune dysregulation.

PP13 maternal blood concentrations steeply increase in preeclampsia compared to normal pregnancy starting in the second trimester, with the steepness correlated to disease severity. This phenomenon is closely related to the ischemic placental stress and the consequent increase in trophoblastic shedding of PP13 immunopositive microvesicles (**Figure 12**). Because of the pro-inflammatory nature of these aponecrotic trophoblast microvesicles and other "toxins" released from the placenta, preeclampsia, especially its early-onset subform, is characterized by an exaggerated maternal systemic inflammation and generalized endothelial dysfunction, leading to kidney damage, proteinuria and hypertension. It is interesting that reduced placental PP13 expression in preeclampsia correlates with altered immune-interactions at the maternal-fetal interface. Similarly, maternal blood PP13 concentrations in the second and third trimesters are elevated in relation to the increased placental stress and maternal systemic inflammation (**Figure 12**). These phenomena have already been utilized for developing a PP13 blood test for predicting preeclampsia, and indirectly for impaired placentation, in the first trimester. The analysis provided here shows that this test may be further used for preeclampsia diagnosis in the third trimester.

Functional studies have just started to assess the *in vitro* and *in vivo* effects of PP13 during pregnancy, showing various functions that PP13 may have at the maternal-fetal interface. *In vitro* studies need to take into account the pleiotropic actions of PP13, which may depend on the activation and differentiation status of the affected cells, the way PP13 is released from the placenta (e.g. free or extracellular vesicle-bound), the redox status of the environment, and the interaction of PP13 with various small molecules. *In vivo* studies, while starting in rodents, may eventually need to be extended to other models, optimally to primates. Nevertheless, the results of the first studies support the importance of PP13 in the regulation of blood pressure and vascular remodelling at the maternal-fetal interface.

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The role of placental tryptophan catabolism

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This review discusses the mechanisms and consequences of degradation of tryptophan (Trp) in the placenta, focusing mainly on the role of indoleamine 2,3-dioxygenase-1 (IDO1), one of three enzymes catalyzing the first step of the kynurenine pathway of Trp degradation. IDO1 has been implicated in regulation of feto-maternal tolerance in the mouse. Local depletion of Trp and/or the presence of metabolites of the kynurenine pathway mediate immunoregulation and exert antimicrobial functions. In addition to the decidual glandular epithelium, IDO1 is localized in the vascular endothelium of the villous chorion and also in the endothelium of spiral arteries of the decidua. Possible consequences of IDO1-mediated catabolism of Trp in the endothelium encompass antimicrobial activity and immunosuppression, as well as relaxation of the placental vasotonus, thereby contributing to placental perfusion and growth of both placenta and fetus. It remains to be evaluated whether other enzymes mediating Trp oxidation, such as indoleamine 2,3-dioxygenase-2, Trp 2,3-dioxygenase, and Trp hydroxylase-1 are of relevance to the biology of the placenta.

Keywords: pregnancy, placenta, intrauterine growth restriction, fetal growth restriction, preeclampsia, vasotonus, feto-maternal tolerance, immunoregulation

INTRODUCTION

L-Tryptophan (L-Trp) is a hydrophobic amino acid with a chemical structure based on an indole ring. L-Trp is the least abundant essential amino acid, and therefore needs to be supplied by nutrients such as meat, fish, milk, eggs, vegetables, nuts, and seeds such as soybeans, sesame, and sunflower seeds. The daily requirement of adults is in the range of 3 mg/kg (1). Apart from protein synthesis, L-Trp is utilized for the synthesis of the neurotransmitter serotonin and the hormone melatonin in the pineal gland. Degradation of Trp in mammals occurs predominantly (>95%) along the kynurenine pathway, leading to synthesis of nicotinamide adenine dinucleotide (NAD^+) (2) (**Figure 1**).

The first step in the oxidative metabolism of L-Trp along the kynurenine pathway is catalyzed independently by three different enzymes: indoleamine 2,3-dioxygenase-1 (IDO1), indoleamine 2,3-dioxygenase-2 (IDO2), and Trp 2,3-dioxygenase (TDO). By incorporating molecular oxygen, these enzymes convert L-Trp to *N*-formyl-kynurenine, which is then converted to kynurenine. L-Trp degradation not only leads to depletion of the amino acid but also to the production of metabolites displaying various biological activities.

TRYPTOPHAN-DEGRADING ENZYMES

INDOLEAMINE 2,3-DIOXYGENASE-1

Indoleamine 2,3-dioxygenase-1 (IDO, indoleamine-pyrrole 2,3-dioxygenase), reviewed in Ref. (3), is a cytosolic heme-containing enzyme sharing some sequence similarity with myoglobin (4). IDO1 has been conserved through 600 million years of evolution (5). The protein is encoded by the IDO1 (also INDO) gene that is located on chromosome 8, contains 10 exons, and a promoter region that includes 2 interferon (IFN) – stimulated responsive elements. Human IDO cDNA encodes a protein of 403 amino acids with molecular weight of about 45 kDa (6, 7). The primary sequence of human IDO1 shows 57 and 58% identity to mouse and rat IDO1, respectively, whereas no sequence homology was found to rat TDO (8). IDO1 requires activation by reduction of its Fe^{3+}-heme form. Early studies suggested that superoxide anion is responsible for this reductive activation (9), although more recent studies indicate formation of Fe^{2+}-IDO1 is accomplished by cytochrome b_5 plus cytochrome P450 reductase and NADPH (10). Despite numerous studies, the mechanism by which IDO1 oxidizes L-Trp to *N*-formyl-kynurenine remains controversial, with both concerted incorporation of the two oxygen atoms and consecutive insertions of single oxygen atoms into the substrate being proposed (11). Fe^{2+}-IDO1 rapidly autoxidizes to the inactive Fe^{3+}-IDO1 (12). In the presence of hydrogen peroxide (H_2O_2), IDO1 takes on a peroxidase activity that can lead to the oxidation of L-Trp to oxyindolylalanine, and protein oxidation leading to IDO1 inactivation (13). IDO1 prefers L-Trp as a substrate but may also cleave D-Trp and other indoleamines such as tryptamine. In contrast to rabbit IDO, however, the human enzyme does not act on serotonin (14). 1-Methyltryptophan (1-MT) is a compound commonly used to inhibit IDO1 activity, although it is now recognized that the enzyme is also capable of metabolizing 1-MT. The L-isoform of 1-MT has been reported to be a more efficient inhibitor of IDO1 than the D-isomer (15, 16). Further IDO inhibitors are discussed in (17, 18). INCB024360 and Amg-1 have been reported to block IDO1 selectively, with no effect on IDO2 and TDO (19, 20).

In humans, high Trp-degrading activity has been described in the lung, the intestine, and particularly in the term placenta, where it was attributed to IDO1 (21). At that time, however, a possible contributory role of extrahepatic TDO and/or IDO2 was not envisaged. IDO1 is also detected in the mammalian epididymis,

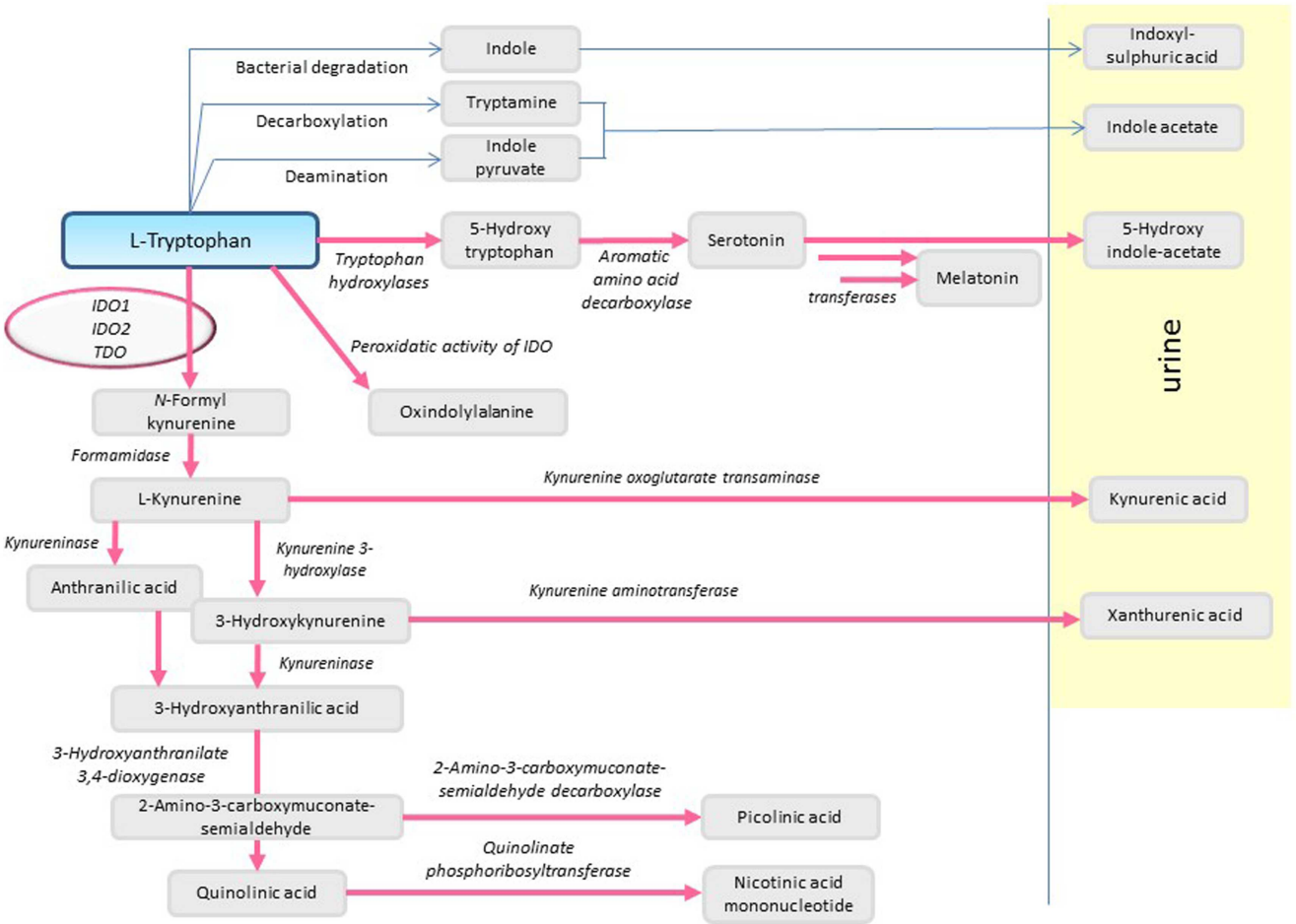

FIGURE 1 | Pathways of Trp degradation.

where its absence generates an inflammatory state and correlates with an increase in abnormal spermatozoa in IDO1 gene knockout ($IDO1^{-/-}$) mice (22). On a cellular basis, constitutive expression of IDO1 has been found in subsets of dendritic cells (DC) (23), including DC of tumor-draining lymph nodes (24). Moreover, IDO1 has been reported in eosinophils (25), in glandular and surface epithelium of the endometrium and Fallopian tubes (26), and in placental endothelial cells (26–28). IDO1 is also present in microvascular endothelial cells of tumors (29) (Blaschitz, unpublished observations for hepatocellular carcinoma) and the heart in human septic shock (30). Regulatory T cells have been reported to induce the expression of IDO1 in vascular endothelial cells of transplanted hearts in rats (31). Diverging inducibility of IDO1 has been reported for different types of normal endothelial cells, as summarized in **Table 1**.

Indoleamine 2,3-dioxygenase-1 can be induced by IFN-γ acting via Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signaling, type I interferons, prostaglandin E2, lipopolysaccharide (LPS), DNA regions containing a high frequency of cytosine nucleotides adjacent to guanine nucleotides (CpG islands), and other factors in a variety of cell types such as DC, macrophages, epithelial and endothelial cells, Langerhans cells, astrocytes, and T lymphocytes. Also hormones such as estrogen (32) and human chorionic gonadotropin (hCG) (33–35) induce IDO1 expression. Upregulation of IDO1 in DC by hCG is independent of IFN-γ (34). The compounds which induce IDO1 expression in DC have been reviewed previously (36). In addition to IDO1 induction, blockade of cyclooxygenase (COX)-2 has been reported to downregulate IDO1 expression in tumors of animal models, suggesting an interplay between these two enzymes (37).

INDOLEAMINE 2,3-DIOXYGENASE-2

Indoleamine 2,3-dioxygenase-2 (IDO-like protein, INDOL1, proto-IDO) was described first in 2007 (38, 39) and has been reviewed recently (40). IDO2 has a molecular weight of 47 kDa, is composed of 420 amino acid residues, and displays 43% identity with IDO1 at the amino acid level. The gene for IDO2 is located on chromosome 8, adjacent to its paralog IDO1, and may have arisen from gene duplication (41). Alternatively spliced transcripts have been described (42), however, it is unclear whether they are all translated into protein. Two genetic polymorphisms in the human gene encoding IDO2 ablate its enzymatic activity, such that about

Table 1 | Expression of IDO1 in various types of vascular endothelium.

Constitutive	Following inflammation *in vivo*	Following cytokine stimulation (IFNγ and/or TNF-α or IL-1β)	Constitutively negative expression following cytokine stimulation not tested	No or little even after stimulation with IFNγ
Chorionic vascular endothelium (26, 28, 59)	Mouse brain vascular endothelium (63, 125)	HUVEC (28, 116)	Iliac vein endothelial cells (28)	HSVEC (IDO upregulated after mycoplasma infection) (116)
Arteries and capillaries of the decidua (26, 28)	Mouse microvascular endothelium in kidney and intestine during cerebral malaria infection or after administration of LPS (63)	HAEC (10, 28)		RAEC (116)
Pulmonary capillaries (Blaschitz, unpublished observations), expression enhanced in hypoxia (62)	Human microvascular endothelial cells in heart and kidney in septic shock (30)	HBMEC (126) Vascular endothelial cells following incubation of porcine, rabbit, rat, and mouse coronary, carotid, and aortic arteries with IFN-γ (63)		IMAEC (116)

HUVEC, human umbilical vein endothelial cells; HAEC, human aortic endothelium cells; HSVEC, human saphenous vein endothelial cells; RAEC, radial artery endothelial cells; IMAEC, internal mammary artery endothelial cells; HBMEC, human brain microvascular endothelial cells.

50% of Caucasians and Asians and 25% of Africans lack functional IDO2 alleles (42).

Expression of IDO2 mRNA has been described in kidney, liver, epididymis, testis, uterus, placenta, and brain (15, 38, 43). IDO2 has also been found in sperm tails (38), pancreatic cancer cell lines (44), and tumors of the stomach, colon, and kidney (45). Similar to IDO1, IFN-γ upregulates IDO2 expression in DC (45), mesenchymal stem cells, macrophages, and astrocytes (43), although IFN-γ does not necessarily induce IDO1 and IDO2 simultaneously (19, 43). Preferential inhibition of IDO2 by a particular 1-MT enantiomer is contentious. An early report of more efficient inhibition by the D-isomer of 1-MT (42) has not been confirmed (16, 46) [for discussion see (40)]. Tenatoprazole has been reported to inhibit IDO2 without affecting IDO1 or TDO, although this compound also displays other biological effects (47).

FURTHER Trp-DEGRADING ENZYMES

Like IDO1, TDO is a cytosolic heme dioxygenase. It is coded for by the TDO2 gene and displays only 10% amino acid sequence identity with IDO1 (48). The structure and function of TDO and IDO1 have been compared previously (49). TDO is a homotetramer with a subunit molecular weight of 103 kDa. In contrast to IDO1, TDO is enantiomer-specific and only cleaves the L-isoform of Trp (48). Although thought initially to be expressed in the liver only, TDO is also present in placenta (50), brain (51), and a variety of human carcinomas. In the mouse endometrium, TDO is induced at the time of implantation (52). The expression of TDO is upregulated by glucocorticoids (53, 54) and by L-Trp (55). 1-MT does not inhibit TDO, while the compound 680C91 has been reported to selectively block TDO but not IDO1 (56).

Tryptophan hydroxylases (Tph-1 and Tph-2) convert Trp to 5-hydroxytryptophan for subsequent synthesis of serotonin and melatonin, rather than being involved in the kynurenine pathway. Tph-1 and Tph-2 are homologous enzymes with 71% amino acid sequence identity, and with their respective genes located on chromosomes 11 and 12. Mast cells are the major source of Tph-1, whereas Tph-2 is expressed predominantly in neuronal cells of the brain stem (57).

PLACENTAL EXPRESSION AND LOCALIZATION OF Trp-DEGRADING ENZYMES

There are several, albeit partly conflicting reports on the localization of IDO1 in the human placenta.

IDO1 IN THE CHORIONIC VASCULAR ENDOTHELIUM

In early pregnancy, IDO1 expression is restricted exclusively to immediately subtrophoblastic capillaries (**Figure 2**), and it increases with advancing gestational age. In term placenta, the endothelium of larger vessels in stem villi and some arteries and veins of the chorionic plate stain positive for IDO1 protein, whereas the vessels of the umbilical cord remain IDO1 negative (28, 58, 59) (**Figure 3**). Similar results for chorionic vascular endothelial expression of IDO1 have been described in rhesus monkeys and common marmosets (60). This increase in protein expression correlates with both the amount of mRNA in the placenta and the increase in the placental kynurenine-to-Trp ratio, a surrogate measure of IDO activity. In term placentas at delivery, the kynurenine-to-Trp ratio measured in the blood obtained from vessels of the chorionic plate is far higher than that in the peripheral blood of healthy blood donors (28). This suggests that endothelial IDO1 within placental vessels is highly active beyond the cessation of placental blood circulation at delivery. Consistent with this, endothelial cells isolated from the chorionic plate of term placenta express IDO1 mRNA, in contrast to endothelial cells isolated from human umbilical vein, iliac vein, or aorta (28). Moreover, expression of the aryl hydrocarbon receptor (AhR)

AhR, a receptor for kynurenine, has been reported for syncytiotrophoblasts, the endothelium of large vessels in the chorionic villi, and in the endothelium of umbilical cord arteries and veins (61).

IDO1 IN VASCULAR ENDOTHELIUM OF THE DECIDUA AND THE UTERUS

In endometrium of non-pregnant women, vascular endothelium does not express IDO1 protein, whereas the protein is expressed in HLA-DR-negative endothelium of spiral arteries and in capillaries. In contrast, the HLA-DR-positive endothelium of veins of the decidua is negative for IDO1 as assessed by immunohistochemistry (**Figure 2**). During mid-gestation, endothelial expression IDO1 extends to the inner but not the outer layer of the myometrium (26, 28). Thus, endothelial IDO1 is increasingly expressed the tissue closer to the feto-maternal interface, similar to the situation in the chorion. It is noticeable that constitutive expression of IDO1 in vascular endothelium is limited to the placenta, the uterus, and the lungs (28, 62) (Blaschitz, unpublished observations). In contrast, IDO1 appears to be more generally expressed in the endothelium under conditions of systemic inflammation (63).

