INNATE IMMUNITY IN NORMAL AND ADVERSE PREGNANCY

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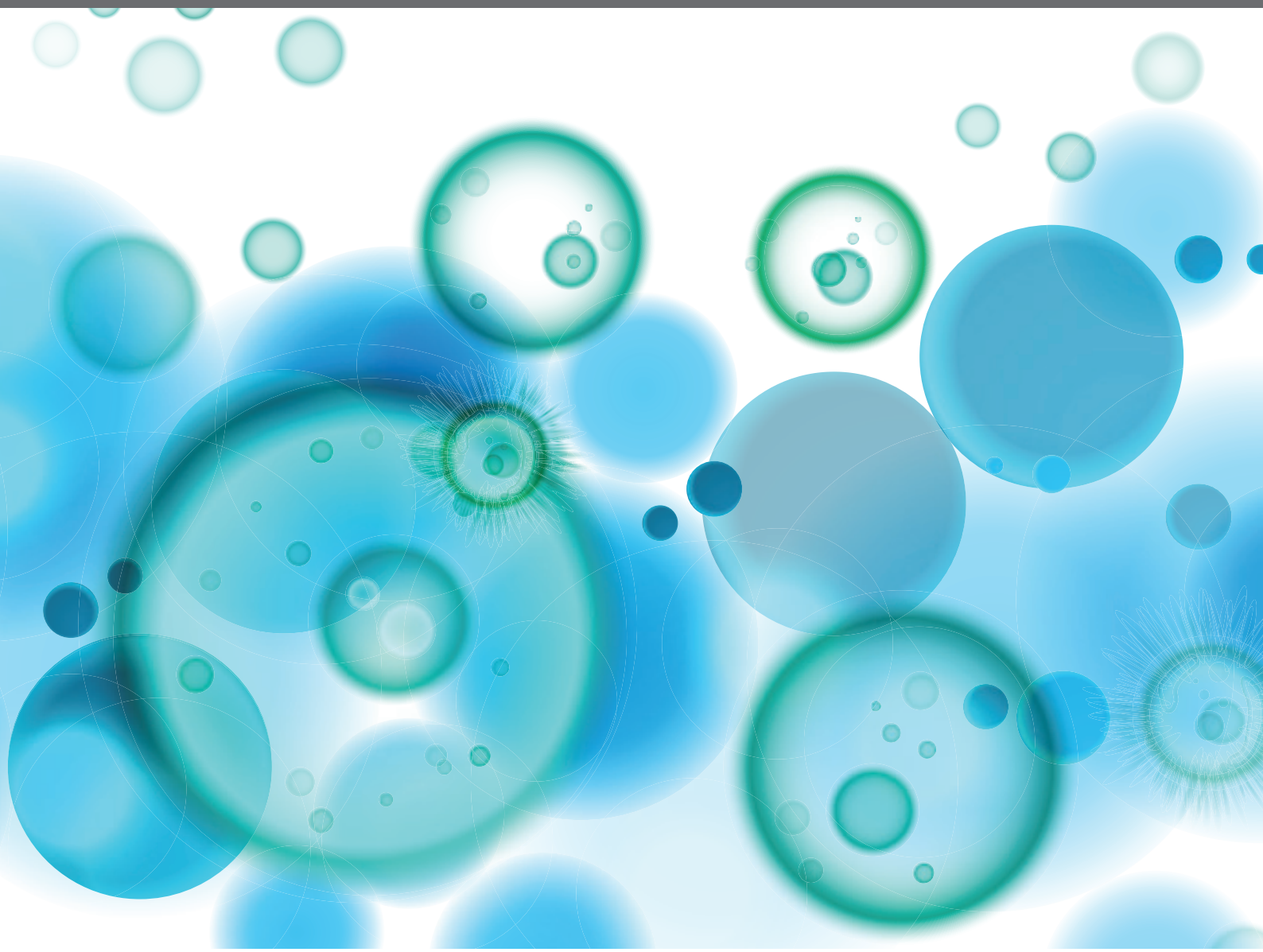

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INNATE IMMUNITY IN NORMAL AND ADVERSE PREGNANCY

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EDITORIAL
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Editorial: Innate Immunity in Normal and Adverse Pregnancy

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Keywords: innate immunity, complement, pregnancy, innate lymphoid cells, infection

Editorial on the Research Topic

Editorial: Innate Immunity in Normal Pregnancy

In pregnancy, successful implantation, placental development and fetal growth, as well as maintenance of both maternal, and fetal health, requires balance of the immune response. Excessive activation of the immune system increases risk of rejection of the fetus and adverse pregnancy outcomes. In addition, disturbances in the immune system can lead to maternal or fetal infection. Due to cross-talk, both innate, and adaptive immunity must be properly regulated. Both our current topic "Innate Immunity in Normal and Adverse Pregnancy" and the parallel topic "Adaptive Immunity in Pregnancy" attracted 10–12 manuscripts. The articles in our topic included original research and comprehensive reviews and fell into 3 general categories: role of complement system, importance of innate immune cells, and an evaluation of innate immunity in infections in pregnancy. Each category evaluated the innate response in orchestrating a normal pregnancy or contributing to pathophysiology of adverse pregnancy outcomes (**Figure 1**). The timeliness of this topic is highlighted by the clear contribution of innate immunity to the pathology of COVID-19 infection and the potential impact on pregnancy outcomes (1, 2). This topic also synchronizes with the recent call by the Surgeon General in the United States indicating "we can—and must—do more for our moms" in reducing morbidity and mortality in pregnancy (https://www.hhs.gov/sites/default/files/call-to-action-maternal-health.pdf).

THE COMPLEMENT SYSTEM IN NORMAL AND ADVERSE PREGNANCY

The complement system is critical to healthy pregnancy due its central role in host defense. Activation of complement proteins provide immediate defense against foreign pathogens through opsonization, inflammation, and cell membrane attack. However, the role of complement in normal pregnancy extends well-beyond host defense. Girardi et al. provide an excellent review on the wide-ranging role of complement throughout pregnancy, including in normal pre-implantation, implantation, placental development, cervical remodeling, and parturition. To complete these normal pregnancy milestones successfully, homeostasis must be achieved by balancing regulation and activation of the complement system. Yet, we remain a long way from fully understanding how complement regulates even fundamental pregnancy processes, such as labor. Livson et al. shed some light on this topic, through original data demonstrating laboring women at term have greater C3 in cervicovaginal secretions, but a lower percentage of C3 activation, compared to non-laboring, or non-pregnant women.

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Complement homeostasis is most critical at the maternal-fetal interface, where excess complement activation may lead to adverse pregnancy outcomes (3). Girardi et al. describe how the complement system becomes dysregulated at various stages leading to early pregnancy loss, placental insufficiency, preeclampsia and preterm birth, with associated long-term adverse effect to both mother and child. This association has been most well-characterized in pregnant women with immune complex disorders, such as systemic lupus erythematosus and antiphospholipid antibody syndrome (4). Chighizola et al. provide a current review describing the role of complement in pregnancy-associated autoimmune disease, expanding our understanding of the role of immune complexes and placental complement deposition in mediating adverse outcomes. Finally, Lokki et al. describe a case of pregnancy-associated atypical hemolytic uremic syndrome, which occurred after delivery for preeclampsia and HELLP syndrome. This case describes rapid clinical improvement and resolution of renal failure following complement (C5) blockade with eculizumab and emphasizes the emergence of complement inhibition as a therapeutic strategy for not only atypical hemolytic uremic syndrome, but potentially other pregnancy disorders characterized by complement dysregulation.

ROLE OF INNATE IMMUNE CELLS IN PREGNANCY

The sentinel innate immune cells in decidua, placenta and fetus include neutrophils, macrophages, dendritic cells, innate B1 cells and innate lymphoid cells (5) (ILCs including NK cells). These innate immune cells protect both mother and fetus from infection throughout pregnancy and are also instrumental in orchestrating a normal pregnancy. Furthermore, these cells play a critical role in educating the adaptive response to induce tolerance. In general, the timeline of pregnancy is thought to cycle through an inflammatory phenotype in implantation to a protective anti-inflammatory state in maintenance of pregnancy, and a return to inflammatory phenotype at labor (6). However, Ono et al. present data in a mouse model indicating that while macrophages are required for implantation, the effective macrophages express an anti-inflammatory, M2-like phenotype. Thus, our simplistic view of inflammation in pregnancy requires modification. Similarly, dendritic cells are known to be essential for successful embryo implantation (7), and Yasuda et al. probes the phenotype of uterine dendritic cells between coitus and implantation to better understand their role. Mendes et al. reviews ILCs focusing on distinctions between peripheral and decidual NK cells. In addition, they highlight a potential role for other ILCs in establishing and maintaining a successful pregnancy and in contributing to adverse pregnancy outcomes. Much work is needed to fully understand the classification and contribution of Group 1, 2, and 3 ILCs and NK cells to pregnancy pathology.

During many pathophysiological conditions such as preeclampsia, both innate immune cells and barrier cells produce an exaggerated and inappropriate pro-inflammatory microenvironment. Aneman et al. discusses the cells contributing to the pro-inflammatory environment, including M1 macrophages, gamma delta T cells, NK cells and B1a B cells. Silva et al. demonstrates a contribution of decidual cells to the proinflammatory microenvironment with increased activation of NLRP3 inflammasome and subsequent IL-1β production in preeclamptic pregnancies without fetal growth restriction compared to normal pregnancies. Hypertensive pregnancy disorders, such as preeclampsia, result in increased risk of later-life cardiovascular disease for both the affected mother and the infant born from the preeclamptic pregnancy (8). Silva's data suggests that the potential benefits of statin treatment in pregnancy may be due to alterations in decidual cholesterol and the NLRP3 inflammasome, reducing risk of subsequent cardiovascular disease.

INNATE IMMUNITY AND INFECTION IN PREGNANCY

The innate immune system changes throughout pregnancy but these adaptations must not compromise the ability of the immune system to protect mother and child from infection. The innate immune response recognition of pathogen associated molecular patterns that interact with molecules such as Toll like receptors (TLRs) is an early line of defense for clearance of microorganisms. In pregnancy, bacterial infection has been associated with spontaneous preterm labor (9). However, the contribution of viral infection to preterm labor remains unclear. Rasheed et al. explore the concept of multi-pathogen induced preterm labor, revealing a synergistic effect of bacterial and viral TLR stimulation on a pro-inflammatory pro-labor response. These data lend support to the idea that multiple hits of viral and/or bacterial pathogens could increase the risk for preterm labor. These studies call for more detailed analyses of multiple pathogens both locally in the reproductive tract and systemically and their association with spontaneous preterm birth.

The review of innate immunity and viral infection during pregnancy by Cornish et al. provides an excellent overview of how acute viral infections challenge the critical balance between immune tolerance and defense against infection during pregnancy. The serious public health challenge posed by SARS-CoV2 infection and the concerns for upsetting a normal pregnancy demonstrates the timeliness of the topic. The fact that influenza and hepatitis E infection increase mortality of pregnant women, makes COVID-19 a clear cause for concern. Cornish et al. focuses on RNA viruses that may cause severe disease in pregnancy. The authors emphasize the importance of understanding the immune response during pregnancy and consideration of pregnancy in the design of vaccine trials. The review by Hoo et al. takes a slightly different view of innate immunity and infection, focusing on the human decidual placental interface during both bacterial and viral infections. How do infections take hold in the presence of this significant placental barrier? A review of the architecture and important innate immune cells is nicely diagramed to highlight both cellular and soluble immune defenses.