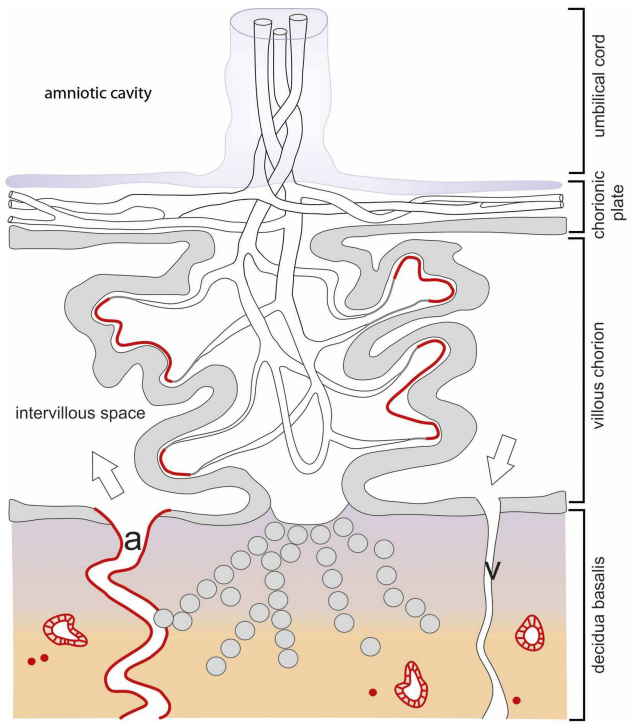

FIGURE 2 | Schematic drawing of the localization of IDO1 in the human placenta during first trimester pregnancy. The chorionic villus is the structural element involved in feto-maternal exchanges. The stem villi originate from the chorionic plate and ramify into villous branches. They consist of a core of mesenchymal connective tissue containing vessels, which are in contact with the fetal vasculature via the umbilical cord. The chorionic villi are covered by a double layer of villous trophoblast (the upper syncytiotrophoblast and the lower cytotrophoblast) separating the fetal closed blood circulation from the intervillous space, which is filled with maternal blood which is supplied via the uterine spiral arteries (a) and discharged via the uterine veins (v). Some of the villi are anchored into the maternal *decidua basalis* by roots built of extra-villous cytotrophoblast cells, which also invade the maternal *decidua*. The IDO1 expression sites are highlighted in red color and refer to the villous subtrophoblastic capillaries, few immune cells of the *decidua*, and the epithelium of uterine glands.

IDO1 IN EPITHELIUM OF THE ENDOMETRIUM AND THE DECIDUA

Expression of IDO1 increases over the course of the menstrual cycle in the surface and glandular epithelium of the endometrium, just as the protein is expressed in cervical glands and epithelium of Fallopian tubes in non-pregnant women. Cervical mucus displays some Trp-degrading activity (26). In first trimester decidua, IDO1 is present in glandular epithelial cells (26, 59).

IDO1 IN THE TROPHOBLAST

There is discrepancy among publications as to whether IDO1 is expressed in trophoblast cells. Earlier publications reported IDO1 to be present in first trimester (59) and/or term placenta syncytiotrophoblast (26, 58, 64) and in extra-villous cytotrophoblast cells (58, 64). Hönig et al. described IDO1 in the invasive extravillous trophoblast in the *decidua basalis* and trophoblast giant cells (58). These observations were challenged in a subsequent publication that also discussed possible reasons for the apparent discrepancies (28). In keeping with this, Wang et al. (65) reported that isolated first trimester trophoblast cells do not constitutively express IDO1 mRNA and protein. However, treatment with polyinosinic–polycytidylic acid [poly(I:C)] (a synthetic

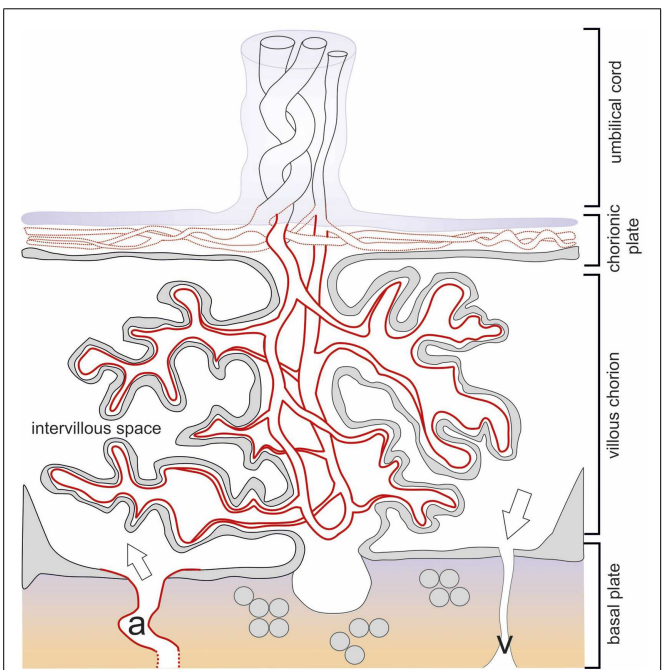

FIGURE 3 | Schematic drawing of the term placenta with the basal plate after delivery. The structures of placental architecture are described in the legend to **Figure 1**. Here, the branching of the villous tree has increased, the villous trophoblast is largely reduced to the syncytiotrophoblast. IDO1 protein is indicated by red color broken red lines indicate partial expression. All endothelia of the vessels of the villous chorion express IDO1, while only part of the vessels of the chorionic plate and none of the umbilical cord vessels are positive. Openings of maternal arteries (a) express IDO1 whereas veins do not.

double-stranded RNA, which mimics viral RNA and is a ligand of the Toll-like receptor-3) induced IDO1 mRNA and Trp-degrading activity in the trophoblasts (65). Conditioned media from poly(I:C)-treated trophoblast cells suppresses T cell DNA synthesis, and IFN-β was identified as the mediator of this effect via the induction of IDO1 (65). In human placental explants, IDO1 mRNA was found after 24 h of culture, the expression increased following LPS stimulation (66).

Recently, expression of IDO1 mRNA was described in cultured third trimester human placental cytotrophoblast cells, with higher expression in male than in female CT cells (67). However, these cytotrophoblast preparations also contained CD34 mRNA (Cvitic and Desoye, personal communication), so that contamination with endothelial cells cannot be excluded. Contaminating endothelial cells may also explain similar findings reported earlier by Dong et al. (68). In mice, placental IDO1 expression was found to be limited to trophoblast giant cells (69).

IDO1 IN OTHER PLACENTAL CELL TYPES

Indoleamine 2,3-dioxygenase-1 expression has been reported in macrophages of the villous stroma (59, 64). However, this finding was contested subsequently by the observation that IDO1-positive chorionic cells consistently co-expressed CD34 (28), suggesting that in the villous stroma IDO1 is restricted to endothelial cells. IDO1 protein is absent from the majority of macrophages and DC in the decidua (70, 71). However, IDO1 can be induced in these cells by treatment with CTLA-4 or IFN-γ (71). Decidual macrophages sorted for $CD14^+$ have been reported to express IDO1 mRNA (72), although the purity of these cells was only 72–90%, so that it cannot be ruled out that contaminating cells rather than macrophages were responsible for the observed presence of IDO1 mRNA. Jones et al. implied the presence of IDO1 in mesenchymal stem cells grown from placentae, based on the observation that these cells suppressed allogeneic T cell proliferation in a manner partly dependent on IDO1 (73). Unpublished data show expression of IDO1 protein in stromal cells of the placental bed *post partum* (Astrid Blaschitz).

TDO AND IDO2

Limited information is available regarding the localization and role of TDO in the placenta. TDO mRNA and protein has been observed in mouse concept and placenta at a time preceding IDO1 expression (50). Dharane et al. reported TDO mRNA to be present in human placental explants (prepared following caesarian section) after 24 h of culture, and its expression increased following *ex vivo* exposure to LPS (66).

Indoleamine 2,3-dioxygenase-2 mRNA has been detected in term and, to a much lower extent, also in first trimester placentae (74). Isolated first trimester and term trophoblast cells as well as the BeWo choriocarcinoma cell line do not express IDO2 mRNA (74). Preliminary observations suggest, however, that both IDO2 and TDO protein are expressed in the human placenta (Astrid Blaschitz, unpublished data).

FURTHER ENZYMES INVOLVED IN Trp DEGRADATION

Kynurenine 3-hydroxylase (KYN-OHase) catalyzes the oxidation of kynurenine to 3-hydroxykynurenine. KYN-OHase has been localized to glandular epithelial cells of first trimester decidua, as well as the syncytiotrophoblast, stroma, and macrophages of first trimester placenta. In term placenta, KYN-OHase expression was confined mainly to vascular endothelial cells of villous blood vessels, and to macrophages within the fetal villus (59). We are aware of only a single report of Tph (is it Tph-1?) in the cytoplasm of human cytotrophoblasts and syncytiotrophoblasts (75).

ROLE OF Trp DEGRADATION

GENERAL ASPECTS

It has been known for decades that IDO1 is induced during infections and displays antimicrobial activity. Originally, induction of IDO has been observed in the lung following application of bacterial LPS (76) and infection with influenza virus (77). Such infection-associated induction of IDO1 was soon found to be mediated by IFN-γ (78). In a variety of different human cell lines, induction of IDO1 by IFN-γ is associated with growth inhibition of intracellular bacteria (such as *Chlamydia psittaci*) and protozoa (*Toxoplasma gondii*), as well an extracellular bacteria (14, 79, 80). In many though not all situations, addition of exogenous L-Trp attenuates growth inhibition, consistent with the notion that limitation of this essential amino acid by IDO1 at least in part explains the antimicrobial activity observed. The antimicrobial activity of IDO1 in human endothelial cells has been reviewed recently (81).

Oxidative degradation of Trp leads to both, a local depletion of Trp and formation of Trp metabolites. Both aspects are biologically relevant and have recently been reviewed (82), see also **Table 2**. For example, the Trp metabolites kynurenine (83) and kynurenic acid (84) are ligands of the AhR. Following ligand binding, this cytosolic transcription factor translocates into the nucleus where it binds to response elements in the promoters of target genes (85). In this way, kynurenine displays immunosuppressive properties by generating regulatory T (T_{reg}) cells (86). The immunogenicity of DC is decreased, as AhR signaling induces DC to express IDO1 and IL10 (86–89). 3-Hydroxyanthranilic acid (3-HAA) as well as the other kynurenine metabolites anthranilic acid, quinolinic acid, and nicotinamide do not directly activate the AhR. Hydroxykynurenine does display an effect which, however, is weaker than kynurenine (86). On the other hand, 3-HAA has been suggested to prime DC for expressing reduced levels of pro-inflammatory cytokines, enhanced levels of TGF-β, and inducing T_{reg} cells (90, 91). The depletion of Trp also triggers amino-acid-sensing signal transduction pathways, such as the GCN2 kinase and inhibition of mTOR (92). The former pathway leads to cell-cycle arrest and functional anergy in $CD8^+$ T cells (93). Lymphocytes are specifically affected by Trp depletion. This is because in these cells, IFN-γ does not induce tryptophanyl-tRNA synthetase so that lymphocytes are inefficient in competing for Trp compared with other cells (94, 95). In T helper cells, Trp depletion inhibits differentiation to Th_{17} cells (96) and it promotes *de novo* T_{reg} differentiation (97). IFN-γ is the main inducer of IDO in DC for the prevention of hyperinflammatory responses, whereas TGF-β confers regulatory effects on IDO independent of its enzymatic activity. In this case, IDO1 appears to act as a signaling molecule, by promoting complex formation of IDO1 with the tyrosine protein phosphatases SHP-1 and SHP-2. This leads to long-term tolerance via activation of SHP-1 phosphatase activity in plasmacytoid DC (98). Moreover,

Table 2 | Pathways of immunomodulation by IDO1 and kynurenine pathway metabolites.

Pathway	Functional consequence	Reference
IDO1 acting as a signaling molecule by complex formation with SHP-1 and SHP-2	Long-term tolerance in plasmacytoid DC	(98)
Depletion of Trp, activation of GCN2 kinase, and inhibition of mTOR in IDO-expressing cells	Cell-cycle arrest and functional anergy in $CD8^+$ T cells	(92, 93)
Binding of kynurenine to AhR in DC and T cells	Decrease in immunogenicity of DC, and generation of T_{reg} cells	(86, 89)
3-HAA acting on DC (possibly via blocking the JNK and p38 MAPK pathways)	Decrease in expression of pro-inflammatory cytokines, increase in expression of TGF-β, and induction of T_{reg} cells	(90, 91)

IDO1 plays an important role in the self-limitation of the immune response. Thus, short-term (4 h) activation of DC with IFN-γ and LPS leads to the induction of pro-inflammatory cytokines, while long-term (48 h) activation favors immunosuppression and tolerance via IDO1 signals (36, 82).

As stated above, on one hand IDO1 generates metabolic products that induce T_{reg} cells, on the other hand T_{reg} cells can induce IDO1 expression (31). This suggests the presence of a positive feedback loop and raises the question of the limitation of this mutual interaction.

Indoleamine 2,3-dioxygenase-1-based suppression of immune reactions against foreign MHC-I molecules and minor histocompatibility antigens mediates feto-maternal tolerance (99, 100) also via induction of T_{reg} cells, which play a critical role in suppressing the anti-fetal immune response (101). The role for this in pregnancy has been questioned based on the fact that matings of allogeneic male and female $IDO1^{-/-}$ mice yield viable offsprings (69). However, IDO2 and/or TDO may compensate for IDO1 and promote Trp metabolism in these mice, particularly as it is increasingly recognized that TDO expression is not limited to the liver. Rather, the enzyme is also present in mouse placenta (50).

Indoleamine 2,3-dioxygenase-1 mediates tolerance against tumors (102), and IDO inhibitors are being tested in clinical trials with patients suffering from cancer and chronic infections (103). Whereas IDO1 has been found in DC of tumor-draining lymph nodes (24), IDO1 could not be detected in regional lymph nodes of uteri of pregnant mice (P. Ack, Astrid Blaschitz, unpublished observations).

Trp metabolites also display non-immunological functions: for example, quinolinic acid and kynurenic acid have neuroactive properties (104–106), and 3-hydroxykynurenine and 3-hydroxyanthranilic acid display antioxidant activity (107). IDO1-mediated degradation of Trp in the endothelium of mice infected with malaria parasites or induced by endotoxemia contributes to the relaxation of arteries and to the control of blood pressure (63). Originally, kynurenine was reported to mediate arterial relaxation under these pro-inflammatory conditions, in part via activation of soluble guanylate cyclase. These findings were based on studies with commercial preparations of kynurenine (63). However, more recently, HPLC-purified kynurenine was found to be inactive, and IDO1-mediated vasorelaxation has been attributed to a yet to be identified Trp metabolite (Proceedings of the British Pharmacological Society at http://www.pa2online.org/abstract/abstract.jsp?abid\protect\kern+.1667em\relax=\protect\kern+.1667em\relax31322). Most recently, IDO1 has been reported to mediate angiotensin II-induced production of reactive oxygen species, apoptosis, and endothelial dysfunction (108).

The biological role of IDO2 is as yet unclear. Its Trp-degrading activity is much lower or even undetectable (15) compared with IDO1 (41), at least in the *in vitro* ascorbate/methylene blue assay commonly used (14). However, the probable physiological electron donor cytochrome b_5 reduces recombinant mouse IDO2 and it increases its activity *in vitro* compared with that observed in the ascorbate/methylene blue assay (16). Human IDO2 expression is not able to rescue a yeast strain auxotrophic for nicotinic acid, suggesting it does not have sufficient activity to supply NAD^+ in yeast (109). On the other hand, chemokine-induced production of kynurenine in human basal carcinoma cells correlated with the induction of mRNA expression of IDO2, but not IDO1 (110). It has been suggested that IDO2 activity is determined by the presence of particular co-factors that may be present only in certain cell types or conditions (40).

The high expression of TDO in the liver (111) makes it the key enzyme regulating circulating concentrations of L-Trp, and it is believed to have a major role in supplying NAD^+ (112). $TDO^{-/-}$ mice display increased plasma concentrations of Trp, leading to increased serotonin biosynthesis and alterations in behavior and neurogenesis (113). In analogy to IDO1, TDO activity also has been implicated in the inhibition of immune responses against tumors (56).

Hydroxylation by Tph-1 may also contribute to the exhaustion of Trp in a microenvironment, and it too has immunoregulatory effects. Tph-1 deficiency breaks allograft tolerance, induces tumor remission, and intensifies neuroinflammation. These effects are independent of the downstream product serotonin (114).

FUNCTIONAL ASPECTS OF PLACENTAL Trp CATABOLISM

Localization of IDO1 in the utero-placental unit leaves us to speculate about its role at this site in particular: IDO1 in the epithelium of the mucosal surface and the glands of the endometrium and the decidua, and secretion of IDO [reflected in Trp-degrading activity in the cervical mucus (26)) may provide a mechanism of innate immunity against ascending infections of the female reproductive tract with intracellular bacteria such as *Chlamydia* but also against extracellular pathogens.

Endothelial cells may act as semi-professional antigen-presenting cells (115) and, as they degrade Trp, may contribute to the suppression of the immune response (31). Inhibition of IDO activity improves the ability of human umbilical vein endothelial cells to stimulate allogeneic T-cell responses. Transfection of these cells or human saphenous vein endothelial cells with the IDO1 gene, stimulates allogeneic T-cell responses and induces anergy in allospecific T cells (116). IDO1-positive endothelial cells of both the fetal and the maternal part of the placenta do not coexpress HLA-DR, which renders their contribution to the establishment and maintenance of feto-maternal tolerance unlikely. In situations where pro-inflammatory stimuli act on and induce MHC-II expression in placental endothelial cells, the ensuing immune response may, however, be modulated by endothelial IDO1. An antibacterial and antiparasitic role of endothelial IDO1 may be anticipated, and this might contribute to protection of the feto-placental unit against infection (81).

Endothelial catabolism of Trp by IDO1 in the villous chorion may also contribute to the regulation of the placental vasotonus. Preliminary data suggest that preconstricted human placental arterial rings relax in response to added Trp, and that this relaxation is partly inhibited by 1-MT (Roland Stocker, Peter Sedlmayr, unpublished observations). As the maintenance of placental perfusion is of crucial importance to the fetus, IDO1-induced relaxation of placental vessels may play an important role for feto-placental growth in the course of pregnancy. Moreover, on the other side of the interface, expression of IDO1 in the endothelium of spiral arteries may induce vasodilation and contribute to feeding blood into the intervillous space. This suggested role of IDO1 at this location might be a phenomenon particularly relevant after the first trimester of pregnancy, once the endovascular trophoblast plugs have vanished.

ALTERED Trp DEGRADATION IN PREGNANCY PATHOLOGY

There are reports of reduced placental IDO1 mRNA, protein, and placental Trp-degrading activity in preeclampsia, including a correlation between reduced placental Trp-degrading activity and the severity of the disease (27, 117–119). Not all studies, however, take into account that the gestational age of preeclamptic placentae needs to be matched to control placentae, as placental IDO1 expression normally increases with gestational age. Whereas the kynurenine-to-Trp ratio in plasma increases during normal pregnancy, in preeclampsia it remains unchanged and similar to that in non-pregnant women (117, 120).

In a model of pregnant mice carrying hemiallogeneic concept, pharmacological inhibition of IDO1 was reported to result in the mothers developing high blood pressure, proteinuria, and impairment of the local placental circulation, analogous to the lesions characteristic of human preeclampsia (121). In this model, 8-hydroxy-2′-deoxy-guanosine (8-OHdG, a marker for oxidative damage to DNA) was found to be higher in preeclamptic than normotensive pregnancies. Moreover, immunohistochemical signals of 8-OHdG inversely correlated with Trp-degrading activity, suggesting that a decrease in the antioxidant activity of IDO1 contributed to the pathogenesis of this disorder (122).

So far, little is known regarding the role of IDO1 in the context of intrauterine growth restriction (IUGR, synonymous with fetal growth restriction). There is one (however not in-depth) report stating that placentae in this disease show decreased IDO activity (123). Current interest focuses on a possible pathogenetic role of endothelial IDO1: in IUGR with and without preeclampsia chorionic vessels show reduced expression of IDO1, as assessed by immunohistochemistry, and a decrease in the relaxation of placental arteries induced *ex vivo* by added Trp (Roland Stocker and co-workers, unpublished).

Indoleamine 2,3-dioxygenase-1 expression in monocytes, macrophages, and DC of the decidua and of peripheral blood increases in normal pregnancy after treatment with CTLA-4 or IFN-γ whereas it decreases in spontaneous abortion (71). In allogeneic pregnancies in mice, application of 1-MT leads to T cell-mediated hemorrhagic necrosis and rejection of the conceptus soon after implantation (99, 100). This situation is similar to that of *in vivo* administration of an antibody against the T cell receptor β chain (124), and may be analogous to early pregnancy loss in humans, also called "chemical pregnancies."