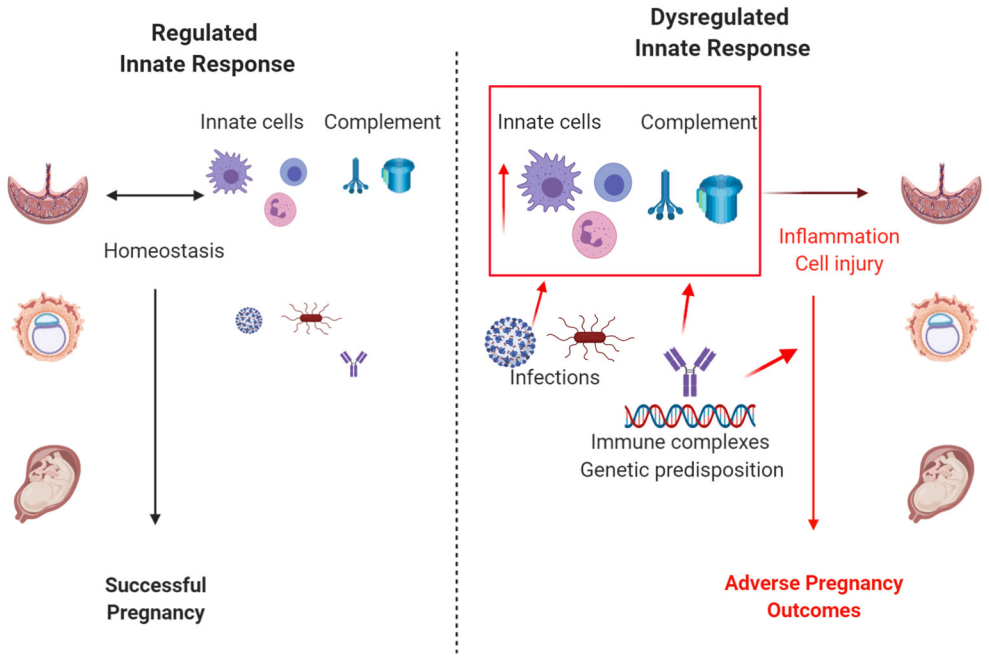

FIGURE 1 | Regulation of the innate immune response influences pregnancy outcomes. When regulated, complement and the innate immune cells, including macrophages, neutrophils, and innate lymphoid cells, control infection and promote a successful pregnancy. Excessive or dysregulated innate responses due to uncontrolled infection, autoimmune disease, genetic predisposition, or other risk factors result in adverse pregnancy outcomes. Size depicts the magnitude of infection and/or activation of the innate immune response. Created with biorender.com.

CONCLUSION

This research topic highlights the diversity of innate immune processes crucial for a healthy pregnancy. More research is needed to enhance understanding of interaction and regulation of innate immunity during pregnancy and to introduce novel therapeutic strategies to address the clinical challenges posed by the complicated pregnancy. Thus, a healthy pregnancy requires an innate cellular and humoral response for proper development, but an inappropriate response results in adverse events.

DISCLOSURE

Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the American Heart Association, Department of Defense or National Institutes of Health.

AUTHOR CONTRIBUTIONS

All authors contributed to the writing of this editorial and approved the final submission.

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CASE REPORT
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Eculizumab Treatment for Postpartum HELLP Syndrome and aHUS—Case Report

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Preeclampsia is a pregnancy-specific disorder affecting ca 3% of all pregnant women. Preeclampsia is the source of severe pregnancy complications. Later life consequences for mother and infant include increased risk of cardiovascular disease. Preeclampsia is caused by the dysfunction of the endothelium with subsequent activation of complement and coagulation systems. HELLP syndrome is considered to be an extreme complication of preeclampsia but it can also present independently. Diagnostic symptoms in HELLP syndrome are Hemolysis, Elevated Liver enzymes, and Low Platelets. Similar phenotype is present in thrombotic microangiopathies (TMAs) and HELLP syndrome is considered part of the TMA spectrum. Here, we present a case of severe preeclampsia and HELLP syndrome, which exacerbated rapidly and eventually led to need of intensive care, plasma exchange, and hemodialysis. The patient showed signs of hemolysis, disturbance in the coagulation, and organ damage in liver and kidneys. After comprehensive laboratory testing and supportive care, the symptoms did not subside and treatment with complement C5 inhibitor eculizumab was started. Thereafter, the patient started to recover. The patient had pregnancy-induced aHUS. Earlier initiation of eculizumab treatment may potentially shorten and mitigate the disease and hypothetically decrease future health risks of preeclamptic women.

Keywords: case report, preeclampsia, aHUS, eculizumab, HELLP, thrombotic microangiopathy

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INTRODUCTION

Preeclampsia is a pregnancy-specific disease affecting 3–5% of all pregnancies (1, 2). It manifests with newly onset hypertension after 20 weeks of gestation and proteinuria. The placenta is central in the pathogenesis of the disease by connecting the mother to the fetus (3). The maternal-fetal interface is the zone where genetically different tissue of fetal origin meets with maternal circulation, endothelium, and immune system. Often called the disease of theories, one generally accepted insight is that preeclampsia affects the maternal endothelium causing disturbance in the function of endothelium, which leads to hypertension and proteinuria (2). In its severe form, preeclampsia may lead to fetal growth retardation, prematurity, and for the mother it might cause organ failure in kidneys and liver as well as eclampsia. Furthermore, preeclampsia may have long-term adverse cardiovascular consequences for the mother and the newborn (4). The development of the placenta is considered inadequate specifically in the process of maternal spiral

artery transformation, where the trophoblast cells invade the maternal side into decidua and transform the arteries into low resistance conduits, also replacing the maternal endothelium (5–7). Typically, this remodeling of the uterine arteries is absent or incomplete especially in severe form of preeclampsia. High resistance in the constricted uterine arteries causes turbulent blood flow in to the intervillous space of the placenta causing oxidative stress and mechanical damage to the placental villous trees. Resulting damage increases the placental shedding of microparticles and inflammatory mediators resulting in generalized endothelial activation and dysfunction (8, 9). These sequential events in the maternal-fetal interface lead to maternal hypertension and other symptoms as described above.

HELLP syndrome was first characterized in 1982 by Weinstein as a separate syndrome, often representing together with preeclampsia but seen also alone (10, 11). HELLP syndrome is characterized by hemolysis, elevated liver enzymes, and low platelets. It often requires intensive care level observation and symptomatic therapy. The pathogenesis of the syndrome is still in the shadows. HELLP syndrome shares common features with thrombotic microangiopathies (TMAs) such as thrombotic thrombocytopenic purpura (TTP) and hemolytic uremic syndrome (HUS). TMAs present in diverse group of diseases with common features of microangiopathic hemolysis, thrombocytopenia, and organ damage resulting from microthrombi. In TTP, a genetic defect of, or as in vast majority of cases, acquired autoantibodies against a disintegrin and metalloproteinase with thrombospondin type 1 motif 13 (ADAMTS13), the enzyme that cleaves activated von Willebrand factor (vWF), cause formation of unusually large activated vWF multimers on endothelial cells leading to platelet thrombi in small vessels and hemolysis. Typical HUS is caused by Shiga-toxin producing bacterial infection (especially *Escherichia coli*) whereas atypical HUS (aHUS) refers to the type of TMA, in which genetic deficiencies in the regulators of the alternative pathway of complement system underlie, which, in the presence of a trigger may cause the clinical disease (12, 13). The FDA approved treatment for aHUS is the infusion of complement C5 antagonist eculizumab, which prevents the formation of the membrane attack complex (MAC) (**Figure 1**). Clinically HELLP shares the same symptoms as classical TMAs: hemolysis, thrombocytopenia, and organ disorder seen in liver. Depending on the TMA classification used, HELLP syndrome is usually categorized as part of secondary or acquired TMAs (**Table 1**).

In this case study, we report a patient with a complicated pregnancy-induced TMA and the successful course of treatment.

CASE PRESENTATION

Twenty-nine year old primigravida was referred at gestational age 34+2 to the Helsinki University hospital outpatient clinic with upper stomach pain. Initially, her blood pressure was modestly elevated (133/91 mmHg) and urinary dipstick positive for protein. The initially reported upper stomach pain was gradually improving. In the ultrasound scan the fetus had normal biophysical profile (BPP), the weight estimation was at the −2 *SD* growth curve. Cardiotocography (CTG) was normal. Blood hemoglobin (Hb) was 115 g/L, platelets 158 E9/L (normal range 150–360 E9/L), alanine aminotransferase (ALT) was normal (23 U/L). The urinary dipstick was positive for protein (+2) and calculated proteinuria was 1.6 g/24 h. A decision was made to initiate cortisone treatment to facilitate the lung maturation of the baby. The patient was discharged with a plan to return the next day for control check-up and second dose of cortisone. As scheduled, she came for control at gestational week 34+4. Blood pressure was 147/87 mmHg, ALT 23, platelets 177, CTG and the BPP of the fetus in the ultrasound scan was normal. She was discharged and another check-up was scheduled. In the afternoon of the same day, the upper stomach pain returned and steadily worsened toward the evening. She returned to the hospital at 2.20 a.m. She was experiencing tight upper stomach pain, restlessness, and she had vomited two times and was feeling tremor. The blood pressure was clearly elevated at 170/94 mmHg, urine protein dipstick was strongly positive, ALT was elevated at 159, Hb 122, and platelets 172. She was admitted to the prenatal ward. At 4 a.m. she was experiencing headache. Antihypertensive medication was started (Labetalol 100 mg thrice). Urine protein excretion peaked in the night being 13 g/24 h. Subsequently, she started vomiting, had upper stomach pain, headache, and the CTG monitoring showed decelerations. The patient was transferred at 7.11 a.m. to the delivery ward and as the cervix was three centimeters dilatated, the fetal membranes were artificially broken for the induction of labor. At the same time the laboratory tests were completed with Hb 122, platelets 172. Lactate dehydrogenase (LD), however, was clearly elevated at 1231 U/L at this time. In the CTG, the decelerations continued and as bradycardia continued an emergency caesarean section was performed. Male infant (1960 g, −2 *SD*) was born at 7.25 a.m. with umbilical artery pH value of 7.05, BE −6.80, Apgar 1/6/8. Blood loss in the operation was 400 ml.

At 9 a.m. after the caesarean section the mother's platelets were low at 49, with Hb of 102. By the afternoon the ALT had risen to 1800, LD 3570, serum creatinine level was 153 (μmol/L), while platelets decreased to 33. There was disturbance in the coagulation indicated by low level of fibrinogen (1.1 g/L, reference values 2–4 g/L) and high level of D-dimer of fibrin (30.2 mg/L, <0.5 mg/L). There was some bleeding from the caesarean section wound, in which additional sutures were placed. At this time, eight units of platelets were administered. Potassium level rose from 4.7 to 5.6 (mmol/L). Hemolysis was clearly observed. Coombs test was negative. Urine excretion was only 10 ml/h. The laboratory test showed clearly a severe disease with signs of damage to both the kidneys and the liver. In addition, there was disturbance in the coagulation system presenting significant coagulation and marked fibrinolysis simultaneously. Magnesium sulfate infusion was started because of hyperreflexia, which is considered a predictive sign of convulsions, a severe complication of preeclampsia. Intravenous dexamethasone 10 mg was started, and the patient was transferred into intensive care unit (ICU).