CONCLUSION

Trp-degrading enzymes in the placenta lead to a deprivation of tryptophan and the formation of biologically active tryptophan metabolites at and near the sites of catabolism. The combination of these two processes has important consequences for the establishment and maintenance of feto-maternal immune tolerance. In addition, it may affect placental circulation and growth, as well as modulate local antimicrobial activity, the precise underlying mechanisms of which await elucidation. In particular, at present we lack detailed information on the expression, localization, and specific roles of IDO2 and TDO in the placenta. The occurrence of allogeneic pregnancies in IDO1$^{-/-}$ mice suggests redundancy for the role of IDO1 in protecting against alloreactive maternal T cells, the mechanism of which needs to be uncovered. This might be done, e.g., by using various combinations of IDO1, IDO2, and TDO double gene knockout mice, perhaps in combination with pharmacological inhibition of the third Trp-oxidizing enzyme where appropriate.

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Regulation of the anti-inflammatory cytokines interleukin-4 and interleukin-10 during pregnancy

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Inflammation mediated by both innate and adaptive immune cells is necessary for several important processes during pregnancy. Pro-inflammatory immune cell activation plays a critical role in embryo implantation, placentation, and parturition; however dysregulation of these cells can lead to detrimental pregnancy outcomes including spontaneous abortion, fetal growth restriction, maternal pathology including hypertensive disorders, or fetal and maternal death. The resolution of inflammation plays an important role throughout pregnancy and is largely mediated by immune cells that produce interleukin (IL)-4 and IL-10. The temporal and spatial aspects of reducing inflammation during pregnancy represent a complex process that if not functioning optimally can lead to persistent inflammation and pregnancy complications. In this review, we examine how immune cells that produce IL-4 and IL-10 are regulated throughout pregnancy as well as the effects that reduced IL-4 and IL-10 signaling has on fetal and maternal physiology.

Keywords: inflammation, pro-inflammatory cytokines, anti-inflammatory cytokines, pregnancy disorders, immune cells

INTRODUCTION

The immunological features of normal pregnancy are unique as the maternal immune system has to accept a semi-allogeneic fetus, a product of two histo-incompatible individuals. Medawar proposed that in order to accept a half-foreign fetus, the mother needs to be in an immunosuppressed state (1). Recent progress in our understanding suggests that the maternal immune system not only needs to be suppressed but at the same time also needs to protect the mother and the growing fetus from infection during pregnancy. Thus, a successful pregnancy depends on the ability of the mother's immune system to become tolerant to paternal antigens as well as the ability to reject the fetus in case of pathogen infection.

The maternal immune response is regulated by a complex array of cytokines to protect the conceptus and promote proper growth and development of the placenta. Wegmann and colleagues suggested that during pregnancy there is a T-helper (Th) 2 bias to promote tolerance to the half-foreign fetus and Th1 cytokines are detrimental to the tolerance of the conceptus, similar to allografts in transplant recipients (2–5). It has been found that during tolerance induction to an allograft there is a decrease in Th1 cytokines such as interleukin (IL)-2 and IFNγ and an increase in Th2 cytokines including IL-4 and IL-10. Conversely, high levels of IL-2 and IFNγ were detected in rejecting allografts (6–8). Existing data suggest that Th2 bias in pregnancy is an oversimplified model and that during the various stages of pregnancy the pro-inflammatory and anti-inflammatory cytokine milieu is dynamically modulated. The first stage of pregnancy, which involves a blastocyst implanting into the uterus, is a predominantly pro-inflammatory phase. Localized activation of inflammatory mediators occurs and the mother's immune system repairs the damage done by the invading blastocyst. The second phase of pregnancy is a predominantly anti-inflammatory phase. Th2 cytokine skewing during the second phase of pregnancy can be systemic or local at the feto-maternal interface. The last phase of pregnancy is parturition, which causes contraction of the uterus and again, the pro-inflammatory milieu is predominant. Inflammation is tightly controlled during all stages of pregnancy, however excessive and persistent maternal inflammatory responses are associated with adverse pregnancy outcomes.

Pregnancy disorders such as preterm birth (PTB), fetal growth restriction (FGR), and preeclampsia (PE) are often associated with infection during pregnancy (9–11). Infection due to bacteria, viruses, and parasites, which normally induce a Th1 immune response can impact placental development and function and ultimately fetal survival (12). Th1 responses induce IFNγ that in turn propagates Th1 responses by up-regulating IL-12 receptor expression and inhibiting Th2 responses.

Preterm birth is associated with increased production of pro-inflammatory cytokines and chemokines such as IL-1β, IL-6, TNFα, and CXCL8 (13). These cytokines induce prostaglandin synthesis in the placental tissues that triggers preterm labor (14, 15). Likewise, maternal inflammation due to infection is an important contributor to the development of FGR (16). Administration of the anti-inflammatory cytokine IL-10 can attenuate FGR induced in rats by lipopolysaccharide (LPS) or infection with *E. coli* (17). The addition of exogenous IL-10 reduces fetal resorption in pregnancies of CBA/J × DBA/2 mice. Anti-IL-10 neutralizing antibodies increase fetal loss and lead to growth defects after birth (18, 19). PE is also associated with an exaggerated maternal inflammatory response. Zenclussen and colleagues demonstrated that adoptive transfer of the Th1 cells into pregnant mice was associated with the development of PE-like symptoms (20). Pro-inflammatory

cytokines are not only increased in PE but the production of the anti-inflammatory cytokines IL-4 and IL-10 are also known to be decreased. In this review, we highlight how immune cells that produce IL-4 and IL-10 are modulated during pregnancy and their role in adverse pregnancy outcomes.

IMMUNE CELLS THAT PRODUCE IL-4 AND IL-10 AND THEIR ACTIONS

Interleukin-4 and IL-10 are pleiotropic anti-inflammatory cytokines that function mainly by suppressing the pro-inflammatory milieu. Several different immune cells that produce IL-4 are activated T cells, mast cells, basophils, eosinophils, and NKT cells (21, 22). IL-4 aids in the polarization of antigen-stimulated naïve Th cells into Th2 effector cells as well as propagates Th2 responses by binding to its receptor, IL-4Rα, and activating the signal transducer and activator of transcription (STAT) six signaling pathway (23–26). STAT6, through the induction of a zinc-finger transcription factor GATA3 (GATA-binding protein 3), might directly suppress Th1 cell development by silencing IFNγ expression (27). Recent studies also indicate that IL-4 enhances Th2 immunity by inhibiting Th1 responses through the repression of IL-12 signaling (28). Several studies implicate a role for IL-4 in regulatory T cell (Treg) development and maintenance IL-4 signaling through STAT6 is important for FoxP3 mRNA expression and protein production in natural Tregs (29–32). IL-4 also induces the formation of inducible Tregs from naïve CD4+ T cells. Thus, IL-4 not only mediates Th2 cell function but also plays a part in the regulation of Tregs which play an important role in successful pregnancies.

Interleukin-10 production was first determined in Th2 cells and was initially thought to be only produced by immune cells, but later studies demonstrated that IL-10 is also produced by non-immune cells (33). Immune cells that produce IL-10 include subsets of T cells such as Th1, Th2, and Th17, as well as monocytes, macrophages, dendritic cells, human B cells, granulocytes, eosinophils, and mast cells. Non-immune cells that produce IL-10 include keratinocytes, epithelial cells, and tumor cells. IL-10 primarily exerts its anti-inflammatory effect by inhibiting pro-inflammatory cytokines such as IL-1, IL-6, IL-12, and TNF as well as chemokines (34). IL-10 also inhibits antigen presentation by blocking MHC class II expression and co-stimulatory molecules such as CD80 and CD86 (35). IL-10 exerts its biological effect by binding to its receptor which is composed of two subunits, IL-10R1 and IL-10R2 (35). Initially, IL-10 binds its cognate receptor IL-10R1 and the binding of IL-10R2 is specific to initiate a signaling cascade. IL-10 then activates Janus kinase (JAK) and STAT pathways. This recruits Tyk2 and Jak1 to the receptor complex and induces phosphorylation of the receptors leading to transcription of IL-10-regulated genes (36). IL-10 production is also associated with other types of immune cells such as macrophages and myeloid-derived suppressor cells (MDSCs). Following Toll-like receptor (TLR) activation in macrophages and MDSCs, the signaling cascade comprising the adaptor molecule TIR-domain-containing adaptor protein inducing IFNβ (TRIF) is activated. The extracellular signal-regulated kinase 1 (ERK1), ERK2, p38, and nuclear factor-κB (NF-κB) pathways are activated leading to the production of IL-10 and several other genes. IL-10 up-regulates its own production by modulating tumor progression locus 2 (TPL2) expression (34). Both IL-4 and IL-10 mediate signaling between immune cells and also regulate recruitment, activation, and suppression of both immune and non-immune cells.

MODULATION OF IL-4 AND IL-10 DURING PREGNANCY

Anti-inflammatory cytokines perform a multitude of functions during normal pregnancy by promoting placental formation, modulating trophoblast invasion and differentiation, inducing placental proliferation and angiogenesis, and inhibiting pro-inflammatory cytokines. IL-4 is detectable at the feto–maternal interface during all phases of pregnancy (37). IL-4 is produced not only by immune cells of the placenta but also by the maternal decidua, amniochorionic membranes, cytotrophoblasts, and both maternal and fetal endothelial cells (38, 39). IL-4 production is increased in the gravid state and levels of IL-4 increase throughout normal pregnancy (40). Progesterone is a known inducer of IL-4 and together they act to inhibit Th1 responses during pregnancy. Given the important role of IL-4 in suppressing inflammation, it is surprising that IL-4-knockout mice have normal pregnancies with respect to fetal growth and development (41). This would suggest that the role of an individual cytokine may not be crucial to the success of pregnancy but rather depends on the complex interplay with other cytokines in a spatiotemporal manner.

Interleukin-10 has been shown to be constitutively expressed in placental villous trophoblasts but not in extravillous trophoblasts (42). Additionally, uterine NK cells (uNK cells), monocytes, and Tregs in the decidua are also important producers of IL-10 (36). IL-10 acts on its receptors (IL-10R) that are expressed on several cell types including placental trophoblasts, decidual stromal cells, macrophages, and uNK cells. In mice, IL-10 is expressed throughout pregnancy and peaks at gestational day 12 (37). To determine the exact role of IL-10 in pregnancy, pregnant IL-$10^{-/-}$ mice were compared to pregnant wild type (WT) mice and no change in litter size or development were noted indicating that IL-10 is not essential for pregnancy (41). However, IL-10 plays a role in placental growth and remodeling because IL-$10^{-/-}$ mice exhibited increases in placental size and maternal blood sinuses (43). IL-10 is not essential for the growth and development of the fetus in mice but rather it plays an important role to inhibit excessive inflammation. Pregnant IL-$10^{-/-}$ mice are susceptible to low doses of LPS and CpG (a TLR 9 agonist) compared to WT mice (44, 45). These results suggest that IL-10 acts as a protective agent during infection and deficiency of IL-10 exacerbates inflammation in mice. Normal pregnant women were determined to have increased IL-10 production during the first and second trimesters but not in the third trimester (46). Moreover, IL-10 production decreases prior to labor and delivery of the fetus and placenta and increases post labor (47). Precise regulation of IL-4 and IL-10 are important to curtail maternal inflammation and allow crosstalk between the placental decidua and the invading fetal trophoblasts at different stages of pregnancy.

IL-4 AND IL-10 IN SPONTANEOUS ABORTION AND FETAL GROWTH RESTRICTION

It has been well documented that the lack of fetal tolerance is largely mediated by Th1 cells. Their recruitment from the maternal

circulation into the feto–maternal interface and their production of pro-inflammatory cytokines coupled with a decrease or lack of increase in anti-inflammatory cytokines can lead to a spectrum of pregnancy disorders (**Figure 1**). Various cells that produce IL-4 and IL-10 including NK cells, T cells, regulatory B cells, and others are dysregulated and fail to increase production of these anti-inflammatory cytokines at the appropriate time and location.

Immunological models of spontaneous abortion and FGR in animals include the female CBA/J × male DBA/2J mating with and without stress, excessive TLR activation in early pregnancy (LPS and poly I:C), and transvaginal rIL-17 administration, and all of these are associated with decreased production of IL-4 and IL-10. Several studies have reported that abortion-prone CBA/J females demonstrate decreased circulating and placental levels of IL-4 and IL-10 (18, 48–52) and that experimental therapeutics at various time points including a B7 monoclonal antibody, adenoviral-mediated heme oxygenase-1 overexpression, progesterone or its derivatives, adoptive transfer of Tregs, or alloimmunization, all decrease abortion rate and this is associated with increased IL-4 and IL-10 and/or Th2/Th1 ratios (18, 48–50, 52–55). A more direct study demonstrated that administration of IL-4 as well as IL-4 and IL-10 decreased the resorption rate in these mice (51). In animals, TLR activation in early pregnancy induces spontaneous abortion or resorption and this is regulated by IL-10 as rIL-10 administration was able to prevent these effects (18, 56–59). Further support for IL-10 and its role in preventing spontaneous abortion was demonstrated in mice administered rIL-17 transvaginally on gestational day 1 which decreased decidual IL-10 levels and induced abortion (53). Adoptive transfer of IL-10-producing Tregs from pregnant mice increased IL-10 levels and decreased abortion rates in these mice (53).

These experimental studies are supported by clinical observations in women who have had recurrent or initial reproductive failures. Production of IL-4 and IL-10 by NK cells, regulatory B cells, T cells, and others is decreased in women who have had spontaneous abortions, recurrent miscarriages, small for gestational age babies, and infertility. Several studies have reported low levels of IL-4, IL-4-producing cells, and Th1 cytokine/IL-4 ratios in women with spontaneous abortions (60–65). With respect to IL-10, numerous studies have reported low levels of IL-10, IL-10-producing cells, and Th1 cytokine/IL-10 ratios in women with spontaneous abortions (40, 61–71). Additionally, women experiencing multiple unsuccessful *in vitro* fertilization cycles have increased TNFα+/IL-4+ and TNFα+/IL-10+ T cell ratios (65). A recent study found that low levels of circulating anti-inflammatory cytokines during early gestation were associated with habitual miscarriages in women (72). Further support for an important role of IL-4 and IL-10 in preventing reproductive failure was provided by a study in which i.v. Ig therapy in women with recurrent spontaneous abortions increased IL-4 and IL-10 levels and decreased the ratio of IFNγ+/IL-4+ T cells (73). Together these data support the notion that a lack of early, appropriate anti-inflammatory responses and excessive inflammation can lead to reproductive failure. Further experimental and clinical studies in which augmentation of IL-4- and IL-10-producing immune cells would determine whether this would be sufficient to induce successful pregnancies.

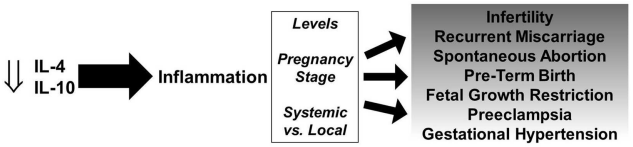

FIGURE 1 | Role of IL-4 and IL-10 in pregnancy disorders. Decreased levels of IL-4 and IL-10 promote persistent inflammation and depending on the levels, stage of pregnancy, and systemic vs. local effects, can lead to a spectrum of gestational complications.

IL-4 AND IL-10 IN PREECLAMPSIA

One of the characteristics and potential cause in some cases of PE is uncontrolled amplification of the maternal immune system. The excessive pro-inflammatory state seen in PE may be partly due to increased production of pro-inflammatory cytokines and/or decreased production of anti-inflammatory cytokines. Indeed, PE-like symptoms develop in rats following infusion of IL-6 or TNFα indicating that pro-inflammatory cytokines contribute to the development of PE (74, 75). Recent experiments from our lab indicate a pathogenetic role of decreases in the anti-inflammatory cytokine IL-4 in PE. We demonstrated that although fetal growth and development was not affected in IL-$4^{-/-}$ mice, mild PE-like symptoms such as hypertension and proteinuria developed during pregnancy. Additionally, deficiency of IL-4 induced systemic and placental inflammation in mice. These experiments implicate that deficiency of IL-4 contributes to mild cardiovascular and renal effects and the protective role of IL-4 is more pronounced during infection. In support, we demonstrated that pregnant IL-$4^{-/-}$ mice exhibited even further increases in inflammation and PE-like symptoms following viral mimetic activation of TLR3. Importantly, non-pregnant IL-$4^{-/-}$ mice did not exhibit hypertension and proteinuria at baseline or following TLR3 activation (76). These observations also correlate well with numerous clinical studies in which women with PE have been reported to have decreased IL-4 levels and increased circulating levels of the soluble IL-4 receptor compared with normotensive pregnant women (77–79). These studies establish a role for decreased IL-4 in the development of PE and also indicate that administration of IL-4 may be a viable treatment option for women with PE.

Similar to IL-4, several studies document the importance of IL-10 in preventing PE. Serum from PE patients induces the clinical features of PE such as hypertension, proteinuria, and FGR in IL-$10^{-/-}$ mice. PE serum induced HIF-1α in the placenta which may have triggered production of the anti-angiogenic factors sFlt-1 and sEng and also induced renal pathology and poor spiral artery remodeling. Endovascular capillary tube formation is also significantly disrupted by serum from PE patients in these IL-$10^{-/-}$ mice. However, serum from healthy women or women with PE administered to non-pregnant animals failed to induce any PE-like features (80). In another study by Lai et al. deficiency of IL-10 coupled with hypoxia induced severe PE-like features including renal pathology, proteinuria, and hypertension. Moreover, increased expression of anti-angiogenic factors, apoptotic pathways, and placental injury were noted. Expectedly, recombinant IL-10 administration reversed the hypoxia-induced features

in pregnant IL-10$^{-/-}$ mice confirming the protective role of IL-10 in PE (81). In our studies, pregnant IL-10$^{-/-}$ mice exhibit mild hypertension, endothelial dysfunction, and proteinuria only during pregnancy. In addition, PE-like symptoms were augmented in IL-10$^{-/-}$ mice following activation of TLR3 during pregnancy (82). Clinical studies further support reduced production of IL-10 from patients with PE (83).

Based on the aforementioned studies, we hypothesized that administration of either of the anti-inflammatory cytokines IL-4 or IL-10 or co-treatment with both recombinant IL-4 and IL-10 may improve outcomes in TLR-activated PE mice. Administration of IL-4, IL-10 alone, or IL-4/IL-10 co-treatment during gestation normalized blood pressure and endothelial function in mice treated with a TLR3 agonist. IL-4/IL-10 co-treatment had the most beneficial effect on fetal development and renal function as well as decreased the levels of the pro-inflammatory cytokines IL-6, IFNγ, and TNFα (84). These studies raise the possibility of using anti-inflammatory cytokines in combination as a therapeutic option for women with PE.

CONCLUSION

The role of inflammation is important and necessary for successful pregnancies, however aberrant and persistent inflammation and the lack of resolution by anti-inflammatory cytokine-producing cells can lead to a variety of pregnancy disorders depending on various factors (**Figure 1**). IL-4 and IL-10 play crucial roles in the success of pregnancy and there is strong evidence that a deficiency in IL-4 and/or IL-10 contributes to infertility, spontaneous abortion, PTB, FGR, and hypertensive disorders of pregnancy.

Most studies to date have aimed to determine the role of each individual cytokine which has generated important findings and improved our understanding of the role of anti-inflammatory mediators during pregnancy; however there is considerable redundancy among cytokines and within an immune response. Integrative studies that take into context the local environment and cytokine milieu, especially during the gravid state, are necessary to determine how cytokine–cell and cell–cell communication influences local and systemic inflammation and the physiological effects during pregnancy. Novel therapies that target the augmentation of multiple anti-inflammatory cytokines including both IL-4 and IL-10 may elicit better effects than a single anti-inflammatory cytokine targeting therapy. The challenge will be in determining when, where, and how to achieve this during gestation in order to produce a healthy, successful pregnancy.

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Innate immune system and preeclampsia

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Normal pregnancy is considered as a Th2 type immunological state that favors an immune-tolerance environment in order to prevent fetal rejection. Preeclampsia (PE) has been classically described as a Th1/Th2 imbalance; however, the Th1/Th2 paradigm has proven insufficient to fully explain the functional and molecular changes observed during normal/pathological pregnancies. Recent studies have expanded the Th1/Th2 into a Th1/Th2/Th17 and regulatory T-cells paradigm and where dendritic cells could have a crucial role. Recently, some evidence has emerged supporting the idea that mesenchymal stem cells might be part of the feto-maternal tolerance environment. This review will discuss the involvement of the innate immune system in the establishment of a physiological environment that favors pregnancy and possible alterations related to the development of PE.

Keywords: preeclampsia, mesenchymal stem cells, immunomodulation, Th1–Th17, Th2-Treg

INTRODUCTION

Preeclampsia (PE), its complications and associated pathologies, have become one of the main causes of maternal and fetal morbidity and mortality in the world (1), causing nearly 40% of premature births delivered before 35 weeks of gestation and complicating around 2–8% of all pregnancies worldwide. Moreover, PE has been strongly associated with an increased risk of later-life death due to cardiovascular disease, independent of other risk factors (2–4).

Preeclampsia is classically defined as the new onset of hypertension during the second half of pregnancy accompanied by significant proteinuria (5). Despite the breakthroughs in the understanding of PE's etiopathogenesis, the physiopathology that triggers the disease is still not clearly elucidated. Nevertheless, it seems clear that the development of PE requires the presence of a placenta, since the clinical syndrome will not develop in the absence of a placenta and it disappears soon after placental delivery (6). It is also widely accepted that the pathophysiological process of PE begins with an abnormal trophoblast invasion early in pregnancy, which produces increased placental oxidative stress contributing to the development of systemic endothelial dysfunction in the later phases of the disease. This leads in turn to the characteristic clinical manifestations of PE.

ETIOPATHOGENESIS OF PE: A SILENCING START EARLY IN PREGNANCY

During the first weeks of a normal gestation, after the blastocyst makes contact with the maternal decidua, cytotrophoblast cells proliferate forming cell columns intruding maternal tissue (7). From the tip of these anchoring villous structures, extravillous trophoblast (EVT) cells derived from this proliferating cytotrophoblast, invade the maternal decidua differentiating further into interstitial and endovascular trophoblast cells. The invasion process begins at the center of the placental bed, and expands progressively to the lateral areas, like a ring-shape spread. During the interstitial invasion, the compact decidual tissue is "swamped" by interstitial EVT cells that, from 8 weeks onward, can be seen both in the inner myometrium zone of the placenta – where they stop the invasive process – and clustered around blood vessels (8). At the same time, endovascular trophoblast cells migrate into the maternal spiral arteries in order to plug these vessels. Around 10–12 weeks of gestation, trophoblast plugs begin to dissolve and endovascular trophoblast replace maternal endothelial lining as far as the inner third of myometrium, degrading the muscular and elastic component of the vessel walls resulting in the formation of low-resistance vessels that are required for adequate uteroplacental circulation and fetal growth (7, 9). Thus, a new onset of maternal blood flow into the intervillous space begins. A deficient trophoblast invasion process and failures in the spiral artery remodeling transformation have been demonstrated to be associated with the development of placental diseases such as PE (10, 11), but the trigger of these altered processes is still not well understood.

Regarding abnormal trophoblast invasion process, in PE the maternal vessels, such as spiral arteries, are poorly remodeled. In these altered vessels, the diameter is diminished in comparison with normal remodeled vessels, and also the extent of remodeling process is decreased. Further, the vascular smooth muscle layer remains surrounding PE remodeled vessels, contributing to a contractile tone of these arteries. This observation is in accordance to the idea that a maternal pulsatile blood flow to the placental bed could induce hypoxia–reperfusion events that can be related to placental hypoxia, and placental oxidative stress observed in PE (12).

The trophoblast invasion process and finally the successful in pregnancy establishment relies on an orchestrated interaction between trophoblast-derived cells and maternal tissue that is crucial for normal pregnancy and that might give clues for the understanding of PE development. In this regard, the maternal immune system plays a key role, allowing the interaction of two immunologically different beings, the embryo and mother.

PREECLAMPSIA DEVELOPMENT AND THE IMMUNE SYSTEM

Several hypotheses have been proposed to explain the abnormal trophoblastic invasion early in pregnancy associated with PE, many of them suggesting that it might be triggered by an altered maternal immune response or a defective development of maternal tolerance to the semi-allogeneic fetus (13–17). Epidemiological evidence supporting this idea has been published by many groups (18–20), suggesting the importance of the maternal immune system in the pathogenesis of PE.

In order to elucidate if the deficient invasion of trophoblast observed in PE might be due to an alteration of the immune-tolerance environment in the decidua, different studies have been performed in order to characterize the immune milieu of these patients. An excessive activation of neutrophils and monocytes in PE patients (circulating and in the decidua) have been described by many groups (21–26). These monocytes have been found to spontaneously synthesize greater amounts of pro-inflammatory cytokines such as IL-1b, IL-6, and IL-8 (27). Furthermore, $CD4^+$ and $CD8^+$ T-lymphocytes along with natural killer (NK) cells and dendritic cells (DCs) have also been found to respond differently in PE women compared to normal pregnancies, tending to a pro-inflammatory response, similar to that seen in non-pregnant women, instead of the immunotolerant and anti-inflammatory response seen in normal pregnancies (28–30). Moreover, DCs demonstrate a pro-inflammatory bias secondary to dysregulation of toll-like receptors (TLRs) (31) and decidual NK cells, which play a particularly important role in regulating cellular interactions in successful placentation by promoting placental development and maternal decidual spiral artery modifications, are found to secrete lower amounts of invasion-promoting factors when taken from decidual tissue from women with altered uterine artery Doppler (non-invasive screening for PE development) (17).

PREECLAMPSIA: A Th1–Th17/Th2-Treg IMBALANCE

Another important immune aspect of PE development is the Th1/Th2 imbalance. Normal pregnancy is considered to be a Th2 type immunological state, which favors an immunotolerant environment for the prevention of fetal rejection (32) (**Figure 1A**). On the other hand, PE pregnancies have been characterized as a maternal pro-inflammatory state with Th1 predominance: increased plasma levels of pro-inflammatory cytokines have been described by different authors, mainly during the second and third trimester of pregnancy (33, 34) (**Figure 1B**). However, the Th1/Th2 paradigm has been proven incomplete to fully explain the functional and molecular changes observed during normal/pathological pregnancies. Recent studies have described several other immune cells involved in this process, expanding the Th1/Th2 paradigm into the Th1/Th2/Th17 and regulatory T cells (Treg) paradigm, introducing Treg as regulators of Th17 lymphocytes and other immune cell types involved in the feto-maternal tolerance (28, 35).

Th17 cells, a relatively novel $CD4^+$ lymphocyte subpopulation associated with Th1 cytokine profile, are characterized by the

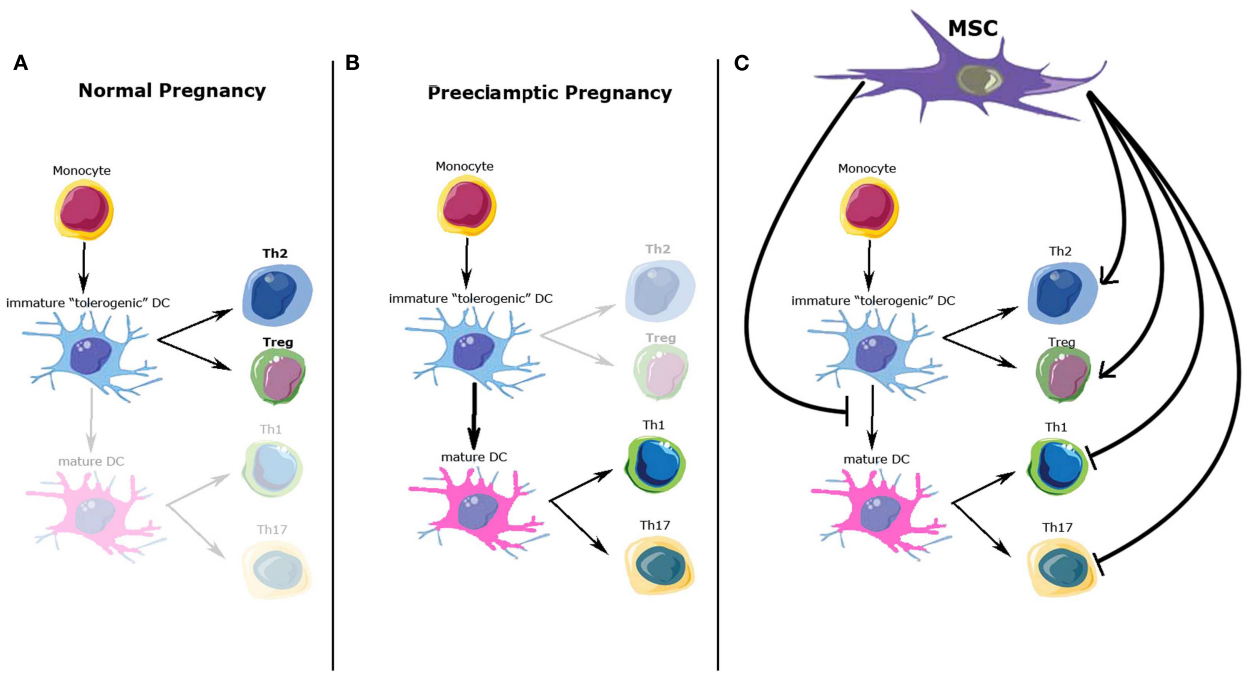

FIGURE 1 | Possible immunomodulatory role of mesenchymal stem cells (MSC) over immune cells involved in normal and preeclamptic pregnancy. **(A)** Normal pregnancy is considered as a Th2 type immunological state, where Th2 $CD4^+$ T-cells and Treg cells response and cytokine profile predominate. **(B)** On the other hand, preeclamptic pregnancies have been considered as a maternal pro-inflammatory state with Th1/Th17 predominance. **(C)** Possible MSC effects over the immune cell types involved in normal and preeclamptic pregnancies. MSC inhibit maturation of dendritic cells, maintaining a tolerogenic DC phenotype; MSC inhibit Th1/Th17 proliferation and function, whiles promote Treg and Th2 differentiation and cytokine secretion. All these effects favor a Th2/Treg phenotype. DCs, dendritic cells; Th2, T-helper 2; Th1, T-helper 1; Th17, T-helper 17; Treg, T-regulators cells.

production of IL-17. An up-regulation of this lymphocyte subpopulation has been related with the development and progression of autoimmune and chronic inflammatory diseases, allergic disorders, and graft-rejection reactions (36). Furthermore, Th17 subpopulation has been described as up-regulated in PE compared to normal pregnancy. Darmochwal-Kolarz et al. have reported that, IL-17-producing lymphocytes are increased in peripheral blood of PE patients in the third trimester of pregnancy, compared to a control group. Moreover, they described a significant correlation between Th17, IL-2- and IFN-g-producing T-cells, and PE development (37). Their data firmly support the idea that the up-regulation of Th17 immunity is related to the activation of a Th1 response in PE, suggesting that regulatory role of Treg could be also altered.

Regulatory T cell is another lymphocyte subpopulation, characterized by the expression of a high level of CD25, cytotoxic T-lymphocyte antigen 4 (CTLA-4), and the expression of the transcription factor FOXP3 (38). Treg plays a crucial role in the development and maintenance of tolerance in peripheral tissues, as well as in the induction of transplantation tolerance, so it has been also proposed as a key factor in the maintenance of maternofetal tolerance (39–41). A low amount and activity of Treg cells has been described in PE, while normal human pregnancy is associated with elevated numbers and immune suppressive effects of these cells (42). Peripheral Treg cells are normally produced within peripheral tissues, such as decidualized endometrium during early pregnancy, and respond to antigens specifically restricted to the tissue where they are found (43–45). Peripheral Treg must meet antigens presented by "tolerogenic" DCs in an appropriate cytokine environment to proliferate, get to functional maturity, and exert their suppressive effects. Tolerogenic DCs are characterized by their immature or semi-mature phenotype, their altered expression of co-stimulatory molecules CD80 and CD86, and the lack of expression of the Th1-inducing cytokine IL-12 (46). Only these DCs possess the functional characteristics of immature DCs and consistently induce Treg cells with immunosuppressive function (**Figures 1A,B**).

An important cell type that induces and maintains the tolerogenic phenotype of DCs, are mesenchymal stem cells (MSC) (47). Furthermore, a growing body of evidence supports the idea that MSC can modulate the behavior and cytokine secretion of all cell types previously described involved in feto-maternal tolerance development (48), suggesting a plausible role for MSC in the regulation of trophoblast invasion, and conversely a potential role in abnormal placentation, a feature of PE.

MESENCHYMAL STEM CELLS: A CELL WITH IMPORTANT IMMUNOMODULATORY POTENTIAL

Mesenchymal stem cells are multipotent mesenchymal stromal cells that proliferate *in vitro* as plastic-adherent cells, have fibroblast-like morphology and can differentiate into bone, cartilage, and fat cells (49). They are found in almost all human tissues and an endometrial mesenchymal stem cells (eMSC) population has also been identified by Gargett et al. These eMSC show high clonogenic properties similar to bone marrow-derived MSC (BM-MSC) (50, 51). Recent studies have described the influencing MSC capacities over immune and inflammatory responses, and especially in the endometrium these cells could be key players in the immune regulation needed for a successful implantation and normal invasion process carried out by the trophoblast. Inversely, an abnormal performance of these cells at this crucial point could lead to an abnormal development of the trophoblast and an impaired placentation.

It has been shown that MSC suppress the differentiation of DCs from monocytes by arresting them in G_0 phase of cell cycle, an effect that is mediated by soluble factors (47). Moreover, MSC interfere in maturation of DCs avoiding a Th1 response typical of mature DCs, and promoting an immature DC phenotype that helps to generate a tolerogenic environment (52). Besides, Jiang et al. have reported that MSC maintain DC in an immature state and that MSC inhibit up-regulation of IL-12p70, a pro-inflammatory cytokine (53). Similarly, it has been reported that MSC can alter the cytokine profile secreted by DC to induce a tolerogeneic microenvironment (54). Specifically, MSC induce the DC-associated production of IL-10, which in turn, induce the secretion of the anti-inflammatory cytokine IL-4 by Th2 cells (55). All these effects depend on the cytokine environment, because it has been shown that an increase of pro-inflammatory cytokines, such as IL-6 and TNF-a, reverse the immunosuppressive effects of MSC over DC cells (47).

Also, it has been described that MSC inhibit Th17 differentiation and function, decreasing the number and activity of these cells in the inflammation site. Moreover, it has been shown that the co-incubation of MSC with Th17 induces "regulatory" features in these cells even in an inflammatory environment. This effect is carried out by the down-regulation of retinoic-acid-receptor-related orphan receptor gamma t (RORgt) transcription factor and by the up-regulation of FOXP3 transcription factor (56). These effects could be associated to the release of soluble factors from MSC, such as prostaglandin E2 (PGE_2), or by the modification of cytokine environment that favors a Treg phenotype (57).

Furthermore, it has been shown that MSC increase the number and the activity of Treg (58). It has been demonstrated that co-culture of $CD4^+$ T-cells with MSC induce the appearance of $FOXP3^+CD25^{High}$ T-cells. Another possible mechanism for the increase of Treg number by MSC is the inhibition of IL-6 production, which is a necessary cytokine in the Th17 differentiation process from Tregs. Also, it has been shown that the influence of MSC over DC cells favors the generation of Treg cells, because of the tolerogenic environment generated both by MSC and by tolerogenic DCs (47, 54).

Mesenchymal stem cell not only can influence the phenotype of the different cells that play a role in the immune environment of pregnancy, but also have a role in the regulation of Th1/Th2 balance. It has been shown that MSC can shift a Th1 phenotype to a Th2. This effect could be performed through the modulation of DC phenotype (shifting from a DC1 or Th1-associated phenotype to a DC2 or Th2-associated phenotype) or by the direct effects over Th1/Th2 cells. In this regard, MSC inhibit $CD4^+$ T-cell proliferation by the inhibition of the entry to S phase of cell cycle (47). This effect is mediated at least in part by soluble factors such as TGF-β, hepathocyte growth factor (HGF), and PGE_2 (54, 59). MSC inhibit proliferation of activated T-cells in respond to: (i) non-specific stimuli such as DCs, phytohemagglutinin (PHA),

and IL-2, (ii) their specific antigen (55). MSC also inhibit Th1 phenotype by the inhibition of IFN-g production, which is necessary for Th1 cells development, and by increasing Treg cell number, that works as a counterpart of Th1 cells. MSC not only suppress Th1 response, but favors the emergence and maintenance of Th2 response by inducing IL-4 production that favor the Th2 differentiation (54, 55, 57). There are several studies that indicate that MSC could positively alter the Th1/Th2 balance. Bai et al., showed in an experimental allergic encephalomyelitis model that MSC induce neurological improvements by the reduction of T-cells infiltration to the brain and by the increased production of Th2 cytokines such as IL-4 and IL-5 production accompanied by the reduction in Th1/Th17 related cytokines such as IL-17 IFN-γ and TNF-α (60).

In summary, MSC regulate immune cell types involved in the feto-maternal tolerance that allows a normal invasion of the decidua by the EVT (**Figure 1C**). Dysregulation of this invasive process is part of the etiopathogenesis of PE, but clear evidence of the involvement of the immunomodulatory properties of eMSC in this process remains to be elucidated.

MOLECULAR MECHANISMS OF MSC IMMUNOSUPPRESSIVE EFFECT

So far, we have discussed about MSC effects on different immune cell types and its potential role in the abnormal placentation observed in patients that develop PE, but the mechanisms underlying these effects need to be explained. Inhibitory effects of MSC over T-cell proliferation could be accomplished by at least two different ways.

CELL-TO-CELL CONTACT-DEPENDENT MECHANISM

It is mediated mainly by PD1–PD1L pathway (61). PD-L1 is a transmembrane glycoprotein and a ligand of the programed cell death protein 1 (PD-1) that is expressed in various cell types, including T-cells, macrophages, DCs, and placenta (62, 63). The interaction of PD-L1 with PD-1 leads to the suppression of the immune response (62). PD-L1 is considered a key suppressor factor in maternal tolerance (64). It has been shown that PD-L1 is up-regulated on decidual T-cells during pregnancy (65), and that their expression on the surface of Tregs is essential to exert their suppressive effect and to control the maternal immune response (66). Moreover, placental MSC express higher levels of PD-L1 than BM-MSC, although IFN-γ treatment proved to have a lower immunomodulatory capacity on T-cell proliferation (67). Furthermore, PD-L1 pathway in BM-MSC mediates suppression of Th17 cell proliferation and IL-17 production (68). However, there is no data to the best of our knowledge about the expression of PD-L1 on the decidua and eMSC of patients that develop PE.

METABOLISM OF THE ESSENTIAL AMINO ACID TRYPTOPHAN

Mesenchymal stem cells express the enzyme indoleamine 2,3-dioxygenase (IDO), a tryptophan-degrading enzyme, that through the consumption of tryptophan amino acid serves as a natural immunoregulatory mechanism for the inhibition of T-cell proliferation. Munn et al. showed that the functional inhibition of IDO resulted in the uniform rejection of allogeneic fetuses, suggesting the crucial role of this enzyme in maternal tolerance maintenance (69). Similarly, it has been shown that placental MSC treated with IFN-g showed an increase in IDO expression, inhibiting autologous T-cell proliferation (70). In PE, IDO expression is increased (71) and this altered IDO expression has been postulated to be associated with the reduction of Treg cell subset, a feature observed in patients that develop PE (72).