Further, laboratory tests were issued for differential diagnostic purposes of other medical emergencies (**Table 2**). The activity of ADAMTS13 was normal 62% (40–130%), which excludes TTP. Serum complement C3 (0.52 g/L, 0.71–1.41 g/L) and C4 (0.07

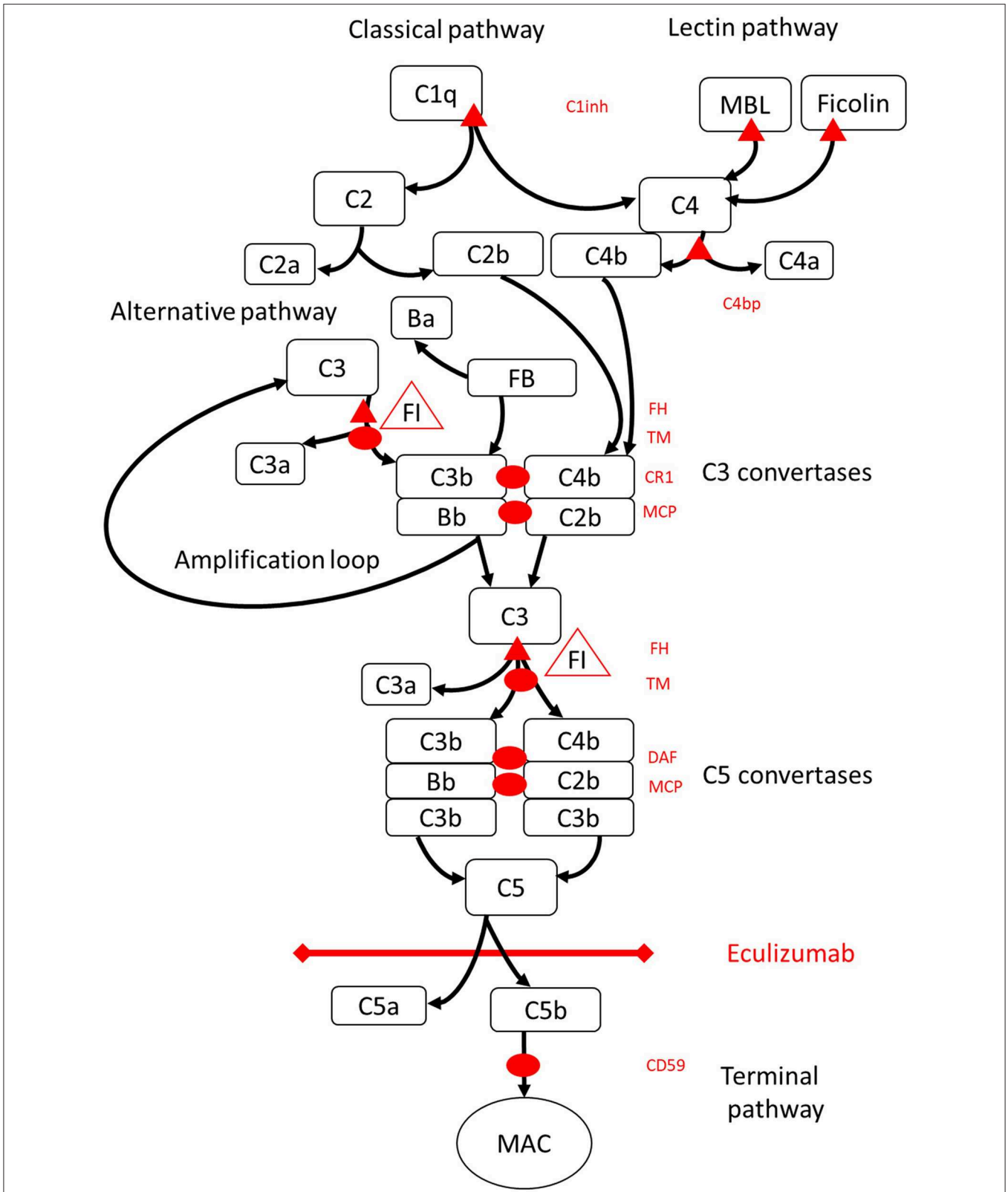

FIGURE 1 | Eculizumab inhibits terminal pathway of complement activation. Complement activation may be initiated via three pathways of activation, that all lead to the formation of C5 convertases that have the capacity to activate the terminal pathway leading to formation of the MAC on the target surface. This schematic

(Continued)

FIGURE 1 | illustration of the complement system shows its most relevant activators (in black font) and inhibitors (in red). The activators may be membrane bound (oval) or soluble (triangle). The alternative pathway activates spontaneously on all surfaces that do not allow for inhibition by the soluble regulator factor H (FH). FH acts as the cofactor for inactivation of C3b to iC3b by factor I (FI). Thrombomodulin (TM) enhances FH cofactor activity. Cleaved factor B (FB) together with the activator C3b forms the alternative pathway convertase, which has the capacity to cleave C3 into C3b creating an amplification loop of alternative pathway activation. Classical pathway of complement activation may be initiated by for example binding of immune complexes to C1q, while the lectin pathway is activated by mannoses binding lectin (MBL) or ficolins binding to for example patterns of carbohydrates on microbes. Lectin pathway activation results in mannose-associated serine proteases 1 and 2 (MASP-1 and MASP-2, not pictured) cleaving complement components C4 and C2 to form the classical pathway convertase C4bC2b. C1 inhibitor (C1inh) and C4bp are the soluble regulators of the classical pathway, while membrane cofactor protein (MCP) and complement receptor 1 (CR1) are membrane bound regulators of early complement pathways. The formation of C5 convertases initiates the terminal pathway of complement activation and cleavage of C5 in the absence of surface bound regulators decay accelerating factor (DAF) and MCP. Assembly of MAC is regulated by the surface bound regulator CD59 (protectin). Cleavage of C3 in the early pathways and C5 in terminal pathway releases anaphylatoxins C3a and C5a and results in inflammation. Eculizumab is a humanized recombinant antibody against the complement protein C5, which inhibits cleavage of C5 by the C5 convertases thereby regulating the prothrombotic and proinflammatory effects of complement activation. The patient described in this case report was tested for genetic mutations in genes coding for Factor H (*CFH*), *FHR5*, and *MCP*, *CFI*, *CFB*, *THBD*, and ADAMTS13 (*ADAMTS13*), a regulator of the vWF pathway of coagulation cascade (not shown). The results of the genetic testing were negative.

TABLE 1 | HELLP and aHUS diagnostic criteria.

	HELLP	aHUS
Hemolysis	Plasma haptoglobin below limit for normal, plasma lactate dehydrogenase > 600 U/L	Non-immunological (Coombs test negative) hemolysis with red blood cell fragmentation > 1–2% in peripheral blood smear, plasma haptoglobin below limit for normal, and increased plasma lactate dehydrogenase
Organ dysfunction	Elevated liver enzymes: alanine aminotransferase > 70 U/L	Positive markers of injury (of any organ, but typically acute kidney injury with serum creatinine over 200 μmol/L)
Low platelets	<100 E9/L	Thrombocytopenia (platelet count below 150 E9/L or decrease over 25% from baseline)

g/L, 0.12–0.34 g/L) levels were low. Level of soluble terminal complex of the complement (C5b-9, 971 ng/mL, <366 ng/mL) was elevated on the first postpartum day. Antiphospholipid antibodies were not detected, the infection serology concerning Hepatitis B and C, and HIV was negative. From stool sample, the pathogens causing typical HUS tested negative.

The patient was treated with plasma exchange treatment on first and second postpartum day and was hemodialyzed altogether three times over the course of her treatment (days 2, 4, and 6 postpartum).

On third postpartum day the patient was stable and transferred back to Women's Hospital recovery room were observation and symptomatic therapy was continued. Hypertension was treated with Amlodipine 10 mg twice a day and Labetalol 200 mg three times a day. On the fourth postpartum day, platelets continued decreasing and the patient was diagnosed with aHUS. Often the differential diagnosis with HELLP syndrome and aHUS lies in spontaneous recovery of HELLP patients usually on third postpartum day. Treatment with eculizumab was started (900 mg IV). Patient received a pneumococcal vaccination and prophylactic antibiotic (penicillin) was started. The patient received all together four weekly doses of eculizumab (900 mg) and she started to recover rapidly. She did not require further hemodialysis after her third hemodialysis on the sixth postpartum day (**Figure 2**). Kidney function corrected gradually, platelet count elevated, and hemolysis resolved. Four weeks postpartum the plasma levels of C3 and C4 were normalized.

In genetic testing, no known gene polymorphisms were identified. She was tested for mutations in complement regulators Factor H (*CFH*), Factor H related protein 5 (*FHR5*), and membrane co-factor protein (*MCP*), complement alternative pathway inactivator factor I (*CFI*), alternative pathway activator factor B (*CFB*), and the following components of the coagulation cascade: *ADAMTS13*, thrombomodulin (*THBD*), and an intracellular enzyme, diacylglycerol kinase E (*DGKE*), whose mutations are a known causes of aHUS (14). Furthermore, antibodies against factor H were not detected either. No *C4* deficiency was detected.

As a summary, our patient had severe preeclampsia and fulfilled diagnostic criteria for HELLP syndrome. Although genetic testing for aHUS remained negative, the clinical course of the disease (especially, severe acute kidney injury) and response to treatment (especially, eculizumab) suggested that our patient had pregnancy-induced aHUS.

DISCUSSION

The primigravida described above was diagnosed with severe preeclampsia, HELLP syndrome, and pregnancy-induced aHUS. No known gene mutations, which could predispose to aHUS were discovered. Differential diagnosis between types of TMA, TTP, HUS/aHUS, and secondary TMAs like HELLP syndrome is important. Especially TTP must be identified early on, as the disease is treated with rapid daily plasma exchange until remission (15).

At present, laboratory analysis methods for testing genetic mutations potentially causing aHUS are able to show mutations in only up to 40–60% of aHUS cases, leaving the possibility of false negative cases. Therefore, a negative test result for mutations does not rule out a true aHUS (16). Rarely aHUS has been induced after pregnancy and parturition. In these cases, during the following three years ∼50% developed chronic kidney disease (CKD), some even end-stage kidney disease (ESKD). When genetic mutations were observed, up to 85% may develop CKD or ESKD (17).

TABLE 2 | Timeline of the disease diagnostics and treatment.

Days	Diagnostics	Treatment	Aim
Ceasarean section	Basic blood count, C-reactive protein, blood chemical values, hemolysis markers, coagulation factors and descriptive, antiphospholipid antibodies, Coombs test, plasma ADAMTS13 activity, and antinuclear antibodies	Transfer to ICU	To exclude TTP, antiphospholipid syndrome, SLE, and autoimmune hemolytic anemia
Postpartum day 1	Plasma C3 and C4 levels, Complement terminal complex-level, C4A and C4B genetic testing	Plasma exchange	
Postpartum day 2	Hepatitis B and C, HIV, and aHUS genetic tests (Complement system)	Plasma exchange, Hemodialysis	To exclude viral hepatitis as a cause of liver damage
Postpartum day 3	Stool sample testing the pathogens causing typical HUS	Transfer back to Women's Hospital recovery room were observation and symptomatic therapy continued	To exclude typical HUS
Postpartum day 4	Basic laboratory tests concerning hemolysis, liver and kidney function, platelets, and coagulation	Hemodialysis, Transfer to the department of Nephrology, first dose of Eculizumab	Diagnosis of aHUS was placed
Postpartum day 5	Basic laboratory tests concerning hemolysis, liver and kidney function, platelets, and coagulation		
Postpartum day 6	Basic laboratory tests concerning hemolysis, liver and kidney function, platelets, and coagulation	Hemodialysis	