On the other hand, MSC can produce immunosuppressive effects by the production and release of immunosuppressive factor such as HLA-G and PGE_2.

- *HLA-G*: it has been shown that MSC express and secrete HLA-G (73, 74). This expression can be up-regulated by progesterone treatment (75) and pro-inflammatory cytokines (76). Furthermore, the induction of HLA-G expression as a strategy to enhance the immunosuppressive properties of MSC in transplantation has been postulated (77). HLA-G is a non-classical MHC class Ib molecule that initially was identified in trophoblast cells. HLA-G has soluble and membrane-bound isoforms (78, 79), and it is recognized by immunoglobulin-like transcript receptor expressed in T-cells, B cells, NK cells, and macrophages (79). The physiological role of HLA-G during pregnancy is to establish immune-tolerance at the maternal-fetal interface, abrogating the cytolytic activity of maternal NK and cytotoxic T-cells against fetal tissue (80). HLA-G exerts a direct suppressive effect on $CD4^+$ T-cells (40) and induces apoptosis in $CD8^+$ T-cells (81). A soluble form of HLA-G also participates in the vascular remodeling of maternal uterine spiral arteries during pregnancy (81). Defective HLA-G expression has been associated with PE (82). HLA-G levels in plasma from women who subsequently develop PE are lower than control patients (83, 84). MSC have been shown to secrete and express HLA-G (73, 74).
- *PGE_2*: it has been postulated that MSC immunosuppression is also mediated by PGE_2 (85). PGE_2 is a bioactive lipid synthesized by cyclooxygenase (COX) enzyme pathway. It elicits a wide range of effects on inflammation process and immune cells. PEG_2 inhibits IFN-g production in $CD4^+$ T-cells, which facilitates development of Th2 cytokine production (86), induces the expression of inhibitory receptors on cytotoxic lymphocytes (87), regulates Th17 differentiation and enhances Th17 cytokine expression (88). PGE_2 also has an effect on innate immune response suppressing proliferation, cytokine secretion, and NK cell-mediated cytotoxicity (89). PGE_2 is produced by decidua and fetal membranes, and is believed to play a role in the onset of labor (90). Secretion of PGE_2 by MSC inhibits inflammation (91) and alters T-cell and NK cell proliferation and cytokine production (92) in effector immune cells. However, the evidence of the involvement of PGE_2 in the development of PE is poor.

All the immunosuppressive properties of MSC have mainly been studied using BM-MSC. However, it has been shown that these cells have different immune behavior than eMSC (93), suggesting that these two MSC types differ in their immunomodulatory and anti-inflammatory effects. Those results converge toward positioning the eMSC as a crucial endometrial cell type that might have a role in uterine physiology and pregnancy. In

order to understand the role of maternal immunotolerant mechanisms and how an alteration in these mechanisms could trigger the development of PE, it would be important to isolate and characterize the immune properties of eMSC. For this, further experimental evidence is needed to unravel the functional role of MSC from endometrial origin, the decidua, and in a pregnancy-associated environment, and the possible alterations that could be related to the development of PE.

CONCLUSION

The physiology of the immune interaction between the fetus and the mother during pregnancy is an unexplored field that has received increasingly attention during the past years. The understanding of immune interactions during normal pregnancy could help guide the research of pregnancy-associated disorders such as PE that finally allow the development and implementation of effective therapeutic tools. In this regard, the study of MSC biology as master immunomodulatory cell, specifically eMSC, might become an important contribution to the understanding of physiological and pathological immune interactions during the establishment and maintenance of pregnancy that could be related to the development of disease states, such as PE.

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Insights into the role of *Helicobacter pylori* infection in preeclampsia: from the bench to the bedside

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Preeclampsia (PE) is defined as a hypertensive and coagulative disorder affecting about 2–8% of all pregnancies and is one of the main causes of maternal and fetal morbidity and mortality. Despite the great amount of studies run in this field, little is known about the precise pathogenic mechanisms behind PE. While endothelial and trophoblast dysfunctions, exaggerated inflammatory response, and hypercoagulative state have been shown to play a key role in the occurrence of PE, the primary trigger is still unknown. One of the hypotheses is that some infectious agents may represent a trigger for PE onset. Consistently, higher seroprevalence of *Helicobacter pylori* (HP) infection, a Gram-negative bacterium with a specific tropism for human gastric mucosa, has been shown in women with PE. Even tighter association has been found between PE and infection with cytotoxin-associated gene-A (CagA)-positive strains of HP. Recent *in vitro* studies have shown that anti-CagA antibodies cross-react with human trophoblast cells and determine a functional impairment in terms of cell invasiveness, thus, providing the first pathogenic model of HP infection-mediated placental damage. Since in the early process of implantation and placental development, trophoblast invasion of maternal decidua is a crucial step, the proposed autoimmune mechanism induced by HP infection, negatively interfering with the fetal side of the early developing placenta, may represent a mechanism explaining the higher seropositivity for HP infection among PE women. However, the contribution of HP infection to the pathogenesis of PE or to the worsening of its clinical presentation need to be further investigated as well as the possible impact of pre-pregnancy screening and eradication of HP infection on the incidence of the syndrome.

Keywords: preeclampsia, *Helicobacter pylori*, infection, placenta, anti-CagA antibody

INTRODUCTION

Pre eclampsia (PE) is generally defined as new hypertension and substantial proteinuria at or after 20 weeks' gestation (1). Complicating 2–8% of pregnancies, PE is a major cause of severe maternal morbidity and mortality and adverse perinatal outcomes worldwide (2, 3).

In the last 20 years, the incidence of PE has risen in the Western Countries, probably due to an increased prevalence of predisposing factors, such as advanced maternal age, chronic hypertension, diabetes, obesity, and the growing use of assisted reproductive techniques (4, 5).

Despite the great socio-economic impact of PE and the amount of studies carried out in this field, the pathogenic mechanisms leading to PE onset still remains unclear as well as an effective preventive intervention is still lacking (6).

The placental origins of PE have long been recognized and then formalized in the two stage model of the syndrome (7). The first stage is represented by inadequate development of the early placenta and its maternal blood supply, called poor placentation, which is established before 20 weeks and before clinical signs appear. During physiological placental development, extensive remodeling of maternal spiral arteries takes place in order to supply increased need of maternal blood in the second two trimesters of pregnancy. That process depends on extravillous cytotrophoblasts that invade the lining of the pregnant uterus from weeks 6 to 18 of gestation, expanding the vascular capacity of the utero-placental circulation (8). In many cases of PE, trophoblast invasion has been shown to be inadequate with poorly remodeled arteries and reduced capacity of the utero-placental circulation (9).

In the second stage, a dysfunctional and hypoxic placenta is considered to release factors into the maternal circulation that cause the clinical features of this condition, including hypertension and proteinuria, as well as clotting and liver dysfunction. These appear to arise from a generalized systemic inflammatory response, of which endothelial dysfunction is a prominent component (7).

Thus, nowadays, one of the biggest challenges in the research field of PE is to identify possible primary triggers of poor placentation, then leading to clinical PE, in order to develop effective preventative interventions. In that scenario, a possible role for infections has been widely suggested.

EPIDEMIOLOGIC ASSOCIATION BETWEEN *HELICOBACTER PYLORI* INFECTION AND PREECLAMPSIA

In the last few years, an epidemiological link between *Helicobacter pylori* (HP) infection and PE has been observed (10–13).

Helicobacter pylori is a Gram-negative bacterium with a specific tropism for the gastric mucosa (14); it is the main cause of chronic gastritis and peptic ulcer, as well as a risk factor for MALT-lymphoma and gastric cancer (15). Only some strains of HP possess determinants of pathogenicity, able to modulate the local and systemic inflammatory response (16), like the cytotoxin-associated gene-A (CagA), which encodes for a hydrophilic, surface-exposed protein (17). CagA-positive strains of HP have been shown to induce an inflammatory response in the gastric mucosa greater than that induced by CagA-negative ones (18). Owing to its capability to stimulate the immune system, HP has also been proposed to play a role in some extra-gastric diseases; in particular, the epidemiological association between HP infection and vascular diseases has been shown, including ischemic heart diseases, primary Raynaud's phenomenon and migraine, all conditions characterized by endothelial dysfunction (19, 20).

Interestingly, anti-CagA antibodies seem to be able to cross-react with antigens localized on the surface of human endothelial cells in either normal or atherosclerotic arteries, thus providing a possible mechanism explaining this association (21, 22).

Daví and co-authors have shown an association between HP infection and high levels of *in vivo* markers of lipid peroxidation and platelet activation, urinary 8-iso-PGF2 and 11-dehydro-TXB2, respectively. Interestingly, successful eradication of HP infection led to a significant reduction in both markers, suggesting a novel mechanism by which an infectious agent could contribute to atherothrombosis (23).

A few years ago, Ponzetto et al. showed, for the first time, higher seropositivity for HP infection in 47 mothers with PE (51.1%) compared with 47 women with uneventful pregnancy (31.9%). The difference was even greater when considering positivity for CagA-positive strains of HP (80.9 and 14.9%, respectively) (10).

This epidemiologic association has subsequently been confirmed by several studies (11, 13) (**Table 1**), and a correlation between persistent and virulent infections (VacA/CagA seropositive patients) for HP and PE complicated by fetal intrauterine growth restriction (IUGR) has also been shown (13).

Thus, since the association between HP infection and PE occurrence has been widely confirmed, we hypothesized that this bacterial infection might have a role as possible trigger in the etiopathogenesis of PE.

Table 1 | Studies investigating the prevalence of HP infection in general, and CagA+ strains HP infection, in particular, in healthy pregnant women (CTR) in comparison with preeclamptic women (PE).

Authors	Population (*n*)	HP+ (%)	*P*	CagA+ (%)	*P*
Ponzetto et al. (10)	CTR = 47	31.9		14.9	
	PE = 47	51.1	0.033	80.9	<0.001
UstUn et al. (11)	CTR = 40	12.5		–	–
	PE = 40	35.0	0.034	–	–
Pugliese et al. (12)	CTR = 25	32.0		28.0	
	PE = 25	84.0	<0.001	80.0	<0.001
Cardaropoli et al. (13)	CTR = 49	42.9		22.4	
	PE = 49	85.7	<0.001	81.6	<0.001

$P < 0.05$: statistically significant.

ANTI-CagA ANTIBODIES CLASS IgG-MEDIATED TROPHOBLAST INVASION INHIBITION: AN *IN VITRO* MODEL OF HP-INDUCED POOR PLACENTATION

To try to answer that question, we investigated whether HP infection might induce an immune humoral response able to trigger an autoantibody-mediated placental cellular damage. In particular, since anti-CagA antibodies are able to cross-react with antigens of endothelial cells (21) and cytotrophoblast cells show an endothelial origin, we tested murine anti-CagA antibodies class IgG – the only class of immunoglobulins able to cross placental barrier on human primary trophoblast cultures in order to find a possible cross-reaction. Interestingly, we observed that anti-CagA antibodies are able to bind, on the surface of trophoblast cells, to β-actin protein, one of the main components of cell cytoskeleton (24). Consistently, immunofluorescence performed on trophoblast cells using either anti-CagA or anti-β-actin antibodies showed an identical pattern of reaction, thus confirming β-actin to be the real cross-reacting protein. Interestingly, actin, in either endothelial or trophoblast cells, is not only important for maintaining the cell structure but it is also crucial for intercellular adhesion (25, 26) and it is now well established that actin-associated adhesions contribute to placental anchorage (26). We observed that anti-CagA antibodies show a dose-dependent binding activity as well and, as biological effect, a dose-dependent impairment of cytotrophoblast invasiveness *in vitro*, a crucial point for PE development. Furthermore, to better understand the molecular mechanisms involved in the antibody-mediated functional impairment of trophoblast cells, we examined the effect of anti-CagA on ERK activation and NF-kB nuclear translocation, two important factors activated during trophoblast proliferation, and we observed that anti-CagA antibodies are able to inhibit the activation of both elements (24).

As a whole, these observations provided a possible autoimmune pathogenic mechanism induced by HP infection, negatively interfering with the fetal side of placental development. This pathogenic model of autoimmune-mediated placental impairment is the first one linking HP infection, poor placentation, and PE (**Figure 1**).

DISCUSSION

Although the cause of PE remains largely unknown, the leading hypotheses strongly rely on disturbed placental function early in pregnancy (27). Impaired remodeling of the spiral arteries has especially been considered as an early defect causing PE (28).

Inadequate placentation may lead to impaired intervillous perfusion and to the establishment of placental hypoxic status, causing oxidative stress of trophoblast cells and the release in maternal circulation of anti-angiogenic factors and trophoblast debris, believed to mediate maternal systemic inflammatory response, endothelial dysfunction, and hypercoagulability in PE syndrome (28, 29).

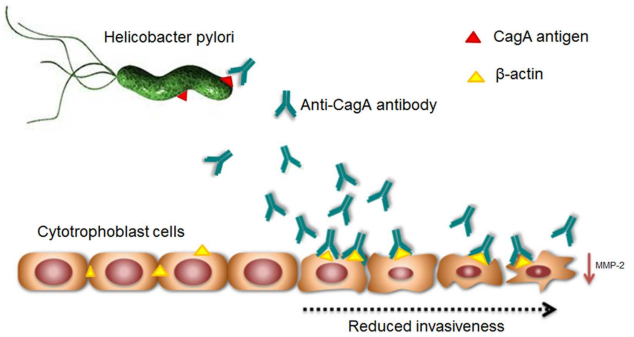

FIGURE 1 | Supposed mechanism of *Helicobacter pylori* infection-induced molecular mimicry leading to cross-reaction of anti-CagA antibodies to trophoblast cells and poor placentation.

Several studies suggested a strong association between PE and HP infections (10–13). Our *in vitro* studies showed an anti-CagA antibody-mediated mechanism of placental impairment at fetal side of early placental development. Thus, it is likely that antibodies directed against bacterial CagA protein might cross-react with antigens expressed on trophoblast cell, and in particular with β-actin, through an immunologic mechanism called molecular mimicry, leading to autoimmune response. This binding could inhibit significantly trophoblast invasiveness, probably negatively interfering with intracellular signaling ad intercellular connections, potentially leading to inadequate placental development. That could represent an intriguing model of infection-induced autoimmune triggering for poor placentation and PE onset, giving an explanation to the higher prevalence of HP infection among women developing PE.

CONCLUSION

More studies are needed to further investigate the impact of HP infection in triggering PE onset or, eventually, in worsening its clinical presentation.

However, it should be considered that, nowadays, in obstetrical practice, diseases with lower incidence than PE, like Rh alloimmunization or Down's syndrome, are commonly screened. Thus, if HP will be confirmed as a contributing factor to PE, it will have important positive implications for the public health system, since the infection is treatable, and the future challenge would be to assess whether pre-pregnancy screening and preventive HP eradication would reduce the incidence of PE or moderate the severity of its clinical presentation.

AUTHOR CONTRIBUTIONS

Giovanni Scambia, Tullia Todros, Francesco Franceschi, Simona Cardaropoli, and Nicoletta Di Simone were responsible for the manuscript concept, design, and supervision. Chiara Tersigni performed the literature searches and extraction of data. Chiara Tersigni, Tullia Todros, and Nicoletta Di Simone drafted the manuscript.

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Controlling the immunological crosstalk during conception and pregnancy: HLA-G in reproduction

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In several years after its discovery in the placenta, the human leukocyte antigen (HLA) class Ib protein, HLA-G, was not given much attention, nor was it assigned great importance. As time has unraveled, HLA-G has proven to have distinctive functions and an unforeseen and possibly important role in reproduction. HLA-G is characterized mainly by its low polymorphism and restricted tissue distribution in non-pathological conditions. In fact, its expression pattern is primarily limited to extravillous cytotrophoblast cells at the maternal-fetal interface during pregnancy. Due to low polymorphism, almost the same protein is expressed by virtually all individuals. It is these unique features that make HLA-G differ from its highly polymorphic HLA class Ia counterparts, the HLA-A, -B, and -C molecules. Its function, seemingly diverse, is typically receptor-mediated, and involves interactions with a wide range of immune cells. As the expression of HLA-G primarily is limited to gestation, this has given rise to the hypothesis that HLA-G plays an important role in the immunological tolerance of the fetus by the mother. In keeping with this, it might not be surprising that polymorphisms in the *HLA-G* gene, and levels of HLA-G expression, have been linked to reproductive failure and pre-eclampsia. Based on recent studies, we speculate that HLA-G might be involved in mechanisms in reproductive immunology even before conception because HLA-G can be detected in the genital tract and in the blood of non-pregnant women, and is present in seminal fluid from men. In addition, HLA-G expression has been found in the pre-implanted embryo. Therefore, we propose that a combined contribution from the mother, the father, and the embryo/fetus is likely to be important. Furthermore, this review presents important aspects of HLA-G in relation to reproduction: from genetics to physiological effects, from pregnancy and pregnancy complications to a short discussion on future possible means of preventative measures and therapy.

Keywords: MHC, HLA class Ib, HLA-G, human reproduction, pregnancy complications

INTRODUCTION

The uterus and the placenta constitute a unique site of immune modulation where the semi-allogeneic fetus is tolerated by the maternal immune system. Both the mother and the fetus contribute to maintenance of tolerance. The mother through the presence of local regulatory immune cells that regulate redundant immune responses and the fetus, possibly among several mechanisms, through expression of non-classical human major histocompatibility complex (MHC) class Ib molecules, human leukocyte antigen (HLA)-E, -F, and -G, on extravillous trophoblast cells that infiltrate the decidua and make a direct contact with maternal immune cells (1–3). To the best of our knowledge, a classical antigen-presenting function, or capacity, of HLA-G has never been described, although the HLA-G molecule can bind peptides (4).

The expression of HLA-G is primarily limited to gestation and it has been widely studied in pregnancy because of its association with pregnancy complications, in particular pre-eclampsia and recurrent miscarriages (2, 5–8). The expression of HLA-G by embryos, as well as in the presence of soluble HLA-G (sHLA-G) in the maternal circulation, is associated with better pregnancy rates (9, 10). Furthermore, a different, possible role of HLA-G has been proposed in the context of remodeling of spiral arteries during placental development (11). However, further studies are needed to confirm this.

An accumulating body of evidence suggests that HLA-G may be an important factor in reproduction even before conception. sHLA-G circulates in the blood of non-pregnant women (and in the blood of male donors), HLA-G is expressed in the female genital tract, and sHLA-G has been identified in seminal plasma (**Figure 1**) (12–16). In addition, HLA-G expression has been found in the pre-implanted embryo. Thereby, a combined contribution from the mother, the father, and the embryo, or fetus, is likely to be important. The aim of the present review is to give an overview of, and to discuss, important aspects of HLA-G in relation to reproduction: from genetics to physiological effects, from pregnancy and pregnancy complications to a short discussion on future possible means of preventative measures and therapy.

THE *HLA-G* GENE

HLA-G POLYMORPHISMS IN CODING REGIONS

The *HLA-G* gene contains eight exons and seven introns (**Figure 2**). The external part of the HLA-G molecule consists of three parts, the α1, α2, and α3 domains (exons 2–4). The HLA-G

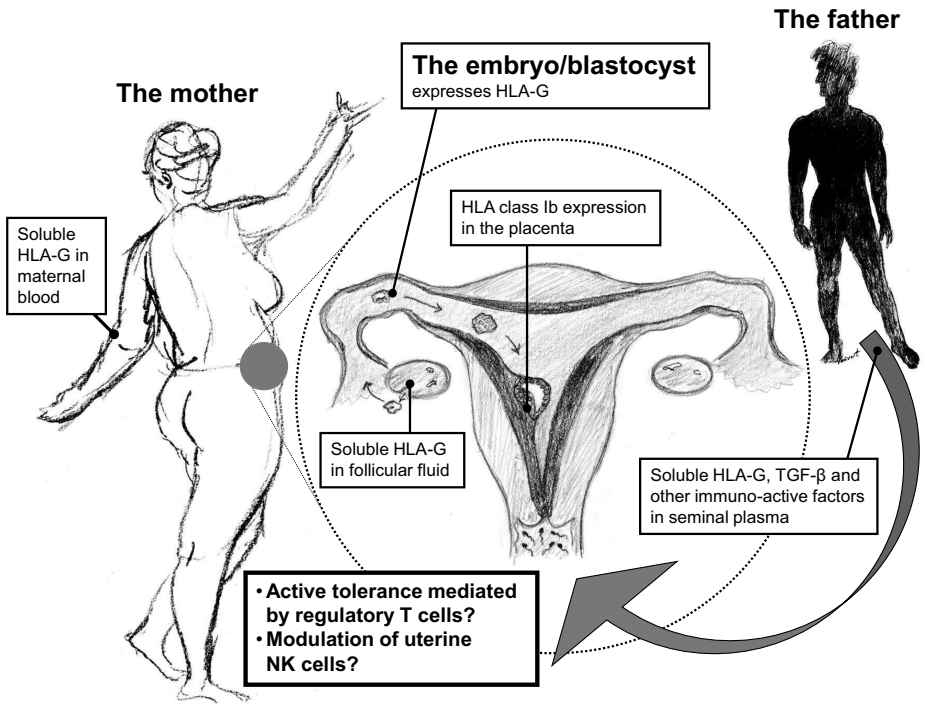

FIGURE 1 | An important and central role of HLA-G in reproduction may be depicted from its wide distribution within the reproductive cycle. HLA-G is expressed in maternal blood, in follicular fluid, and in seminal plasma prior to implantation, and after fertilization in the blastocyst/embryo and in the placenta by the trophoblast cells. The continuous expression of HLA-G in the reproductive cycle may in particular modulate local immune cells in the female reproductive system for immunological acceptance of the semi-allogenic embryo.

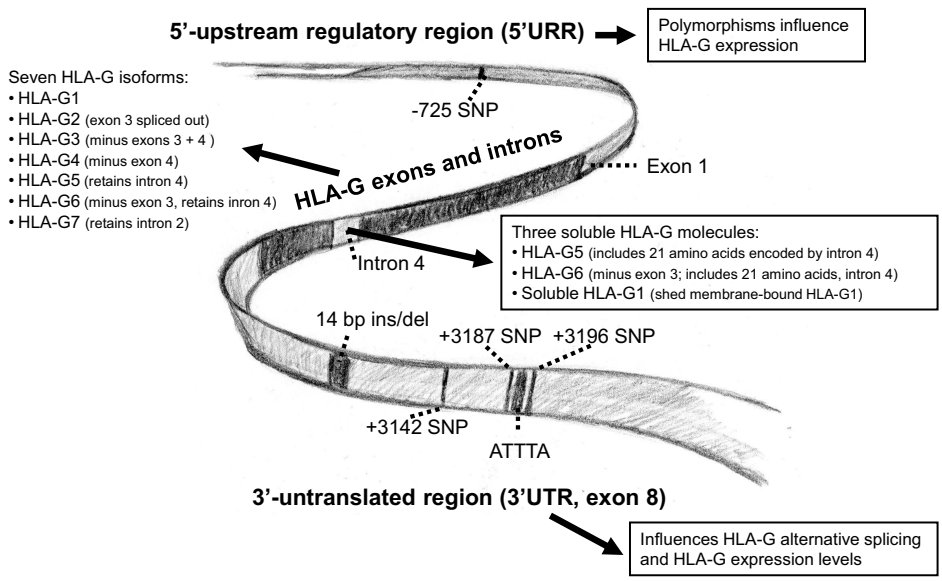

FIGURE 2 | A schematic representation of the *HLA-G* gene. Some of the most intensively studied gene polymorphisms in the 5′-upstream regulatory region and in the 3′-untranslated region. An overview of the different HLA-G isoforms is included.

full-length membrane protein is anchored in the cell membrane by the transmembrane region (exon 5). The cytoplasmic domain is encoded by exon 6 and the very first, short part of exon 8 (3, 17). Polymorphisms in the coding region of *HLA-G* are relatively scarce but evenly distributed between exons 2, 3, and 4, as well as in introns (6, 7, 18–20). Most polymorphisms do not alter the protein sequence, and the ones that do, allow for a grouping in major allele groups: *G*01:01:xx:xx*, *G*01:01:xx*, *G*01:02*, *G*01:03:xx:xx*, *G*01:04:xx*, *G*01:05N* (null allele), *G*01:06*, and *G*01:07* to *G*01:18*. However, polymorphisms that define these allele groups have probably no effect on the secondary structures of the heavy chains, and the functional relevance of this nucleotide variability remains unclear. In total, 50 alleles and 16 allele groups representing an amino acid substitution have been described in the *HLA-G* gene sequence [WHO Nomenclature Committee for Factors of the HLA System and the International Immunogenetics Information System (IMGT)/HLA Database].

The polymorphic deletion of the first basepair (bp) of codon 130 or the third of codon 129, which results in a frameshift, defines the null allele (*G*01:05N*), and this null allele does not encode functional full-length HLA-G protein isoforms (HLA-G1 and -G5, see below) (18, 21). Nonetheless, studies show that these isoforms are not essential for fetal survival, indicating that expression of other HLA-G isoforms or HLA-E and/or -F, which are also involved in immune modulation in the placenta, may compensate for the lack of HLA-G1 and -G5 protein (22–24).

HLA-G POLYMORPHISMS IN NON-CODING REGIONS

The functional mRNA level of *HLA-G* is governed by the rate of synthesis, mainly driven by the promoter region, or 5′-upstream regulatory region (5′-URR), as well as by the rate of degradation, stability, localization, and translation of the mRNA (25) (**Figure 2**). The rate at which pre-mRNA is produced is partly mediated by pre-transcriptional events, e.g., binding of transcription factors to regulatory motifs in the promoter region. HLA class I promoters sequences are generally conserved, but the *HLA-G* promoter is somewhat unique. Although its nucleotide sequence and structure is similar to other class I genes in several aspects, peculiarly, most regulatory motifs in the HLA-G promoter region are non-functional (20). Of importance, two main regulatory modules are flawed. First, the interferon (IFN)/Enhancer A region is blemished by a 16-bp deletion (17, 26), and second, the SXY module that mounts the transcriptional apparatus, represents a divergent sequence that does not allow for appropriate binding of the class II transactivator (CIITA) (27).

The 3′-untranslated region (3′UTR) of the *HLA-G* gene also exhibits several regulatory elements including AU-rich motifs and a poly-A signal to influence mRNA stability, turnover, mobility, and splicing pattern (20, 28). Polymorphisms in these regions may thus affect the expression of HLA-G by altered regulation of gene transcription or by destabilizing the mRNA transcript (28–30). Indeed, several important polymorphisms have been described in the 5′URR and the 3′UTR of the *HLA-G* gene (28, 29, 31–34).

THE 5′-UPSTREAM REGULATORY REGION OF THE *HLA-G* GENE

Polymorphisms in the HLA-G 5′URR are close to regulatory elements and CpG sites, and are likely to alter binding of transcription factors and/or promoter methylation, and as a consequence influence the rate of transcription. Although sequence variation affecting transcription would be expected inside regulatory elements, most variable sites are not found in known motifs (20). Interestingly, an accumulating body of evidence indicates a balancing selection on the *HLA-G* promoter, and thereby indicates a preference for heterozygosity in which, possibly, individuals with both high- and low-expressing promoters are privileged (19, 29, 33, 35). Few studies address the direct association between HLA-G promoter SNPs and a differential HLA-G expression. One variation, a SNP at position −725 (rs123334), is associated with sporadic miscarriages and differential HLA-G expression (32, 36), and others, SNPs at position −1305, −964, and −486, are associated with yet other conditions like vitiligo, asthma, and acute allograft rejection in end-stage renal disease (37–39).

THE 3′ UNTRANSLATED REGION OF THE *HLA-G* GENE

In contrast to the coding region, the 3′UTR of the *HLA-G* locus presents a rather high degree of variation. Since the 3′UTR of the *HLA-G* gene exhibits several regulatory elements including AU-rich motifs, a poly-A signal, as well as signals that regulate the spatial and temporal expression of mRNA, the polymorphic sites may influence mRNA stability, turnover, mobility, and splicing pattern (20, 28, 30). A 14-bp ins/del (rs66554220) located in exon 8 is the best studied polymorphism in the 3′UTR, and has been shown to influence *HLA-G* mRNA transcript size and stability (19, 28, 30, 31, 40–42). The presence of the 14-bp-insertion sequence introduces an alternative splice site that generates a 92-bp deletion in the 3′UTR of the *HLA-G* mRNAs, and this alternative splice form seems to have an impact on the expression levels of HLA-G (5, 28, 40, 41). The positions of polymorphism in the 3′UTR of the *HLA-G* gene are in the current review numbered according to the publication by Castelli et al., which includes the 14-bp sequence in the reference sequence (43). At least three other SNPs in the 3′UTR are associated with *HLA-G* mRNA regulation and differences in sHLA-G levels: one positioned at +3142 (rs1063320) substituting a C to a G, another at +3187 (rs9380142) substituting an A to a G, and the third at position +3196 (rs1610696) where a C is substituted with a G (**Figure 2**). Studies show that polymorphisms in the 3′UTR probably act as targets for microRNAs, thereby controlling *HLA-G* mRNA stability and expression levels (8, 43–47). Furthermore, the +3187 and the +3196 SNPs are located just before and after an AUUUA motif associated with mRNA stability (28, 43).

COMBINED 5′URR AND 3′UTR *HLA-G* HAPLOTYPES

In some cases, polymorphism in the *HLA-G* 5′URR/promoter region may be in linkage disequilibrium with *HLA-G* 3′UTR variants (19, 33, 37), and some of them might influence alternative splicing and mRNA stability (30, 48). The −725 SNP located in the 5′URR is possibly in linkage disequilibrium with the 14-bp ins/del, the +3142, and the +3187 polymorphic sites, and is suggested to influence the stability of mRNA transcripts (36). Recently, the full combinations of 5′URR haplotypes, *HLA-G* WHO nomenclature alleles, and 3′UTR haplotypes in the HLA-G gene have been elucidated in a Brazilian population (33). The DNA polymorphisms in these extended *HLA-G* haplotypes may influence *HLA-G* expression and the stability of *HLA-G* mRNA transcripts in combination.

Investigating the 5′URR and 3′UTR *HLA-G* extended haplotypes instead of evaluating single polymorphisms could determine the significance of allelic variants of *HLA-G* more accurately (33). A study correlating 3′UTR extended haplotypes with HLA-G soluble levels in a Brazilian and French cohort, showed that some haplotypes were associated with high sHLA-G levels (named UTR-1) and some with low sHLA-G levels in blood plasma from healthy donors (named UTR-5 and UTR-7) (47). However, full consensus does not exist in these studies, as another French study reported conflicting results (46). These different *HLA-G* haplotypes differ at the 14-bp ins/del, the +3142, the +3187, and the +3196 polymorphic sites in the 3′UTR, as well as in polymorphic sites in the 5′URR.

UNIQUE CHARACTERISTICS OF HLA-G

A characteristic unique to HLA-G is the post-transcriptional alternative splicing of the mRNA from the single *HLA-G* gene. HLA-G1 represents the full-length isoform, whereas the other isoforms are formed by out-splicing of exons. This result in seven isoforms, four of which are membrane-bound (HLA-G1, HLA-G2, HLA-G3, and HLA-G4), and three of which are soluble (sHLA-G5, sHLA-G6, and sHLA-G7) (40, 49, 50) (**Figure 2**). HLA-G1 and HLA-G5 are the most studied isoforms. In contrast to other *HLA class I* genes, exon 6 of the *HLA-G* gene encodes a pre-mature stop codon that results in a truncated cytoplasmic tail (17). The truncated cytoplasmic tail results in a reduced endocytosis and thus a low cell surface turnover of the HLA-G molecule (51, 52). The soluble isoforms lack the transmembrane region due to a stop codon in intron 4 (40, 50). In the presence of metalloproteinases, HLA-G1 loaded with peptide can be shed from the surface by proteolytic cleavage, also resulting in a soluble molecule. HLA-G1 and sHLA-G5 are both capable of forming heterodimers with β2-microglobulin (β2m) (53).

HLA-G EXPRESSION AND FUNCTION IN RELATION TO REPRODUCTION

We propose that HLA-G might be involved in mechanisms in reproduction even before conception because HLA-G can be detected in the genital tract and in the blood of non-pregnant women, and is present in seminal fluid from men.

The function of HLA-G seems to be diverse, involving interactions with NK cells, cytotoxic T lymphocytes, regulatory T cells (Tregs), and it may be involved in regulating angiogenesis and cell migration (**Figure 3**). HLA-G is expressed by the extravillous trophoblast cells in the placenta, where the molecule has been attributed an important role in early placentation and maintenance of successful pregnancy (2, 3). Also, HLA-G is expressed in tissues important for the reproductive cycle. HLA-G is expressed in the

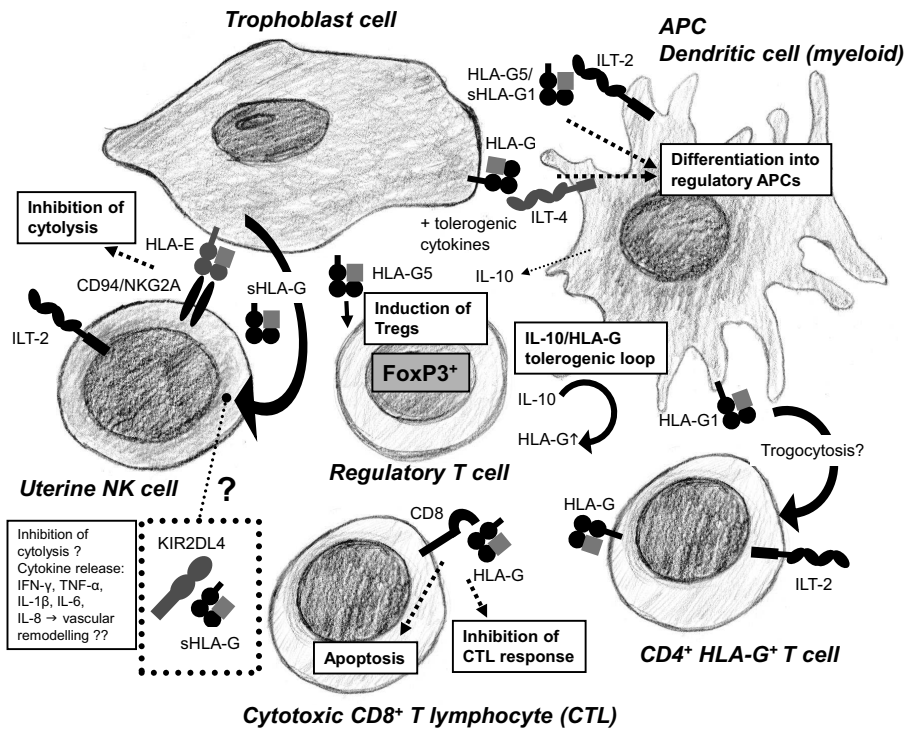

FIGURE 3 | Human leukocyte antigen-G is a key mediator of the tolerogenic loop arising from the crosstalk between immune cells in the placenta. HLA-G promotes differentiation of DCs into tolerogenic DCs secreting IL-10, TGF-β, and expressing HLA-G. IL-10 and TGF-β induce Tregs. Tregs stimulate trophoblast cells to further upregulate expression of HLA-G. HLA-G can be acquired by $CD4^+$ T cells through trogocytosis, increasing the pool of regulatory immune cells in the placenta. Cytotoxic $CD8^+$ T cells are inactivated and undergo apoptosis by binding of HLA-G to the CD8 co-receptor. HLA-E presents HLA-G derived signal peptides and bind to the CD94/NKG2A receptors on uterine NK cells inhibiting cytotoxicity. Soluble HLA-G from trophoblast cells accumulates in $KIR2DL4^+$ endosomes in uterine NK cells, which may result in active secretion of proangiogenic and proinflammatory cytokines, although this is controversial.

follicular fluid and in the genital tract (15, 54, 55). Furthermore, HLA-G and sHLA-G has been detected in the male reproductive system and in semen (14, 56). After fertilization, HLA-G is expressed by the blastocyst and the early embryo (57, 58). HLA-G is also expressed at other immune privileged sites such as the cornea and thymic epithelial cells (59, 60). Finally, sHLA-G can be measured in peripheral blood from healthy female and male donors, and during pregnancy the concentration raises two to five times compared to what is observed in non-pregnant women (9, 12, 61, 62). One of the sources of soluble HLA-G5 in the blood of non-pregnant women and in men is most likely monocytes; though $CD4^+$ and $CD8^+$ T cells and B cells seem to be able to secrete HLA-G5 as well, although in lower amounts (63). However, during pregnancy a substantial amount of sHLA-G may be derived from the placenta by shed HLA-G1 and possibly secreted HLA-G5. sHLA-G levels in blood are associated with *HLA-G* gene polymorphisms and *HLA-G* haplotypes (19, 64).

The blastocyst implants into the uterine wall and the fetal-derived extravillous trophoblast cells invade the decidua and are involved in the remodeling of the spiral arteries. The maternal blood flow and tissue leukocytes are hence in direct contact with fetal trophoblast cells that express HLA-G (2, 65).

Still, it seems to be controversial, whether or not the soluble HLA-G5 and -G6 isoforms are secreted by trophoblast cells (66). Nonetheless, first trimester trophoblast expresses HLA-G5 and -G6 mRNA transcripts (28). One study has suggested that the major sHLA-G isoform in maternal serum should be HLA-G6 (or soluble HLA-G2) only consisting of heavy chains (67). However, to our knowledge these results have not been reproduced.

REGULATION OF HLA-G EXPRESSION

The regulation of HLA-G expression has been studied on different levels, and the majority of studies have focused on the importance of the genetic polymorphisms in the *HLA-G* gene as previously mentioned. Especially the 14-bp ins/del polymorphism in the 3′UTR, exon 8, of the *HLA-G* gene has been implicated in mRNA stability and hence the overall HLA-G production. However, conflicting reports on whether the 14-bp-insertion allele is associated with high or low level of expression of HLA-G exist. Experiments with *HLA-G* transductants showed that K562 cells with the 3′UTR 14 bp-insertion sequence had more stable mRNA compared to transductants lacking the 14-bp insertion. The study also looked at the functional impact of the 14-bp insertion on NK cell cytotoxicity, and found that K562 transductant cells carrying the 14-bp-insertion sequence were significantly less sensitive to NK cell cytotoxicity as compared to K562 cells that did not carry the 14-bp-insertion sequence (42). However, this study only investigated the isolated role of the 14-bp sequence in experiments performed with the use of a cell line, and the importance of the 14-bp-insertion sequence may be modulated or influenced by other linked polymorphisms in the 3′UTR, and in future studies it is important to study different *HLA-G* haplotypes. A study by Martelli-Palomino et al. showed that the 14-bp deletion allele correlated with higher blood plasma sHLA-G levels compared to the 14-bp insertion in extended 3′UTR *HLA-G* haplotypes (47). This is in line with previous studies, which have shown that the 14-bp ins/14 bp ins HLA-G genotype is associated with lower blood plasma and serum sHLA-G levels, compared to the 14-bp del/14 bp ins genotype and the 14-bp del/14 bp del genotype (12, 13). MicroRNAs have also been shown to regulate HLA-G expression and thereby function. In a study by Manaster et al., two microRNAs, miR-148a and miR-152 were shown to down-regulate HLA-G expression and thereby reduce the binding of HLA-G to its cognate inhibitory receptor immunoglobulin-like transcript 2 (ILT-2). And interestingly, in the placenta, the cellular content of miR-148a and miR-152 shown to reduce HLA-G expression was very low compared to other tissues. Therefore, it can be speculated that this might be one of the reasons for high tissue restricted expression in the placenta (45). The HLA-G suppression by miR-152 in JEG-3 cells, followed by increased susceptibility to NK cell-mediated cytolysis, has also been shown in a previous study (68). Soluble factors, like immune-modulatory hormones and cytokines have shown to influence the transcription of HLA-G. The rate of HLA-G transcription is increased by the cytokine IFN-β. The indoleamine 2,3-dioxygenase (IDO), which is an enzyme catabolizing tryptophan, has shown to increase the shedding and expression of HLA-G in myeloid dendritic cells (DCs), which contribute to a tolerogenic milieu (69). In fact, IDO has shown to promote maternal tolerance toward the fetus by catabolizing tryptophan and thereby suppressing the T cell activity in mice (70).