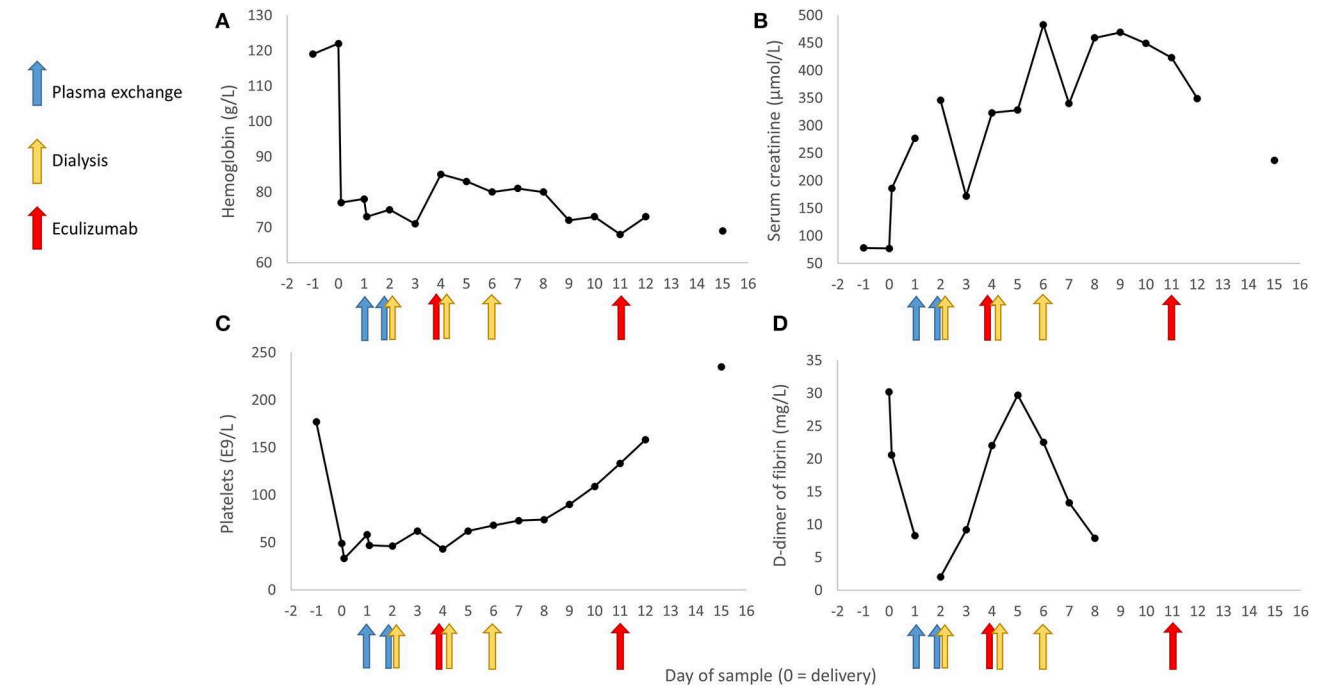

FIGURE 2 | Selected laboratory values observed during the early stages of the disease and the timing of plasma exchange, hemodialysis and administration of eculizumab. In panel **(A)** is represented the development of blood hemoglobin measurements, in panel **(B)** the serum creatinine level, in panel **(C)** the number of platelets, and in panel **(D)** D-dimer of fibrin values across the 17 day follow-up period.

It is generally accepted that in hypertensive disorders of pregnancy, placental inflammation results in endothelial dysfunction. If the integrity of the endothelium is disturbed this results in activation of complement and coagulation (18). Disrupted maternal endothelium has also been hypothesized to contribute to the later life maternal morbidity associated with severe preeclampsia and other hypertensive pregnancy disorders (2, 19). Damaged endothelium has been shown to function abnormally even years after the initial diagnostic symptoms subside (20–23).

In up to 46% of HELLP patients, gene mutations have been described in the regulators of the alternative pathway of the

complement system (24, 25). In the early stages of pregnancy, when the placenta is developing, activation of complement system is seen in elevated levels of Bb in the serum in patients who later developed preeclampsia (26). Activation of the complement system has been observed in severe preeclampsia and HELLP syndrome, and elevated levels of terminal complex (C5b-9) have been detected in urine of patients with severe preeclampsia. In patients with HELLP syndrome, increased activation of complement system was shown by functional test and, furthermore, by deficiencies of expression in CD55 and CD59, leading to decreased regulation and exacerbated activation of the complement system (27–29).

There are many similarities shared between HELLP syndrome and aHUS. In both conditions, endothelium disturbance is clearly involved followed by complement and coagulation activation. In severe preeclampsia and HELLP syndrome, eculizumab given at the early signs of severe disease and TMA would be beneficial for the protection of the kidneys and maternal endothelium (30). Preserving endothelial integrity may potentially protect the patient from long term health risks such as cardiovascular diseases. Typically, in pregnancy-related TMAs, clinical findings of hemolysis, and thrombocytopenia resolve in ∼3 days. If disease activity lasts longer, differential diagnostics are to be continued and only then is alternative treatment (e.g., eculizumab) considered. Introducing eculizumab treatment earlier would benefit women by preventing kidney damage and minimizing the turbulence in endothelium and systemic inflammation. Eculizumab, given early enough and as few as one or two doses, could possibly be sufficient to stop the turbulence and be enough to stop the cascade of events (31). On one hand, eculizumab is a very expensive medication, but on the other, the cost of intensive care treatment, plasma exchange, hemodialysis, possible kidney transplantation, not to mention the emotional consequences for the mothers and families remain inestimable.

As a conclusion, our patient was severely ill, experiencing hemolysis, disturbance in the coagulation, liver damage, and kidney failure needing admission to ICU and hemodialysis. It is possible that eculizumab, if initiated earlier, at the first signs of HELLP syndrome, might have been beneficial during the later course of the disease, potentially mitigating kidney injury, and thus preventing need for hemodialysis and later CKD. Therefore, in this era of modern immunological medicine, could we do more for the mothers and families than just wait and hope for the best?

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AL and JH-E developed the idea to the manuscript and wrote the manuscript. JH-E and MH were involved in the diagnostic and therapeutic care of the patient. All authors reviewed and edited the manuscript and approved its final version for publication.

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frontiers in Immunology

REVIEW
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Essential Role of Complement in Pregnancy: From Implantation to Parturition and Beyond

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The complement cascade was identified over 100 years ago, yet investigation of its role in pregnancy remains an area of intense research. Complement inhibitors at the maternal-fetal interface prevent inappropriate complement activation to protect the fetus. However, this versatile proteolytic cascade also favorably influences numerous stages of pregnancy, including implantation, fetal development, and labor. Inappropriate complement activation in pregnancy can have adverse lifelong sequelae for both mother and child. This review summarizes the current understanding of complement activation during all stages of pregnancy. In addition, consequences of complement dysregulation during adverse pregnancy outcomes from miscarriage, preeclampsia, and pre-term birth are examined. Finally, future research directions into complement activation during pregnancy are considered.

Keywords: innate immunity, complement, pregnancy, preeclampsia, preterm birth, pregnancy loss, fetal development

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INTRODUCTION

The complement system or alexin was identified more than 100 years ago by Jules Bordet for its ability to "complement" the role of heat stable antibody in protecting the host and lysing bacteria (1). Since then numerous activities of this powerful enzymatic amplification cascade have been extended beyond host defense and immunopathology to defining a role for the complement system in homeostasis and normal development. Once viewed as simply a humoral component of the immune system, the functions of complement have been recognized far beyond an extracellular system that lyses bacteria. This review will focus on the role of complement in helping to orchestrate a normal pregnancy, and the evidence that control of the system is important to prevent pathology in the mother and rejection of the semi-allogeneic fetus. In addition, control of the complement system is essential for normal placental and fetal development to avoid lifelong adverse consequences for offspring of those pregnancies. We will provide background on the complement system, as well as an evaluation of the literature to date that shows a role for complement in the following events: pre-implantation, implantation, and placental development, as well as normal development of the fetus to parturition and labor. Studies demonstrating dysregulation of the complement system in recurrent pregnancy loss, preterm birth, preeclampsia, hypertensive disorders of pregnancy, and intrauterine growth restriction will be reviewed. Clearly, problems in pregnancy can lead to adverse effects in offspring of that pregnancy. Thus, we will extend our discussion to the role of the complement system in neurodevelopmental and behavioral disorders as well as the risk of cardiovascular and metabolic disease in the offspring following adverse pregnancy outcomes.

IMMUNITY VS. REPRODUCTIVE SUCCESS

To guarantee survival and maximize reproductive success, resources are subject to trade-offs within the organism (2, 3). A successful pregnancy requires lengthy periods of time and considerable amounts of energy. In addition, the fetus demands additional energy from the mother; therefore, the utilization of resources need to be adapted during gestation. The assignment of available resources to reproduction occurs at the expense of other systems like immune function (4). Reproduction results in reduced immunity, and conversely, infection, and immune responses reduce reproductive success. A successful pregnancy requires the development of a maternal inflammatory reaction that is thought to control exaggerated fetal demands. However, an excessive inflammatory reaction has been associated with adverse reproductive outcomes. The complement system, part of the innate immune response, plays a crucial role in normal pregnancy from conception to delivery (5). However, uncontrolled complement activation results in several pregnancy complications such as miscarriage, preeclampsia and preterm birth (6, 7). This is in agreement with the trade-off paradigm; increased immune responsiveness restricts reproduction.

THE COMPLEMENT SYSTEM: EXTRACELLULAR AND INTRACELLULAR

Extracellular

Jules Bordet's concept of complement has been elucidated over the years to reveal the intricacies of complement activation, regulation, and clinical significance. A brief overview of the cascade and its inhibitors are discussed below. Complement can be divided into three extracellular pathways (**Figure 1**) and an intracellular pathway (**Figure 2**) that differ in activation and inhibition.

The first pathway to be fully characterized was rightfully coined the Classical complement pathway. The Classical pathway

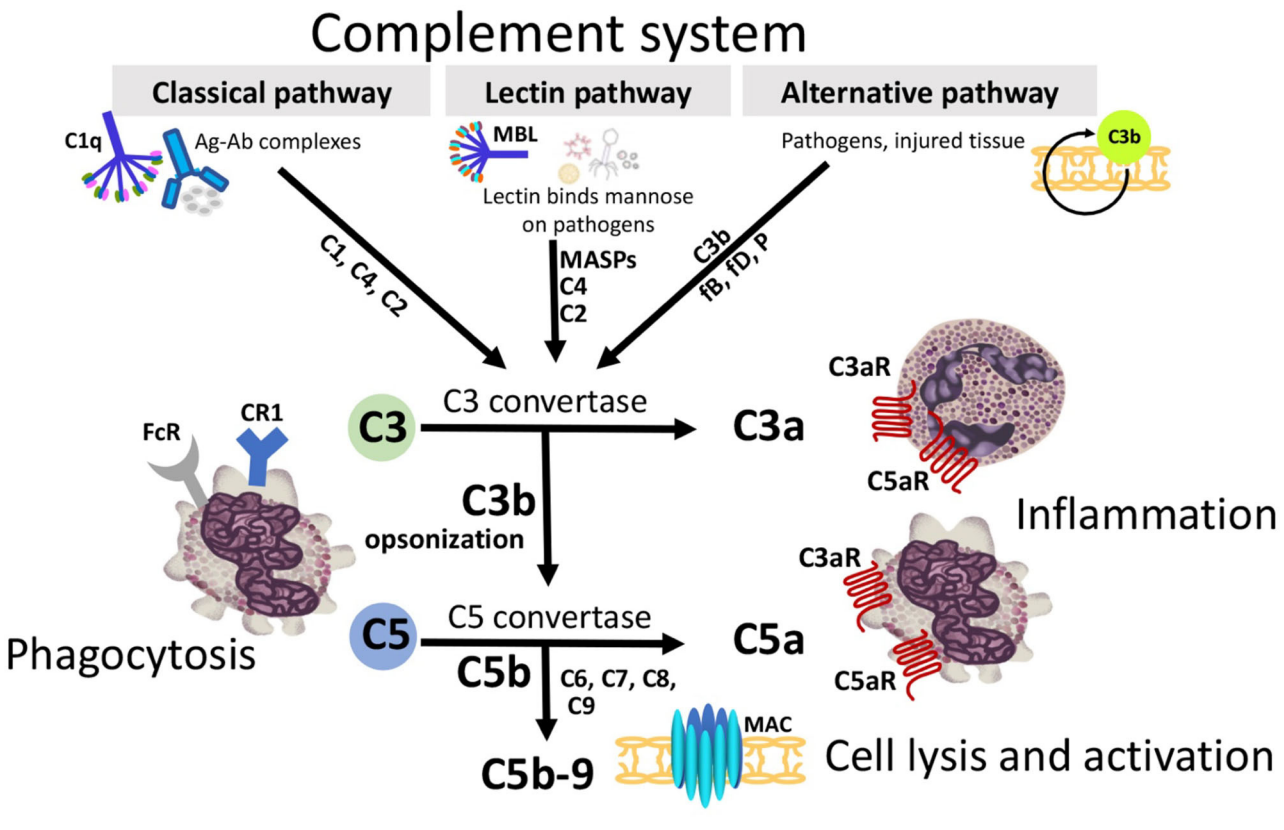

FIGURE 1 | Three extracellular complement initiation pathways culminate in a common terminal pathway. Grey boxes identify initiation and terminal pathways with complement components identified along the arrows. The Classical pathway is activated by antigen/antibody complexes, recognized by C1q in complex with C1r and C1s. Proteases C1r and C1s cleave C4 and C2 to generate the Classical pathway C3 convertase C4b2a. The Lectin pathway is triggered by binding of mannose-binding lectin (MBL) or ficolins to carbohydrates on the target membrane. The MBL-associated serine proteases (MASPs) then cleave C4 and C2 generating the C3-convertase C4b2a. The Alternative pathway, an amplification loop, is triggered when the C3b protein directly binds a microbe, foreign material, or damaged tissue. C3b also binds factor B (fB) to form C3bB. FB is cleaved by factor D (fD) to form Alternative pathway C3-convertase, C3bBb. This convertase is stabilized by properdin (P). C3b opsonizes targets for phagocytosis and B-cell activation. All 3 initiation pathways converge on C3 with distinct C3 convertases which cleave C3 to generate the anaphylatoxin C3a, and more C3b to form the C5-convertases (C4b2a3b and C3bBb3b). C5-convertase then cleaves C5 into C5a and C5b. C3a and C5a can attract and activate inflammatory cells and contract smooth muscle through receptors (C3aR, C5aR1, and C5aR2). C5b binds C6, C7, C8, and multiple copies of C9 forming the membrane attack complex (MAC) complex. MAC pores can cause cell death by osmotic flux.

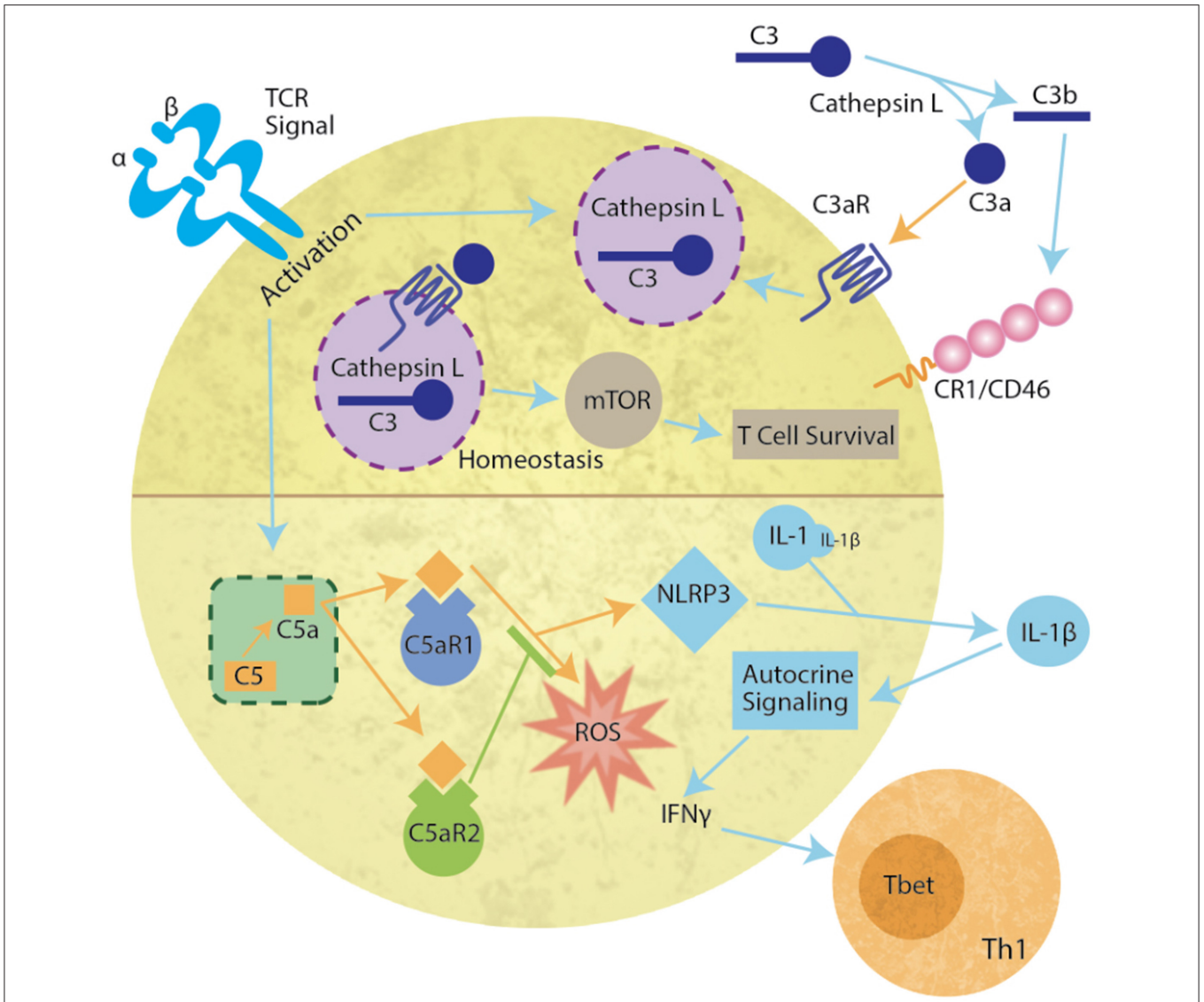

FIGURE 2 | Intracellular complosome activation in the T cell. During homeostasis, lysosomal Cathepsin L cleaves C3 to C3b and C3a. C3a binds to C3aR on the vesicular membrane to activate mTOR resulting in T cell survival (upper panel). Cognate antigen engagement by T cells results in lysosome fusion with the membrane, release of a Cathepsin L and C3 containing vesicle resulting in extracellular Cathepsin L-mediated cleavage of C3. C3b binds complement receptor 1 or CD46 (MCP) and C3a binds C3a receptor (C3aR) (Upper right) inducing T cell proliferation and differentiation. In addition, upon TCR activation, intracellular C5 is cleaved by an unknown protease allowing C5a to bind either intracellular C5aR1 or C5aR2 (lower half of diagram). C5aR1 induces reactive oxygen production (ROS) and NLRP3. NLRP3 cleaves pro-interleukin 1 (IL-1) producing IL-1β. The autocrine signaling induces IFNγ release and Th1 T cell differentiation.

is initiated by a single IgM or multiple IgGs complexed to antigen or a pathogen-associated molecular pattern (PAMP). The antibodies then change conformation to expose a binding site for the first protein in the classical cascade, C1 (8). C1 is composed of C1q, the antibody binding molecule, and $C1r_2s_2$, initially inactive serine proteases. C1q recognition of the antibody/antigen complex induces a conformational change in C1r, transforming it to an active state which activates C1s (9, 10). Once both serine proteases are activated, the zymogenic cascade works quickly, cleaving C4 and C2. The cleavage products form the complex C4b2a, the C3 convertase. As the name suggests, C4b2a then cleaves C3 into C3a, an anaphylatoxin, and C3b which joins C4b2a forming the C5 convertase, C4b2a3b. As the previous pattern suggests, the newly formed C5 convertase cleaves C5 into C5a, another anaphylatoxin, and C5b, the initiator of the Membrane Attack Complex (MAC). Occurring even more quickly than the aforementioned processes, C6 joins the deposited C5b, which is quickly followed by C7, C8, and an amplified quantity of C9 to form a well-designed, yet asymmetrical pore that leads to targeted lysis of the pathogen (8, 11).

Outside of the Classical pathway, C1q also further modulates the immune system and plays a role in development (12). Upon binding the cC1qR on an immature dendritic cell, C1q

induces NF-κB translocation to the nucleus and the successive production of IL-10, IL-12, and TNFα followed by dendritic cell maturation (13). Additionally, C1q has an antiproliferative effect on T cells and other peripheral blood cells, with the exception of erythrocytes (14). These data suggest that C1q may be critical to tolerance of peripheral antigens. C1q also functions in angiogenesis and the clearance of apoptotic cells (12).

Fifty years later, another complement cascade was discovered by Pillemer et al. (15). Pillemer proposed an Alternative pathway which was not formally accepted until almost a decade later (8). A unique feature of the Alternative pathway is autoactivation through the hydrolysis of a disulfide bond on a complete C3 protein to form C3(H_2O) (16). C3(H_2O) then binds and changes the conformation of Factor B in a Mg^{2+}-dependent manner (17). The serum protein, Factor D cleaves the altered Factor B, and the Bb fragment remains associated with C3(H_2O) to form the Alternative pathway C3 convertase, C3bBb. This complex is stabilized by another serum protein, Properdin (18), and proceeds to cleave another molecule of C3 forming the Alternative pathway C5 convertase, C3bBbC3b (19). The Alternative pathway C5 convertase also cleaves C5 to C5a and C5b. Despite the striking differences in molecular composition of the convertases, both pathways proceed down the same terminal pathway after cleavage of C5 to form the MAC. Due to the antigen-independence of the Alternative pathway, it is often considered to function as an amplification loop for the Classical pathway or the next extracellular pathway we will discuss, the Lectin pathway.

The third extracellular complement activation pathway was discovered nearly 40 years later (20). While remarkably similar to the Classical pathway, the Lectin pathway instead activates the complement cascade through pattern recognition molecules [either Mannose-Binding Lectin (MBL) or a ficolin] that recognize monosaccharides exposing 3′ and 4′ hydroxyl groups, such as glucose, mannose, and *N*-acetyl-glucosamine (10, 21). Either MBL or a ficolin will engage with an array of monosaccharides in a similar manner to C1q-IgM recognition. The Lectin pathway proteases, MASP-1 and MASP-2, are activated sequentially with MASP-1 autoactivation when MBL binds the target carbohydrate, and subsequent activation of MASP-2. While there are differences between studies, under physiological conditions, the cascade continues by either MASP-1 or 2 cleaving C2 and MASP-2 cleaving C4 prior to the rest of the zymogenic cascade following along the same path as the Classical pathway (10, 22).