The acquirement of HLA-G by HLA-G-negative cells has been shown to be possible via trogocytosis. This mechanism is characterized by the transfer of surface molecules from one cell to another through cell–cell contact. Activated $CD4^+$ and $CD8^+$ T cells can acquire HLA-G from antigen-presenting cells (APCs) through this mechanism and thus contribute to an immune suppressive milieu without expressing HLA-G themselves, but only temporarily displaying it (71).

The trophoblast cells have an alternative HLA expression profile compared to all other cells in the human body, in that they only express the non-classical MHC class Ib (HLA-E, -F, and -G) molecules, and to some extend the classical MHC class Ia HLA-C. Usually, an altered HLA expression profile is associated with a pathological condition, such as a virus infection or in malignant transformation, and thus induces an immune response mediated by the engagement and cytolytic killing of the cell in question by NK cells. HLA-G, however, engages inhibitory molecules on leukocytes rendering them anergic toward the trophoblast cells, thereby protecting the allogeneic fetus (2, 65, 72). HLA-G interacts with the ILT-2 and ILT-4 receptors, the co-receptor CD8, and maybe the killer cell immunoglobulin-like receptor (KIR) 2DL4 (73).

THE HLA-G RECEPTORS

The uterine NK (uNK) cells, identified by being $CD16^-CD56^{bright}$, as opposed to peripheral $CD16^+CD56^+$ NK cells, express KIR2DL4, which is described as a receptor for HLA-G, although a recent study has made this controversial (74, 75). KIR2DL4 has an immunoreceptor tyrosine-based inhibitory motif (ITIM) at its cytoplasmic tail, and is characterized by its expression in endosomes. Despite having an ITIM, KIR2DL4 when possibly bound to HLA-G shows weak inhibition of uNK cell. Because of its endosomal expression, at least in steady state conditions, KIR2DL4 seems to bind sHLA-G, which may activate the uNK cell to secrete

cytokines and chemokines important for angiogenesis (76). However, further studies are certainly needed to clarify the possible interactions between HLA-G and KIR2DL4.

Immunoglobulin-like transcript 2 and ILT-4 are inhibitory receptors expressed on leukocytes. These receptors also bind other HLA class I molecules, however, preferentially bind HLA-G (77). ILT-2 and ILT-4 contain three ITIMs at their cytoplasmic tail. ILT-2 (also named LILRB1) is expressed by monocytes, macrophages, $CD4^+$ and $CD8^+$ T cells, B cells, and myeloid DCs and engages only heterodimers of HLA-G1 or sHLA-G5 and β2m. ILT-4 (LILRB2) expressed by monocytes, macrophages, and myeloid DCs can also interact with HLA-G monomers. The discrepancy in HLA-G interaction with ILT-2 and ILT-4 has been clarified with the use of crystal structures showing that ILT-2 cannot recognize the β2m-free form of HLA-G, whereas ILT-4 preferably bind the α3 domain of the HLA-G heavy chain (78). In fact, by binding to its cognate inhibitory receptors, HLA-G has shown to up-regulate the expression of ILT-2 and ILT-4. The functional consequence of inhibitory receptor up-regulation by HLA-G was hypothesized to be an increased sensitivity toward inhibition mediated, not only by HLA-G, but also by classical HLA class I molecules known to bind ILT-2 and ILT-4 (79). The co-receptor CD8 expressed on cytotoxic T cells is also known to bind HLA-G. This interaction causes apoptosis of the activated $CD8^+$ T cells mediated by the FAS ligand/FAS pathway (80).

HLA-G IN REPRODUCTIVE IMMUNOLOGY

Cell–cell interactions between leukocytes and trophoblast cells mediated by HLA-G and its above-mentioned receptors are of great interest in order to understand the immune regulation at the feto-maternal interface (**Figure 3**). So far, several studies have tried to elucidate the strict immune regulation taking place at this anatomical site. A range of different studies indicate that HLA-G might have a central position in the immune regulation at conception and during pregnancy.

NK CELLS, DCs, AND T CELLS

It has become clear that DCs and T cells, especially Tregs, in the decidua are important contributors to the tolerogenic milieu in pregnancy and several studies have described such cells and their implications in healthy and in complicated pregnancies. Some of the described cell types have overlapping features. However, the NK cells are by far the most abundant cells in the uterus.

The early decidua is characterized by an abundance of uterine $CD16^{-/dim}CD56^{bright}$ NK cells that are in close contact with the fetal-derived extravillous trophoblast cells. uNK cells possess ILT-2, ILT-4, and KIR2DL4 receptors that bind HLA-G expressed on the surface of the infiltrating trophoblast cells. The $CD16^{-/dim}CD56^{bright}$ NK cells are the largest population of lymphocytes in the uterus, they constitute 50–90% of lymphocytes in human uterine decidua in early pregnancy. They are phenotypically and functionally distinct from conventional $CD16^+CD56^{dim}$ NK cells that circulate the periphery (81). They are recruited in large numbers through the first and second trimester and participate in the modification of the uterine spiral arteries, which increases blood flow to the fetus (82). The crosstalk between DCs and NK cells has been shown to be modulated by sHLA-G in cell cultures. DCs cultured with sHLA-G showed a reduced ability to induce NK cell activation (83). HLA-G non-amers have shown to be presented by HLA-E, which stabilizes the HLA-E molecule (84).

Gregori et al. have shown that a specialized tolerogenic type of DCs is accumulating in the human decidua during pregnancy. These cells express HLA-G and are named DC-10 because they secrete high amounts of IL-10. DC-10 can induce type 1 regulatory T (Tr1) cells, which are characterized by their cytokine profile – secretion of IL-10 and TGF-β among others (73, 85).

Thymic stromal lymphopoietin (TSLP) is expressed by the epithelial cells of Hassall's corpuscles in the thymus and induce DCs to stimulate Treg differentiation. During pregnancy though, the function of the thymus is reduced, and the Treg expansion during pregnancy has been proposed to take place in the placenta since TSLP is produced by the trophoblast cells. The trophoblast cells are in close contact with DCs around the spiral arteries and TSLP likely activate the $CD11^+$ DCs to secrete IL-10 and TGF-β and instructing them to induce the differentiation of immature T cells into $CD4^+CD25^+FoxP3^+$ Tregs that also secrete IL-10 and TGF-β. The Tregs further induce the trophoblast cells to express HLA-G, which causes the decrease in uNK cytotoxicity. This tolerogenic loop was recently described by Du et al. (86). The DCs in the study by Du et al. secreted IL-10 as well as the HLA-G-expressing DC-10 described by Gregori et al. and it can be speculated if they might be part of the same DC subset. Also, HLA-G presenting $CD4^+$ T cells have been identified at the feto-maternal interface, where they may contribute to the tolerogenic milieu (87).

In mice, it has been shown that the overall $CD4^+CD25^+$ suppressive T cell pool increases during pregnancy, that a third of the $CD4^+CD25^+$ T cells in the pregnant uterus express FoxP3, and that depletion of $CD25^+$ T cells results in gestation failure (88).

HLA-G POLYMORPHISMS IN PRE-ECLAMPSIA AND RECURRENT MISCARRIAGES

Pre-eclampsia is a multisystemic pregnancy disorder that is manifested clinically in the late second and third trimester of pregnancy. The etiology of pre-eclampsia is unknown, although a substantial number of studies favor a theory based on a maladapted immune system, with the more specific attributes of low levels of immune regulatory cells and a low expression of HLA-G. The low expression is hypothesized partly to be a consequence of genetic variability. In support of this, a reduced level of *HLA-G* mRNA is observed in pre-eclamptic placentas, which directly correlates with the *HLA-G* genotype (5). Especially, the 14-bp ins/del 3′UTR polymorphism in exon 8 has been extensively studied, and this polymorphism has been found to be associated with severe pre-eclampsia in several studies (5, 6, 8, 89, 90). However, some studies have not observed an association between the 14-bp ins/del polymorphism and pre-eclampsia (91–93). Few studies address the issue that pre-eclampsia presents in a mild and a severe form, and furthermore, that it can be defined based on early- and late-onset, and importantly, that these forms potentially have distinct etiologies (94). Other polymorphisms associated with pre-eclampsia are typically present in the 5′URR and 3′UTR (8, 95).

HLA-G polymorphisms have been investigated in relation to recurrent miscarriages, also, with contradicting results (64, 96, 97). Two meta-analysis have addressed the possible association

between the 14-bp ins/del and recurrent miscarriages: the first performed by Wang et al. including 14 studies, 1464 cases, and 1247 controls, found that the 14-bp ins/del is significantly associated with unexplained recurrent miscarriage, and suggests that the 14-bp-insertion increases the risk of recurrent miscarriage (98). These findings were challenged by another meta-analysis performed by Fan et al. including 17 studies, 1786 cases, and 1574 controls. The authors concluded that the body of evidence to demonstrate a conclusive association between the 14-bp ins/del with the risk of recurrent miscarriages is inadequate (99). In a subgroup analysis, however, they did find an association between the 14-bp ins/del polymorphism and risk of recurrent miscarriage in women, who suffered three or more miscarriages. Furthermore, they criticize the meta-analysis by Wang et al. for including two different studies based on the same study group (99).

An increasing amount of studies acknowledge that the etiology of various pregnancy complications is based on the unique immunogenetic combinations of the mother and the father. Paternal immunogenetic factors may indeed contribute to the risk of development of pre-eclampsia. One study shows that the paternal HLA-G *G*01:06* contribution significantly increases risk for pre-eclampsia in multigravidas, who do not carry this allele (100).

REGULATORY IMMUNE CELLS IN PRE-ECLAMPSIA

Hsu et al. have recently published a study with the purpose of describing the role of immune regulatory cells in pre-eclamptic women (101). $CD4^+HLA\text{-}G^+$ T cells in the periphery and the decidua from healthy pregnant women, and from pre-eclamptic cases and non-pregnant women, were measured. In previous studies, APCs characterized by being $CD14^+DC\text{-}SIGN^+HLA\text{-}G^+$ and ILT-4^+ have been described. These could be the same DCs described by Gregori et al. (87). Like Tregs, this $CD4^+HLA\text{-}G^+$ T cell subset may play an important role in immune tolerance during pregnancy. In the periphery, increase of $CD4^+HLA\text{-}G^+$ T cells during healthy pregnancies was observed compared to non-pregnant controls. Pre-eclamptic women had a significantly lower fraction of $CD4^+HLA\text{-}G^+$ T cells than healthy pregnant women. $CD4^+HLA\text{-}G^+$ T cells seem to be more mature compared to $CD4^+HLA\text{-}G^-$ T cells because of their expression of CD80 and CD86. Also, the $CD4^+HLA\text{-}G^+$ T cells expand in the decidua compared to the periphery, but whether it is a local expansion of the T cell pool or recruitment from the periphery is not known. Again, pre-eclamptic women had a lower expansion of $CD4^+HLA\text{-}G^+$ T cells than healthy pregnant women. It was demonstrated that the $CD4^+HLA\text{-}G^+$ T cells acquire their HLA-G by trogocytosis. In this case from $CD14^+DC\text{-}SIGN^+$ DCs expressing HLA-G and ILT-4 (101).

FUTURE ASPECTS AND CONCLUSION

As mentioned, HLA-G exists in a monomer form and a dimer form, the latter by forming an intermolecular disulfide bridge between two cysteine residues of the α1 domains of two HLA-G molecules (102). Studies indicate that the dimer is the most active form; it has a higher affinity than the monomer to ILT-2 and ILT-4, and the dimer enhances the ILT-2-mediated signaling at the cellular level (103). In line with this, focus has been drawn to HLA-G dimers and synthetic dimer HLA-G molecules for possible therapeutic use (104). In mice, recombinant sHLA-G and synthetic HLA-G molecules have been shown to inhibit the early stages of arthritis in a rheumatoid arthritis disease model and to significantly prolong the acceptance of skin grafts (104, 105). It can be speculated that synthetic sHLA-G analogs might find a place in the treatment of certain pregnancy-related disorders, such as pre-eclampsia and assisted reproduction. However, a better fundamental understanding of the pathophysiology in these disorders is needed before proceeding to such enterprises.

Also, in *in vitro* fertilization (IVF) treatments, the measurement of sHLA-G in the embryo culture medium can be used as a marker for improving successful assisted reproductive technology, by choosing the fertilized oocytes with highest potential, as sHLA-G positive culture medium correlates with pregnancy success (10, 58, 106).

In conclusion, for obtaining a successful conception and a pregnancy in terms of optimized immune modulation, a combined expression of HLA-G from several sources seems to be important: by the mother in the blood, in follicular fluid, and in the genital tract, by the embryo and the trophoblast cells in the placenta, and by the father through the presence of sHLA-G in semen. One of our recent studies even revealed a significant association between HLA-G genotype and the amount of sHLA-G in seminal plasma (16). More studies are needed to elucidate the precise roles and the importance of these different sources of HLA-G in relation to uncomplicated pregnancies and in pre-eclampsia, in recurrent miscarriage and in assisted reproduction, especially with respect to the control of HLA-G expression involving *HLA-G* gene polymorphisms together with molecular and cellular immune interactions.

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The promising potential of menstrual stem cells for antenatal diagnosis and cell therapy

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Menstrual-derived stem cells (MenSCs) are a new source of mesenchymal stem cells isolated from the menstrual fluid. Currently, there is a growing interest in their clinical potential due to fact that they are multipotent, highly proliferative, and easy to obtain in a non-invasive manner. Sampling can be repeated periodically in a simplified and reproducible manner devoid of complications that no existing cell source can match. MenSCs are also free of ethical dilemmas, and display novel properties with regard to presently known adult derived stem cells. This review details their distinctive biological properties regarding immunophenotype and function, proliferation rate, differentiation potential, and paracrine effects mediated by secreted factors. Their possible role in antenatal diagnosis is also discussed. While more insight on their immunomodulatory and diagnostic properties is needed, the impact of clinical and epidemiological factors, such as age, use of contraceptives, or hormonal status still requires further investigations to properly assess their current and future use in clinical application and diagnosis.

Keywords: menstrual stem cells, stem cells, menstrual blood, cell therapy, mesenchymal stem cells

INTRODUCTION

Mesenchymal stem cells (MSCs) are pluripotent progenitor cells with self-renewing capacity and potential ability of differentiating into various specialized cell types under specific conditions. Adult stem cells are derived from different sources, such as bone marrow, adipose tissue (AD), or post-natal tissues such as umbilical cords and placenta. MSC have recently received a great deal of attention because of their therapeutic potential for treating immune mediated or neoplasic human diseases. However, the difficulty of isolating adult stem cells from diverse tissues due to the invasiveness of the extraction methods and the need for *in vitro* expansion are limiting points in their clinical applications. Therefore, many studies have focused on the search for novel stem cells that can be effectively used for therapeutic purposes without these limitations. While each clinical application will have its own selection criteria for choosing the most appropriate MSCs source, a representation of a decision tree based on six sources of MSCs and five different criteria related to their availability, isolation procedure, and different properties is presented in **Figure 1**.

A study published in 2007 identified and characterized a new source of stem cells within the menstrual fluid. They showed that menstrual-derived stem cells (MenSCs) are a highly proliferative stem cell population that is able to differentiate under standard laboratory conditions into specific-tissue cells of three germ layers (1). These cells present a good alternative to MSCs present in other sources such as bone marrow, adipose, and post-birth tissues due to the fact that they have higher proliferation rates and are of easy access with no need for surgical procedures or hospitalization, a feature that none of the existing sources can match. They are also free of ethical dilemmas and display novel properties with regard to the presently known adult derived stem cells.

ARE MenSCs JUST ANOTHER MSCs SOURCE?

A detailed characterization of the MenSCs is a pre-requisite for a head-to-head comparison with related cells from other sources. This will pave the way for evaluating possible advantages of MenSCs and also their safety/efficacy profile for clinical applications.

PROLIFERATION, SENESCENCE, AND MIGRATION

Meng et al. showed that MenSCs from the menstrual fluid of young healthy women grew at a rate of one doubling every 19.4 h, which is twice faster than bone marrow-derived MSCs (BM-MSCs), estimated at 40–45 h in early passages (1). In an effort to understand such a high proliferation rate, one should look back at their origin and physiological function. The endometrium consists of the epithelial layer and the underlying lamina propria. This layer is structurally and functionally divided into the functionalis – with glands extending from the surface epithelium – and the lower basalis (2). The upper two-thirds of the functionalis are shed during menstruation and are a major part of the collected menstrual fluid. Recent studies have provided ample evidence for the existence of stem/progenitor cells in human endometrium. Human uterine endometrial cells were once established as a feeder layer to maintain the undifferentiated state of human embryonic stem cells, since the high expression of embryotrophic factors and extracellular matrices plays a vital role in their growth (3). Human endometrium thus contains a population of stem cells responsible for this remarkable regenerative ability, and menstrual

FIGURE 1 | Schematic representation of a decision tree based on six sources of MSCs and five different criteria related to their availability, isolation procedure and different properties.

fluid include a population of such cells that can be expanded in culture and still remain able to express the phenotype of multiple lineages.

A good proliferation rate is essential for clinical applications since cell-based therapies are dose dependent, preferably with cells from lower passages. In most human trials, one million/kg is the dose of choice; however, when allogenic or repeated usage seems possible, escalating the yield of cultures becomes of utmost importance. Nonetheless, a high proliferation is also a two-edged sword that could lead to genetic instability or the exhaustion of a specific stem cell pool. In fact, these MenSCs have been largely expanded *in vitro* without any mutation or visible abnormality at the chromosomal level reported so far. They maintained a telomerase activity greater than 50% even at passage (P) 12 compared with human embryonic stem cells (4), and also appear to mildly express the chemokine receptor CXCR4 and the respective receptor for stromal cell-derived factor-1 (SDF-1), which play a significant role in the mediation of MSC migration (5). More interestingly, in our hands these cells did not show any sign of stem cell exhaustion evidenced by a steady expression of stromal stem markers, a stable proliferation rate, and colony-forming-unit (CFU) potential when comparing early (P3) versus old (P12) passages (unpublished data). Such a high proliferative rate in the face of genetic stability, with apparent preservation of multipotency, indicates this new type of stem cell could present unexpected therapeutic properties, a fact that is also implied by their extensive differentiation capabilities.

IMMUNOPHENOTYPE

MenSCs have been shown to be positive for mesenchymal stem cell markers including CD9, CD29, CD105, and CD73, and negative – as expected – for hematopoietic markers such as CD34, CD45, and CD133 (6). However, some groups have reported positive expression of embryonic markers such as SSEA-4 and Nanog in MenSCs that were not found on MSCs from other sources (7–10). This raises the question whether these cells presenting earlier markers of stemness represent a more primitive progenitor than MSCs from other sources. Nonetheless, a second group of researchers showed a different pattern of expression in cells isolated and cultured under comparable conditions (1). In **Table 1**, we list an exhaustive comparison of published phenotyping profiles from all available published studies. In our Lab, we have further characterized these cells, not only for mesenchymal and embryonic markers, but also for endothelial and epithelial traits, as other cell types might represent a source of contamination of the MenSCs culture. These quality-control parameters are essential when comparing similar cells from different sources.

DIFFERENTIATION POTENTIAL AND REGENERATIVE PROPERTIES

The ability of MenSCs to differentiate into adipose, bone, cartilage, cardiac, neural, hepatic, and pancreatic cell types has been shown using standard differentiation techniques and media. A study by Hida et al. using coculture with fetal mouse cardiomyocytes evidenced immortalization mediated by human telomerase reverse transcriptase (hTERT) on MenSCs (13). They also demonstrated spontaneous beating upon cardiogenic differentiation. When their cardiac differentiation potential in a scaffold culture system differentiated, MenSCs exhibited higher expression of cardiac marker (TNNT2) when compared with induced BM-MSCs (14).

In addition, these multipotent cells had the ability to differentiate into respiratory epithelial cells, neurocytes, myocytes,

Table 1 | Comparison of the different immunophenotypic profile of MenSCs.

Markers	Cellular expression	Meng et al. (1)	Borlongan et al. (11)	Patel et al. (4)	Allickson et al. (8)	Cui et al. (9)	Khanjani et al. (12)	Mou et al. (10)
CD14	Myelomonocyte	(−)				(−)		
CD34	Hematopoietic progenitor and stem cell, endothelial cell	(−)		(−)	(−)	(−)		(−)
CD38	Variable levels on hematopoietic and no-hematopoietic cell	(−)		(−)				
CD45	Leukocyte	(−)		(−)	(−)	(−)	(−)	(−)
CD133	HSC/endothelial progenitor cell	(−)		(−)		(−)	(−)	
STRO-1	MSC	(−)						
SSEA-4	ESC	(−)		(+)	(+)			
Nanog	ESC	(−)	(+)					
CD9	MSC, hematopoietic, and epithelial cell	(+)		(+)				
CD29	MSC	(+)		(+)	(+)	(+)		(+)
CD73	B and T-lymphocyte, MSC, endothelial cell	(+)				(+)		(+)
CD41a	Megakaryocyte and platelet, found in MSC	(+)						
CD44	MSC, hematopoietic cell (except platelet)	(+)		(+)	(+)	(+)		
CD90	Hematopoietic, MSC, T-lymphocyte	(+)		(+)	(+)	(+)		(+)
CD105	MSC, vascular endothelial cell, myeloid, and lymphoid leukocyte	(+)		(+)	(+)	(+)	(+)	(+)
OCT-4	ESC	(+)	(+)		(+)		(+)	
CXCR4	Stem cell chemotaxis		(+)	(+)				
CD166	MSC, activated leukocyte			(+)	(+)		(+)	
CD49f	ESC			(+)				
MHC I	All cells (except erythrocyte)			(+)		(+)		
MHC II	APC			(−)		(−)		(−)
LIN				(−)				
CD117 (c-kit)	HSC/Germ cell			(+)	(+)	(−)		
CD13	Myelomonocyte, endometrial stromal cell, MSC					(+)		
CD54	MSC					(+)		
CD55	MSC, hematopoietic cell					(+)		
CD31	Endothelial cell					(−)		
CD50	Leukocyte, endothelial cell					(−)		
CD106	Macrophage, endothelial cell						(−)	
Vimentin	MSC						(+)	

Abbreviations: MSCs, mesenchymal stem cells; APC, antigen-presenting cells; ESC, embryonic stem cells; HSC, hematopoietic stem cells.

endothelial cells, pancreatic cells, hepatocytes, adipocytes, and osteocytes (1). A recent paper, showed a comparable hepatic differentiation ability of MenSCs with bone marrow-derived MSCs (BM-MSCs) through the expression of many hepatic markers such as albumin (ALB), cytokeratin 18 (CK-18), and tyrosine aminotransferase suggesting this new source as a safe alternative to BM-MSCs for cell-based therapies in chronic liver diseases (12). Furthermore, the differentiation potential of MenSCs into glial lineage was compared with bone marrow-stem cells (BMSCs), where both sources showed up regulation of glial fibrillary acidic protein, Olig-2, and MBP and down regulation of Nestin protein (15). Li et al. showed that MenSCs present a new source for the generation

of induced pluripotent stem cells (IPS) with a high reprograming efficiency (16).

However, it is important to note that these experiments were conducted exclusively *in vitro*, and that differentiation status was determined only phenotypically using specific antibodies (1). A thorough investigation needs to be undertaken to validate these claims *in vivo* and show that the differentiated cells possess as well functional properties.

IMMUNOMODULATION PROPERTIES

Mesenchymal stem cells exert extensive immunomodulatory effects *in vitro* and *in vivo*, since they have been shown to inhibit mixed lymphocyte reaction (MLR), promote regulatory T cell generation (Tregs) (17), and to curb T helper (Th) 1 and Th17 differentiation among other suppressive effects. The fact they remain hypoimmunogenic or immune privileged has allowed their successful therapeutical use even in allo or xenogenic conditions. However, their action seems somewhat complex, since they have been shown to abrogate or conversely to exacerbate different (or even the same) autoimmune disease model under varying experimental conditions (18). While extensive progress has been made to decipher the immune features of MSCs, the description of the functional and immune effects of MenSCs is still only in its initial stages. Bearing in mind the similarity, but also the differences displayed by the MSCs, we isolated from menstrual fluid – as opposed to bone marrow – a simple extrapolation of functional or regenerative properties seems unwarranted. More so since the exploration of specific functional properties and safety issues are considered a pre-requisite to reach clinical application.

Of note, the endometrium is known to be an integral part of the mucosal immune system. It seems uniquely poised to initiate antigen specific effector as well as immunosuppressive actions, leading to responses that are protective from infectious pathogens while preserving the integrity of the fetus (19). It is therefore not unexpected that these newly discovered stem cells might exert potent immune mediated effects. Nonetheless, there is an understandable dearth of clinical or even pre-clinical data at the present time, given the recent identification of these cells. Zhong et al. reported the feasibility of allogeneic transplantation of MenSCs into four compassionate cases of patients with multiple sclerosis, where no related side effects were found after a year of follow-up, though no immune function studies were reported (20). In fact, we are not aware of any description of the use of MenSC in autoimmune human or animal models of disease (7). However, in a preliminary report of beneficial effects in a murine model of critical limb ischemia, MenSCs were shown to suppress lymphocyte MLR and the production of interferon gamma (IFN-γ) and tumor necrosis alpha (TNF-α) in a dose dependent manner *in vitro* (21).

The complete assessment of the effects of MenSCs on lymphocyte proliferation and alloreactivity in a contact dependent and contact independent manner in transwell experiments, in comparison with BM-MSCs is required to fully unravel their immunomodulatory effect. One published report indicates that MenSCs would exert opposite effects on the MLR response at different target: MenSCs ratios (22). This emphasizes the need for further studies providing insight into the mechanisms involved in this potentially new cell therapy-based application. This includes the evaluation of immunostimulatory molecules such as MHC I and II, CD40, and CD80/86. While BM-MSCs have been described to express antigen presenting (MHC I and even low level MHC II) in response to IFN-γ, they still remain immune privileged since they do not express co-stimulatory (CD80/86) molecules that are required to shift the immune response from a tolerogenic to an effector phenotype (23). Indeed, the main effect of IFN-γ on MSCs is the final "licensing" or activation of their immunosuppressive and reparative properties that tend to occur mainly in the presence of tissue damage. Thus, IFN-γ, concomitant with TNF-α or other proinflammatory cytokines (IL-1α or IL-1β) or mitogens (LPS), triggers a cascade of cellular events responsible for many of the immunosuppressive effects of MSCs both *in vitro* and also *in vivo* (24–27). These entail the upregulation of several chemokines (i.e., CCL-2/MCP-1), adhesion molecules (VLA-4, VCAM, and the SDF-CXCR4 axis among others), and of inducible nitric oxide synthase (iNOS) in the case of murine MSCs. Lymphocytes then migrate into the proximity of MSCs, where T cells are suppressed by nitric oxide (NO) (27). In the case of human MSCs, suppression appears to be exerted by exhaustion of tryptophane, mediated by indoleamine dehydrogenase (IDO) instead of NO (28). In addition, non-contact dependent factors also contribute to the immune effects of BM-MSCs, including prostaglandin E2 (PGE2), IL-6, IL-10, Galectin-1, and TNF-α-induced protein 6 (TSG-6) (29). These broader or even the species specific mechanisms have not yet been analyzed in the case of MenSCs.

Furthermore, and in an effort to understand the contrasting clinical effect reported for MSCs in mouse models of human rheumatoid arthritis (18, 30) and SLE (31, 32), our group has recently evaluated the role of BM-MSCs in the differentiation of Th1, Treg, and Th17 cells (33, 34). The balance or dysregulation of these $CD4^+$ helper subpopulations is a critical factor governing disease pathogenesis and clinical response in several immune mediated diseases including murine and human SLE (35). Finding a possible explanation for the disparate clinical results of cell therapy, we initially described that MSCs suppressed Th17 cells under resting conditions, but surprisingly, expanded them once activated (33). In further transwell experiments, we evidenced the need for cell contact to suppress Th17 proinflammatory cell function (34). This methodology is currently under investigation in our group, for the full evaluation of Th1/Th17/Treg modulating properties of MenSCs, which are presently unknown.

THE SECRETOME AS "CELL-FREE" THERAPY

The potency in tissue restoration mediated by paracrine factors of a broad range of bioactive molecules (secretome) produced by MSCs has raised interest in further exploring this aspect for potential therapeutic applications. This mechanism includes various main actions: immunomodulation, anti-apoptosis, angiogenesis (36), and support of the growth and differentiation of local stem and progenitor cells, and chemoattraction (37, 38). This secretion of factors or secretome could be exploited to extend the therapeutic possibilities of MSCs for treatment of a variety of diseases. The administration of MSC-released factors or conditioned medium (CM), could avoid some of the limiting factors associated with cell therapy such as immune incompatibility, tumorigenicity, costs,

and waiting time for *ex vivo* expansion. This would provide an alternative option with affordable costs, excellent quality-control, consistency, and reproducibility. A wide range of different growth factors, cytokines, and extracellular matrix proteins (ECM) have been identified as constituents of the *in vitro* cultured MSC secretome. Additionally, several reports also showed that MSCs are able to secrete large amounts of micro and nanovesicles such as exosomes (39). The exosomes, released by most cells, are potent mediators of cell–cell communication due their ability to transfer proteins, lipids, and functional genetic material such as mRNA and miRNA (40, 41). Exosomes are released from cells constitutively, or following activation that significantly increases their secretion. To date the best MSCs characterized secreted proteins are those released by umbilical cord MSCs (UC-MSC) (42), AD (43), and BM-MSC (44). Several authors have documented that cells increase the liberation of vesicles in response to different types of stresses, such as hypoxia, acidosis, oxidative stress, thermal stress, and cytotoxic drugs (45). Since MenSCs niche, homeostasis and physiological condition are different from the sources mentioned above, one can speculate that they might possess a specific secretome signature that will differentiate them from MSCs found in other environmental condition.

For example, the necessary activity against pathogens in the endometrium could condition their secretome, probably through the release of antimicrobial factors. Krasnodembskaya et al. determined in a pneumonia mouse model that in response to stimulation by *Escherichia coli* inhibiting bacterial growth (46).

At the paracrine level, little is known regarding the factors secreted by MenSCs. Meng et al. described that MenSCs secrete matrix metalloproteinases (MMP3 and MMP10), cytokine growth factors [granulocyte macrophage colony-stimulating factor, GM-CSF; platelet-derived growth factor (PDGF)-BB] and angiogenic factors (angiopoietin-2, ANG-2) *in vitro*, in quantities 10–200,000 times higher than UC derived cells (1). However, no difference was observed with others angiogenic factors like VEGF, HGF, and EGF. While the regenerative and therapeutic potential of MenSCs-conditioned media have not been fully evaluated in an animal model yet, a study of an *in vitro* stroke model of oxygen glucose deprivation (OGD) determined that OGD-exposed primary rat neurons that were co-cultured with MenSCs or exposed to MenSCs-conditioned medium (MenSCs-CM) exhibited a significant decrease in cell death (11). It has been recently shown that MensCs can reverse hyperglycemia in diabetic mice most probably through paracrine factors since human insulin-producing cells was not detected in the pancreas of the injected mice (47).

In our hands, MenSCs showed a stronger supportive potential for hematopoietic stem cell (HSC) cultures, than BM-MSCs under cell-to-cell contact conditions (submitted data). We also showed that the non-contact condition (transwell) resulted in the $CD34^{+}CD133^{+}$ HSCs expansion although it was lower than that of the direct cell interactions with the stromal cells. These results suggest that MenSCs might display a quantitative and/or qualitatively distinct "secretome," or panel of surface molecules capable of exerting distinct contact and paracrine effects on their targets. Furthermore, their protein expression profile can also be modified through the overexpression of factors of interest as they were shown to be permissive for retroviral transduction (48).

Taken together, these studies suggest that MenSCs share some properties with other MSCs but might functionally produce factors that are specific to them. This can be investigated through a comparative analysis of their secretome under different stimulation conditions, including a profiling of their exosome content.

SAFETY CONCERNS AND CLINICAL APPLICATIONS

From a safety perspective, concerns have emerged around the procedure of collecting sterile samples, as under many countries regulations, cell and tissue collection and storage must be done in sterile conditions. This has been circumvented by a pre-treatment of the collected sample with antibiotics prior to culture, and by working in a sterile area under good manufacturing practice (GMP) conditions with proper product release criteria. Another concern is the development of endometriosis and the possibility of activation or progression of dormant tumors. To address this aspect, we performed a chronic tumorigenicity and toxicity studies, where progressive doses from 1 to 10^6 MenSCs were injected subcutaneously in both male and female immunocompromised NOD/SCID il2rγ null mice. No sign of tumor development or toxicity was detected after a 16 weeks follow-up (unpublished data). In a different experimental setting (12), injected 2×10^6 MenSC in nude mice (12). According to the histological examination, no evidence of tumor growth was found in inoculation site and the examined tissues had no morphological characteristics of tumor as judged by H & E staining. Moreover, to assess whether MenSCs modulate tumor growth, a rat glioma model was used. The injected cells showed a substantial inhibition of the tumor growth when compared to the control group (49).

The first report of clinical usage of MenSCs involved the allogenic injection of four patients with Multiple sclerosis, with a total dose of 16–30 million cells. Treated patients showed no apparent physical or serological abnormalities at follow-up (20). More recently, Medistem, a stem cell company, launched a phase II clinical trial with MenSCs, planning to enroll a total of 60 patients with congestive heart failure, receiving escalating doses up to 200 million cells from a universal donor. According to the published report in 2013, 17 patients have been injected with no treatment associated adverse events reported (50). Medistem has also obtained FDA clearance to begin Phase I trials in the US for treatment of critical limb ischemia, an advanced form of peripheral artery disease.

In all the MenSCs studies mentioned in this review, cells were isolated from healthy donors. There are no published reports yet characterizing the property changes of MenSCs isolated from epidemiologically different background donors. Thus the effects of age, hormonal status (post-puberty versus pre-menopausal), or prior contraceptive usage remain unexplored. Since stem cells are sensitive to environmental changes and stress conditions, one can only speculate if these variations might affect their function and properties. While it is known that proliferation and therapeutic potential are greatly impacted by the pathological conditions of the donors, little is known on the extent of the effect of these physiological changes on MenSCs. An epidemiological study comparing the secretome, phenotype, and immunomodulatory among other properties would present a valuable guide for the formulation of inclusion and exclusion criteria of donors for a stem cell-based therapy.

MenSCs AS A DIAGNOSTIC TOOL?

As MSCs properties are modulated by environment factors, it also becomes important to analyze the role of these changes in pathological conditions.

Of the 130 million newborns each year, 8 million die before their first birthday. A contributing factor in many of these deaths is poor pregnancy outcome as a result of a complication of pregnancy, including hypertensive syndromes (e.g., pre-eclampsia – PE); poor fetal growth (e.g., intra-uterine fetal growth restriction – IUGR); gestational diabetes and preterm birth. Each occurs with an incidence of 5–10% and are responsible for the majority of obstetric and pediatric morbidity and mortality and can permanently impact on lifelong health. As an example, PE has become one of the main causes of maternal and fetal morbidity and mortality in the world, and has also been strongly associated with an increased risk of later-life death due to cardiovascular disease, independent from other risk factors (51–53). On the other hand, over the past 15 years, much has been discussed and published about the profound effects that sub-optimal health conditions during pregnancy, especially during early stages, have on the predisposition of the newborn to adult diseases (i.e., developmental origins of disease paradigm). Therefore, the understanding of the early processes during implantation and early stage embryo development, will not only impact on the outcome of contemporaneous pregnancies (including, early pregnancy loss, pre-eclampsia, intrauterine growth restriction, pre-term birth, gestational diabetes, and maternal death) but also on newborn morbidity and mortality and their susceptibility. These evidence highlights the need of accurate diagnosis of the pre-disposition to, or early detection of disease during pregnancy, or even before that, allowing the implementation of effective treatments to prevent the occurrence of the disease.

It is now clear that the physiopathological process of many pregnancy diseases begins with an inadequate trophoblast invasion early in pregnancy (54). Several hypotheses have been proposed to explain the abnormal trophoblastic invasion early in pregnancy, e.g., PE or IUGR, many of them suggesting that it might be triggered by an altered maternal immune response or a defective development of maternal tolerance to the allogeneic fetus. Epidemiological evidence supporting this idea has been published by many groups, suggesting the importance of the maternal immune system in the pathogenesis of placental originated diseases. Different studies have been performed to characterize the local and systemic immune milieu of these patients as an explanation for the abnormalities of placentation observed in PE (55–57). Normal pregnancy is considered as a (T helper) Th2 type immunological state that favors an immunosuppressive environment in order to prevent fetal rejection (58). Since, MSCs have been widely implicated in immunosuppressive mechanisms targeting a range of target cells, in the context of antenatal screening, one area of great interest is to identify if MenSCs are also implicated in these complications. This could be achieved through a comparative study of the changes in their immunomodulatory and paracrine factors in comparison to MenSCs isolated from donors with uncomplicated pregnancy history.

Recent data, suggest that microvesicles (MV) are released from the placenta and their concentration in maternal plasma increases during normal pregnancy (59, 60). They contain placenta-specific proteins and miRNA and, as such, may be differentiated from maternally derived MV (61). The concentration of exosomes has been reported to increase in association with pre-eclampsia and we have also established that MVs release is changed when placental cells are exposed to different environment (submitted data). Moreover, we have been able to demonstrate that the content, proteins, and miRNA. Therefore, complications of pregnancy that affect placental perfusion or exposure to abnormal concentrations of factors that modulate the release of MVs will be reflected in their concentration and cargo in the maternal blood. It has been shown that MSCs are among cells that produce high amount of MVs (39), with a known therapeutical effect in myocardial ischemia injury (62), liver fibrosis (63), and other diseases (64, 65). Since abnormal concentration and content of placental-derived MVs in maternal blood is a surrogate measure of placental dysfunction, it will be of great interest to analyze the content of MVs isolated specifically from MenSCs and study their predictive biomarker properties in the diseases and whether they can be established as an early diagnostic test in pre-symptomatic patients.

CONCLUSION

Although MenSCs have been tested only in very limited disease models, these cells have been shown to possess various regenerative properties under physiological and pathological conditions. From a translational point of view, MenSCs appear to have practical and also biological advantages over other stem cell sources. While some clinical research group and companies launched clinical trials using these cell, these fast developments in the face of lacking data, underscore the need to characterize the differentiation potential and immunological properties of well defined populations of MenSCs. The need for this type of information is decisive with respect to the development of safe and effective cell therapies for clinical application in human diseases.

The other to be investigated property of MenSCs is their potential as biomarkers that could be highly informative of the risk of asymptomatic early pregnant women subsequently developing complications of pregnancy. Such tests will offer valuable clinical information that will provide an opportunity for timely and appropriate intervention.

Future research and new evidence would greatly contribute to propulse MenSCs to the top list of best proven source of MSCs for new therapies and novel diagnostic tools.

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