Intracellular

More recently, an intracellular system was identified and primarily characterized in human CD4+ T cells (23, 24). Since this initial discovery of the "complosome," many non-immune cells have been identified as containing functional intracellular complement components, including mesenchymal stem cells (25), intestinal epithelial cells (26), and pancreatic β cells (27). The complosome (**Figure 2**) not only includes intracellular C3, but CD4+ T cells also contain a C3a receptor (C3aR) on the lysosome and Cathepsin L in the lysosome. Cathepsin L constitutively cleaves C3 into C3a and C3b. It is proposed that the intracellular membrane C3aR and C5aR signal similar to vesicular signaling by other G protein coupled receptors (GPCR) (28, 29). Importantly, the signals produced by vesicular receptors may differ from those on the plasma membrane (30). During homeostasis, the C3a-C3aR system sustains low-level mTOR activity, thereby promoting T cell survival *in vivo* (24, 29). However, upon TCR activation, the intracellular C3 system that is normally confined to the lysosome, translocates to the plasma membrane, allowing extracellular release of both C3a and C3b and signaling through their membrane-bound receptors, C3aR and CD46 (MCP) or CR1. This induces IFNγ production, and the development of T_H1 cells (31). In addition, upon TCR engagement, intracellular C5 is cleaved by an unknown protease (23). Activation of the intracellular C5a receptor (C5aR1) increases production of reactive oxygen species (ROS) and induces the NLRP3 inflammasome. The formation of the inflammasome induces cleavage of IL-1, producing IL-1β that signals in an autocrine fashion to increase IFNγ. However, C5aR2 can also bind C5a to negatively regulate NLRP3 inflammasome activity and reduce the Type 1 response produced by intracellular C5a (32). Intracellular complement systems have also been implicated in intestinal damage during ischemia/reperfusion events (26). These data are particularly critical at the highly vascular interface between the mother and the fetus as prenatal hypoxia has severe implications for neonate cognition and development (33).

Regulation

Considering the zymogenic nature of the complement cascade and the risk for self-activation, it is easy to understand why multiple complement regulatory molecules evolved. Two main regulatory systems to control complement activation have been identified: membrane bound regulators and soluble regulators (**Figure 3**). A subset of membrane bound regulators are quite effective in accelerating decay of the C3 convertase. Decay Accelerating Factor (DAF; CD55) affects convertases of both the Classical and Alternative pathway. DAF is globally expressed on many somatic cells and functions to protect them from complement activation (8, 34). Similarly, complement receptor 1 (CR1) also inhibits the Classical and Alternative Pathway. Unlike DAF, CR1 is expressed mostly on antigen presenting cells, erythrocytes, and phagocytes. CR1 functions as a cofactor for Factor I, discussed below. With limited expression in mice, membrane cofactor protein (MCP), otherwise known as CD46, functions similarly to CR1 as a Factor I cofactor. However, MCP specifically aids the degradation of C3b bound by protein rather than those bound by other acceptor molecules (35). Additional membrane bound regulators prevent the formation of the MAC and attenuate target cell damage. Vitronectin, clusterin, and CD59 (protectin) prevent the culminating step of all three pathways. Vitronectin and clusterin inhibit insertion of the C5b-7 complex or C7 and C8, respectively into the MAC (36, 37), while CD59 prevents the insertion of C9 into the membrane. CD59 is expressed on nearly every cell in the human body (38) and stops the complement cascade at C5b-8, saving somatic cells from inattentive MAC formation. C4-binding protein (C4BP) inhibits the enzymatic activity of the Classical C3 convertase,

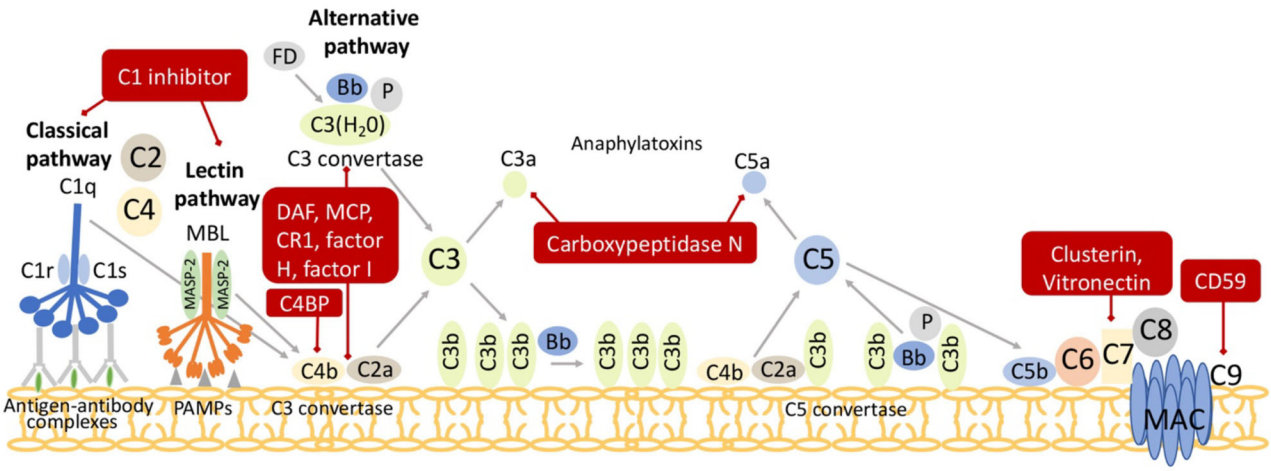

FIGURE 3 | Regulators of the complement pathway. Both surface and soluble complement regulatory molecules identified in the red boxes inhibit critical junctures of the cascade and degrade C3 convertases (C4b2a or C3bBb) or C5 convertases (C4b2a3b or C3bBbC3b) and the anaphylatoxins C3a and C5a. C1 inhibitor prevents excessive activation of both Classical and Lectin pathways. The Classical and Lectin pathway C3 convertase, C4b2a, is regulated by complement receptor 1 (CR1), C4 binding protein (C4BP), decay accelerating factor (DAF), membrane cofactor protein (MCP), and factor I. The Alternative pathway C3-convertase, C3bBb, is regulated by CR1, factor I, factor H, DAF, and MCP. The anaphylatoxins, C3a and C5a, are degraded by carboxypeptidase N. Finally, Vitronectin and Clusterin inhibit the C5b-8 complex while CD59 (protectin) inhibits C9 insertion into membrane attack complex (MAC).

C4b2a (39), while Factor H is a soluble cofactor that competes with Factor B for binding to C3b in the Alternative pathway. In conjunction with co-factors [C4 binding protein (C4BP), Factor H, MCP, and CR1], Factor I regulates all three extracellular pathways by cleaving C3b and C4b and preventing the formation of active C3 and C5 convertases. Another soluble regulator is the anaphylatoxin inactivator, plasma Carboxypeptidase N. Carboxypeptidase N cleaves a terminal arginine from C3a and C5a to generate their des Arg derivatives, C3a des Arg and C5a des Arg, altering their biological activities and potency at the C3aR and C5aR (8, 40).

Complement Regulation at the Maternal-Fetal Interface Is Essential for a Favorable Pregnancy Outcome

The maternal-fetal interface is rich in complement inhibitors, suggesting they have evolved to protect the placenta and control complement activation (41), hence preventing adverse pregnancy outcomes. In humans, DAF (CD55) and MCP (CD46) control C3 activation early on in the complement cascade, whereas CD59 acts in the terminal pathway to prevent formation of MAC (42). In mice and rats, an additional widely distributed complement regulator not found in other species is produced. It is known as Complement receptor 1 related protein y (Crry) (43). Crry is structurally similar to MCP and DAF, with complement inhibitory activities similar to CR1 (44). Molina and colleagues deleted the gene encoding Crry in mice and discovered that homozygous $Crry^{-/-}$ mice died *in utero* (43). In $Crry^{-/-}$ mice, C3 deposited on the embryo and in the ectoplacental cone, suggesting that the absence of Crry resulted in complement-mediated embryonic death. Breeding Crry heterozygotes ($Crry^{+/-}$) with mice deficient in C3 to generate $Crry^{-/-}$ on a C3 deficient background rescued the pregnancies confirming that uncontrolled complement activation was responsible for loss of $Crry^{-/-}$ embryos. Importantly $Crry^{-/-}$ mice on the C3 deficient background survived gestation and were born healthy (43). In addition, treatment of the BPH mouse strain that exhibits high blood pressure and frequent fetal loss with Crry targeted to placental C3b deposition resulted in a decrease in placental inflammation and increased favorable pregnancy outcomes (45). Together these studies demonstrated that favorable pregnancy outcomes required complement regulation at the maternal-fetal interface. In contrast to Crry, DAF deficiency in the mouse did not affect reproductive outcomes (46) suggesting DAF at the maternal-fetal interface was not critical for embryo survival. Thus, while unregulated complement activation is a threat to pregnancy, some complement components favor both the success of a pregnancy and normal fetal growth at multiple steps throughout gestation, from pre-implantation to placental formation and labor and parturition.

ROLE OF COMPLEMENT IN PRE-IMPLANTATION

In the early 1990s, discovery of the ability of C3b and CD46 to facilitate sperm oocyte interactions prompted numerous investigations of the importance of complement in development as reviewed in Anderson et al. (47) and Hawksworth et al. (48). In the early stages of pregnancy, the fertilized egg makes its way down the fallopian tube and into the uterus, with cell division along the way resulting in formation of 4 and 8 cell stage embryos and eventually the blastocyst. Blastocyst implantation in the uterine wall occurs at about day 9 in humans

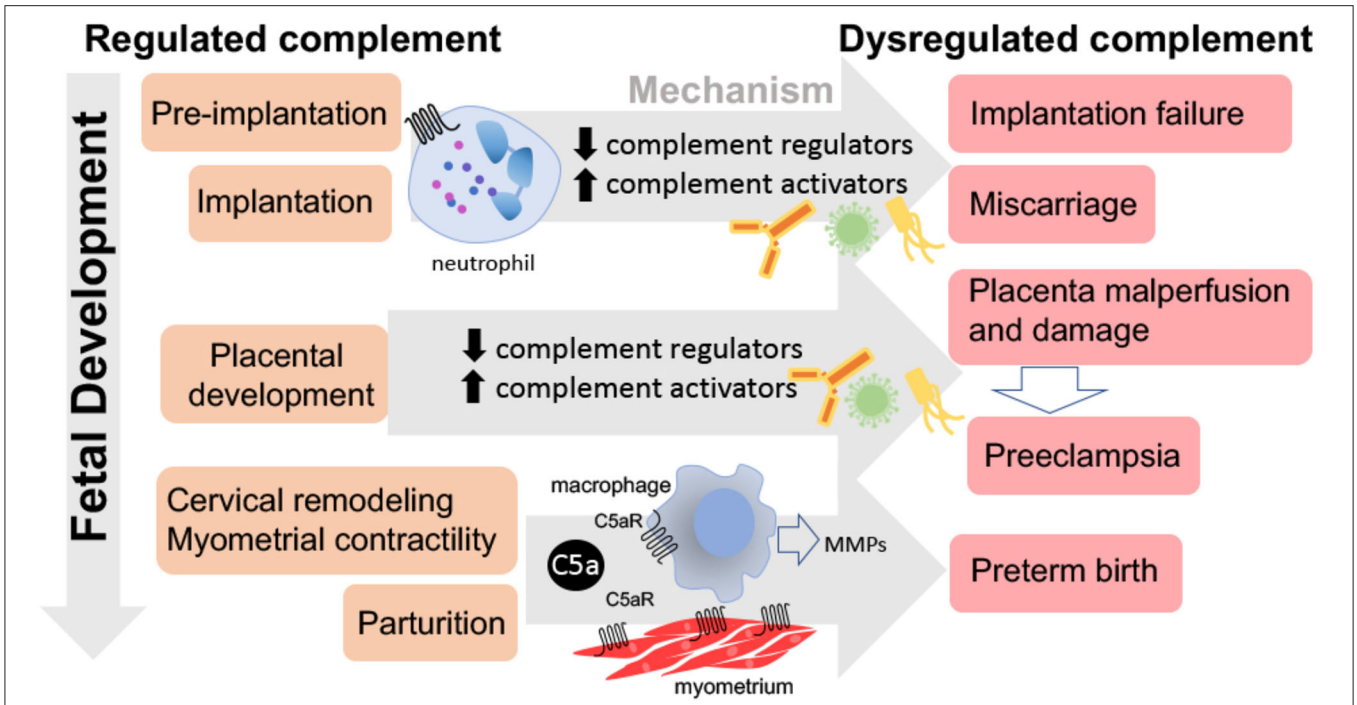

FIGURE 4 | Consequences of dysregulated complement throughout pregnancy. Dysregulated complement activation from pre-implantation through parturition may lead to pregnancy complications. Excessive complement activation and neutrophil infiltration or lack of complement during pre-implantation or implantation may result in implantation failure or miscarriage. Insufficient complement regulation may lead to placental malperfusion, placental damage, and/or preeclampsia. While the C5a-C5aR axis stimulates macrophage production of metalloproteinases (MMPs) leading to normal parturition and labor, inappropriate timing may result in preterm birth.

and day 4 in mice and rats (49). Complement components are found in mucosal secretions in the fallopian tubes, cervix, and uterus. Thus, as a semi-allogeneic collection of cells, the embryo is subject to complement attack before and after implantation in the uterine wall. A recent elegant study by Reichhardt et al. investigated complement targeting of the embryo prior to implantation, as well as the ability of the embryo to produce complement components (50). Cryopreserved human embryos not needed for *in vitro* fertilization were cultured to 4 or 8 cell stage and examined for expression of complement proteins and for evidence of activation of complement on their surface. Complement activation on the surface of the embryo was evident, indicating that pregnancy failure could potentially result from excessive complement activation in the pre-implantation stage (**Figure 4**). Inadequate or dysfunctional complement regulators could also contribute to excessive complement activation at this early stage. Reichhardt demonstrated embryonic expression of complement regulators at the pre-implantation stage that likely limit excessive activation and loss. Genetic mutations in complement regulators have been associated with recurrent pregnancy loss (51), so inadequate complement regulators at the pre-implantation stage could be a cause of pregnancy failure. In Reichhardt's study, no C5 was detected on the embryo surface, suggesting that the embryonic complement regulators were normally able to successfully limit continuation of complement activation after C3. Soluble complement regulators such as C4BP and Factor H were also evident. The CD55 and CD59 expression was seen primarily at cell junctions, suggesting a role in cellular interactions. C5 on the zona pellucida was very evident, suggesting that extensive complement activation occurred on this glycoprotein membrane surrounding the early embryo. In addition, results of this study demonstrated that oocytes and early 4 and 8 cell stage human embryos were capable of generating all of the complement components needed for C3 and C5 activation. Thus, the potential for intracellular activation of complement as well as the traditional extracellular activation is present. This allows one to speculate that intracellular signaling of complement in the embryo may play an important role similar to that demonstrated in T cells (52), where complement functions intracellularly to regulate basic metabolic processes and cellular differentiation.

The ability of the embryo to generate C3 and iC3b may play an important function in promoting embryonic growth and protecting from autophagy. C3 is an embryotrophic factor, primarily through conversion to C3b or iC3b (53–55) and likely important for embryonic growth prior to development of the placenta in both humans and rats (56). In addition, intracellular complement component C3 upregulation in human pancreatic β cells is cytoprotective and involved in autophagy regulation, contributing to β cell survival and the protection from autophagy provided by C3 in the pancreatic β cell (27).

C3 may play a similar role in fetal development of the pancreas and other organs.

Endothelin is a potent vasoconstrictor that is best known for its potential role in high blood pressure. However, use of endothelin antagonists during pregnancy is contraindicated because of teratogenic effects, with known adverse developmental effects on the kidney (57). Jeoung et al. hypothesized that endothelins are required in the oviduct to facilitate early embryonic development, and they demonstrated that antagonism of endothelin resulted in a reduction in the number of two cell embryos developing (58). Our studies with the endothelin 1 antagonist, atrasentan, in the third trimester of rat gestation demonstrated that this inhibitor down-regulated the message for complement regulators in the placenta, suggesting that endothelin was important for controlling complement activation by influencing the expression of complement regulators (59). Thus, endothelin in normal embryonic development may ensure up-regulation of expression of complement system regulators to protect the embryo. One of the adverse effects of endothelin antagonists on development could be due to reducing complement regulators in the placenta or fetus, thus compromising placental and fetal development and increasing susceptibility of the placenta and fetus to attack by complement.

COMPLEMENT FROM IMPLANTATION THROUGH PLACENTAL DEVELOPMENT

A recent publication by Pierik et al. reviewed animal and human studies regarding dysregulation of complement activation in placental dysfunction and preeclampsia (60). Thus, review of that literature in this review article will not be repeated, but some notable more recent publications considered. A recent prospective study by He et al. (61) also looked at complement activation in preeclampsia across the course of pregnancy with evidence that dysregulation of the classical and alternative pathways occurred as early as the first trimester in preeclampsia, and alterations in C3a and C5a were evident throughout. Nevalainen et al. demonstrated that C7 was upregulated in severe early onset preeclampsia compared to late onset (62), demonstrating the heterogenous nature of preeclampsia and indicating that the role of complement may differ. Our continued review will focus on early events in implantation that might contribute to fetal loss, intrauterine growth restriction and placental dysfunction.

Miscarriage includes all pregnancy losses before 24 weeks of gestation. Some miscarriages occur very early at the stage of implantation and other miscarriages occur in later stages of placental development. Implantation involves trophoblast invasion of the decidua to begin the spiral artery remodeling, a critical event in normal placental development. Problems with implantation can easily cascade or present as problems with placental development and eventual placental insufficiency (63).

Mice genetically deficient in complement components have difficult or non-viable pregnancies, and as such, pregnancy failures could be due to multiple reasons. Pregnancy in the absence of C3 is one of the best studied. Mice lacking C3 have smaller blastocysts, suggesting pre-implantation events are compromised by the lack of C3, perhaps due to the lack of C3 embryotrophic properties or the importance of C3b in sperm oocyte interactions. Since C3 is an embryotrophic factor, embryonic growth prior to development of the placenta may be compromised (56). In C3 knockout mice, a normal number of implantation sites is evident at day 8, suggesting the initial implantation process is not affected (64). The placenta is fully formed by 18–20 weeks in the human and by GD11 in the rat (65). The number of implantation sites in C3 knockout mice are reduced at day 15 with more resorptions, smaller fetuses, and reduced placental size indicating the importance of C3 in normal placental development (64). Thus, the implantation sites at day 8 do not result in normal placental development by day 15, and inadequate fetal growth is realized. In humans, patients who had 3 miscarriages had higher C3 concentrations than women who successfully had a live birth after 2 miscarriages (66), suggesting that too much C3 could be detrimental just as too little C3 is associated with adverse pregnancy outcomes.

Mohlin investigated the importance of gene variants of C3 (67), as well as complement regulators CD46, CD55, and C4BP (51) in recurrent spontaneous pregnancy loss. In each case they found rare variants of the C3, CD46, and C4BP that could result in a potentially dysfunctional protein. An association with miscarriage was suggested but not significant, and needs to be evaluated in larger cohorts, similar to studies that identified complement variants important in atypical hemolytic uremic syndrome. A different study found that a polymorphism in Factor H was associated with a decrease in risk of recurrent pregnancy loss (68). Factor H in part controls complement activation on cells by binding to sialic acid. A fetus without sialic acid is not viable because of maternal complement system attack (69). Thus, lack of sialic acid would reduce the ability of Factor H to protect the fetus from complement attack, leading to uncontrolled C3 activation. If C3 is depleted in the absence of sialic acid, fetal viability is restored. In the kidney and retina, VEGF action on endothelial cells results in upregulation of Factor H and reduced complement activation. In preeclampsia, an increase in sFlt-1, a decoy VEGF receptor (VEGFR-1) is observed which would effectively reduce VEGF signaling, limit Factor H and theoretically result in increased placental complement activation. Karumanchi's group demonstrated that placental sFlt is associated with complement activation in the placenta of preeclampsia patients and could be responsible for the trophoblast damage seen (70).

C1q deficient mice have reduced litter size, suggesting a significant effect on fetal viability. This reduced fetal viability could be due to the placental insufficiency observed (71) or altered vascular function demonstrated in offspring of preeclamptic like pregnancies (72–74). In humans, another avenue of research associated with complement and recurrent pregnancy loss relates to development of anti-C1q antibodies. These antibodies are detected in lupus nephritis as well as in anti-phospholipid syndrome. C1q is critical to formation of a normal placenta (60), and the hypothesis is that interference with C1q action would interfere with placental development, but anti-C1q

could also result in excessive complement activation leading to pregnancy loss. Ohmura et al. (75) conducted a very interesting study demonstrating increased anti-C1q antibodies in women experiencing recurrent pregnancy loss, but also demonstrated that anti-C1q administration to a pregnant mouse in the third trimester led to miscarriage and increased complement activation. In patients with SLE or antiphospholipid syndrome, the extent of complement activation may predict the risk of adverse pregnancy outcomes (76).

In pregnancies resulting from *in vitro* fertilization and embryo transfer, the incidence of adverse pregnancy outcomes tends to be greater. Zhao et al. (77) hypothesized that this may be due to changes in complement and/or coagulation pathways. Placentas were obtained in the first trimester following *in vitro* fertilization and embryo transfer and processed for microarray analysis. They found upregulation of numerous complement components and downregulation of regulators such as CD59, predisposing the placenta to increased complement activation compared to normal pregnancy.

A great deal of work has been done in the abortion prone mouse model CBA/J X DBA/2 mouse demonstrating complement involvement and this has been reviewed in the past (7, 78). In addition, the BPH mouse model and the pregnant Dahl SS rat represent a superimposed preeclampsia model; a mildly hypertensive animal that develops preeclamptic like symptoms when pregnant. In the BPH model, data indicate complement involvement in the implantation stage consistent with complement involvement reported in other models. However, the BPH model has not realized widespread use (45, 79–81).

COMPLEMENT IN PARTURITION AND LABOR

Evidence of a role for complement in normal parturition and labor is primarily obtained from studies demonstrating a role for complement in the pathophysiology of preterm birth (PTB). Each year, almost 15 million premature children are born worldwide. Complications of PTB are the leading cause of death in children younger than 5 years of age worldwide (82), and premature infants are particularly vulnerable to brain injury. Increasing evidence suggests that labor and delivery are triggered by inflammatory reactions including the complement system (83). Mouse and human studies underscore the role of complement activation in the initiation of labor, cervical remodeling as well as in uterine contractions. Two mouse models of PTB have provided evidence for the importance of complement activation in cervical remodeling and PTB (**Figure 4**). In one model, PTB was induced by vaginal administration of lipopolysaccharide (LPS) to mimic one of the most common clinical scenarios of ascending infection and inflammation (84). In the other model, PTB was induced by administration of progesterone antagonist RU486 that induces inflammation leading to cervical ripening in mice and women (84, 85). Results in both models demonstrated increased cervical C3 deposition, macrophage infiltration, and serum C3a des Arg and C5a des Arg levels in PTB when compared to gestational age-matched controls.

Results in both models of PTB demonstrated increased cervical distensibility with histological studies revealing a significant degradation of collagen and increased matrix metalloproteinase 9 (MMP-9) activity in the cervix (**Figure 4**). However, neither LPS nor RU486 treatment caused increased MMP-9, cervical remodeling or PTB in C5aR deficient mice. These data indicate that C5aR is required for the cervical remodeling that precedes PTB (84). In response to C5a or cytokines, macrophages release MMP-9 leading to collagen digestion, cervical ripening, and increased distention leading to preterm parturition. Progesterone is one of the few treatments available to prevent PTB in women with short cervix. Vaginal progesterone both decreases the risk of preterm birth and improves perinatal outcomes, with no apparent adverse effects on childhood neurodevelopment (86). Interestingly, animal studies suggest that the protective effects of progesterone might be related to the complement system. Progesterone reduced C5aR on the macrophage surface, inhibited the release of MMP-9, reduced cervical remodeling and prevented PTB (84). In addition, in LPS treated mice, depleting macrophages also prevented cervical remodeling and PTB. Also, C5a-C5aR interaction was required for MMP-9 release from macrophages (84) as well as for the cervical remodeling that leads to PTB, suggesting that complement inhibition may be a therapeutic option to prevent this serious pregnancy complication. Patients with paroxysmal nocturnal hemoglobinuria (PNH) have defective complement regulators, CD55 and CD59, and an increased incidence of adverse pregnancy outcomes. The anti-C5 antibody, eculizumab is used to control the RBC hemolysis in these patients with good outcomes (87). Eculizumab has also been used in PNH patients during pregnancy with favorable pregnancy outcomes (88). However, controlled studies are needed to determine if eculizumab affects incidence of PTB in pregnancies of mothers with fully functional complement systems.

The transformation of the cervix from a closed rigid structure to one that relaxes sufficiently for birth i.e., cervical ripening, depends at least in part on C5a-C5aR. This dynamic process begins long before the onset of labor and must be synchronized with uterine contractions to propel the fetus out of the uterus (expulsion stage). Interestingly, a role for complement activation in myometrial contractions was also demonstrated (89) *in vitro* in mouse and human myometrium. Increased C5a was detected in the myometrium of mice that received intravaginal LPS to induce preterm birth but not in myometrium from age-matched controls or myometrium harvested at term. In human and mouse isolated uterine myometrium, C5a increased contraction frequencies and expression of connexin 43 (Cx43) suggesting that C5a is a uterotonic molecule. Cx43 is a myometrial contraction-associated protein involved in uterine contractility and onset of labor (89). Pravastatin prevented cervical remodeling, myometrial contractions, and preterm labor in a mouse model of PTB (89), and also increased the synthesis and expression of DAF in the cervix, thus inhibiting complement activation (89). These data

suggest that statins may be beneficial in complement-mediated pregnancy complications.

Molecules of the innate immune system termed collectins include surfactant proteins SP-A, SP-D, and mannan-binding lectin (MBL). Collectins are found in amniotic fluid and at the maternal-fetal interface. SP-A, SP-D, and MBL reach maximum concentrations at term in amniotic fluid, suggesting they may play a role in pregnancy maintenance and parturition. Other studies suggest that SP-A and SP-D are involved in onset of labor. The recombinant forms of SP-A and SP-D increased CX43 expression and contraction of a human myometrial cell line, ULTR, when grown on collagen matrices (90). In addition, SP-A and SP-D increase the expression of proinflammatory cytokines, IL-8 and IL-6, that are found in high concentrations in serum from women with increased risk of spontaneous preterm birth (91).

SP-A expression in mouse fetal lungs and its secretion in amniotic fluid represents a signal for the onset of parturition (92, 93). In pregnant mice, injection of purified SP-A into the amniotic fluid stimulates IL-1β production and subsequent preterm delivery. These results were further verified by injecting antibodies to SP-A into the mouse amniotic sac leading to a delay in parturition (92). These studies provide evidence that SP-A and SP-D play an important role in modulating events prior to labor by inducing the synthesis of myometrial contraction-associated proteins and pro-inflammatory cytokines changing the quiescent uterus to a contractile uterus.

Multiple studies in humans underscore the important role of complement activation in the pathogenesis of PTB. Women with increased complement factor Bb in early pregnancy were 4-fold more likely to have PTB compared to women with lower levels (94). In the absence of intraamniotic infection, preterm parturition increases plasma concentrations of complement fragment Bb (95). Interestingly, this activation does not occur in spontaneous labor at term suggesting that the mechanisms leading to pre-term and term labor fundamentally differ with regards to a role for complement activation. This is consistent with mouse studies of PTB (96). In addition, elevated levels of C3a in the first trimester of pregnancy are predictive for PTB and premature rupture of membranes (PPROM) (97). A follow-up study by the same group found higher concentrations of C3a in PTB cases compared to term controls, reinforcing the concept that complement plays a role in the pathogenesis of premature delivery (94). Finally, elevated concentrations of complement factors C3a, C4a, C5a, and Bb have been detected in the amniotic fluid of women with PTB with microbial invasion of the amniotic cavity (98).

Recent studies of the microbiome in pregnancy found that the vaginal bacterial taxonomic composition might be associated with the time of delivery. Lactobacillus-deficient vaginal communities and elevated *Gardnerella* and *Ureaplasma* species were associated with a higher risk of PTB (99). Dysregulation of complement system by a specific uterine microbiome may lead to infection and PTB. It is also proposed that additional pathogenic bacterial species which are not detectable by traditional culture-based methods may initiate complement dysregulation and produce inflammatory mediators to cause cervical remodeling, increased uterine contractility, and increasing the risk for preterm birth. Supporting the role for pathogens in the onset of PTB, intrauterine infection is a definitive risk factor for PTB. However, targeting infection with the use of antibiotics has not reduced the risk of PTB. One potential cause of the antibiotic failure is the polymicrobial flora in the reproductive tract with unknown virulence, susceptibility and antimicrobial resistance (100).

Overall, most of the evidence for the involvement of complement in preterm birth comes from studies in mouse models where preterm birth is induced by a bacterial product, LPS, or by the progesterone antagonist. Clinical studies of women experiencing PTB reinforces the mouse data. Studies have focused on the collectins SP-A and SP-D as well as the complement activation products, without any clear studies in humans delineating whether the complement activation products are an indicator of the problem, or a cause of the PTB. Studies connecting PTB with the microbiome are promising and suggest that manipulations of the microbiome may be fruitful, but much more needs to be learned about the competitive interactions of bacterial species and the influence on pregnancy.

Role of Complement in Fetal Brain Injury in Preterm Birth

Premature babies are particularly vulnerable to brain injury. As previously described, premature labor has many hallmarks of an exaggerated inflammatory response including complement activation. Therefore, we speculate that the inflammatory mediators that induce cervical ripening and myometrial contractions, including complement cleavage products also affect fetal brain development. Animal models characterized fetal brain injury that was associated with inflammation-induced preterm birth and revealed deleterious effects on fetal brain morphology and function. Indeed, a mouse model of inflammation-induced PTB not only demonstrated signs of cortical brain injury but also demonstrated a crucial role for complement activation in this injury (101). Specifically, complement component C5a, involved in cervical remodeling and myometrial contractions was also involved in fetal brain injury, particularly, cortical brain damage (84, 89, 101). Abnormal cortical development can result in the long-term cognitive, behavioral, attentional or socialization deficits observed in children born preterm. Disruption of cortical neuron cytoarchitecture, characterized by shorter dendrites and axons, was observed in PTB-mice (101), and C5aR ($C5aR^{-/-}$) deficiency protected fetuses from this cortical brain damage (101). Treatment with antibody to C5 preventing generation of C5a also prevented cortical fetal brain injury, providing further evidence for a role for complement.

The detrimental effects of C5a on fetal cortical neuron development and survival has also been demonstrated *in vitro*. Glutamate is the primary excitatory neurotransmitter in the brain, and excess glutamate can cause excitotoxicity and brain injury. C5a caused increased glutamate release in fetal cortical neurons in culture (101), and blockade of C5aR not only prevented the glutamate increase but also restored dendritic and axonal growth and survival. *In vivo* studies using non-invasive

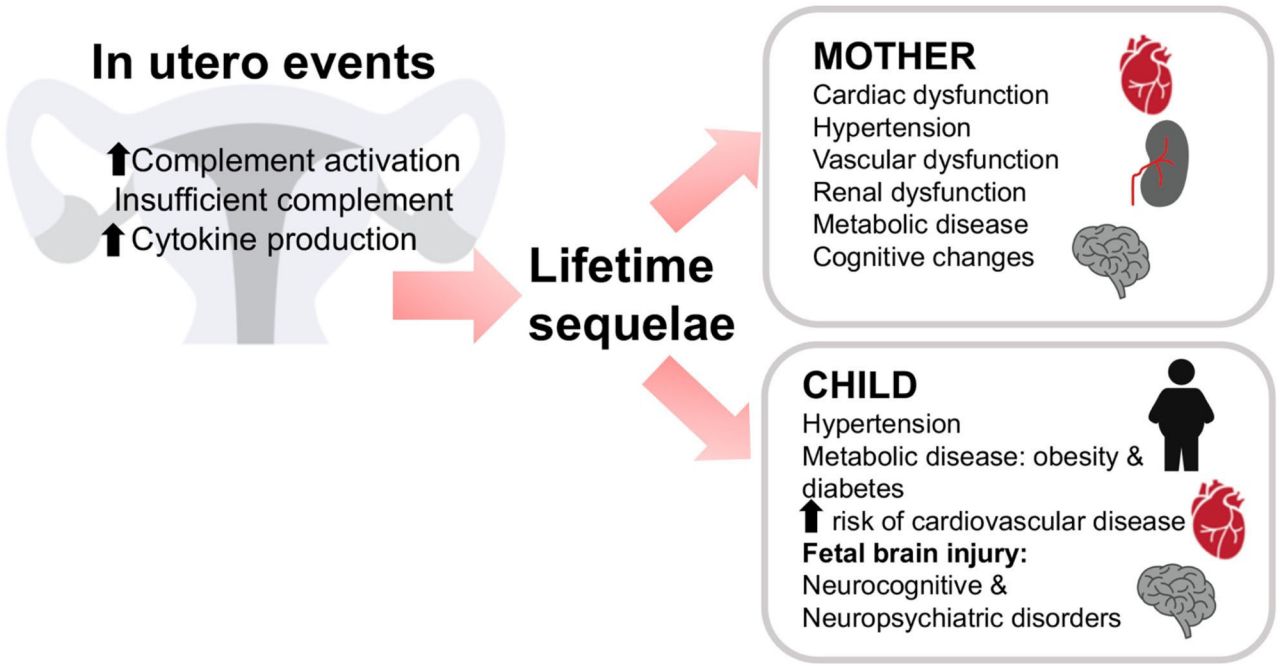

FIGURE 5 | *In utero* events can have lifelong consequences. Insufficient or dysregulated complement may result in lifelong sequelae in both mother and child. Either a lack of complement activation or excessive activation of complement or cytokine production *in utero* may lead to problems throughout the life of the mother or child. The mother may experience cardiac, vascular, or renal dysfunction, hypertension, metabolic disease, or cognitive changes. The child may also experience hypertension, metabolic disease, diabetes, or cardiovascular disease. In addition, fetal brain injury may result in cognitive and/or psychiatric disorders in the offspring.

proton magnetic resonance spectroscopy imaging demonstrated increased glutamate in PTB-fetuses compared to age-matched controls (101). If glutamate receptors were blocked, adverse effects of C5a on isolated fetal cortical neurons were prevented, confirming that the neurotoxic effects of C5a on the fetal brain are mediated by glutamate (101). Interestingly, increased C5a is found in the cerebrospinal fluid of newborn human infants born preterm compared to those that were born at term and these observations were independent of systemic infection (102).

COMPLEMENT IN FETAL DEVELOPMENT

Brain Development

In addition to its well-documented role in immune surveillance and host defense, the complement system plays other roles in the central nervous system. Like cells in many other organs, brain cells can produce complement proteins and receptors (103, 104). Similar to the placenta, the role of the complement system in the brain is described as a double-edged sword (105). Complement activation can result in either protective or deleterious effects on the brain (106). On one hand, complement proteins and receptors aid in brain developmental processes such as neurogenesis, neuronal migration and synaptic remodeling (107–109). On the other hand, deleterious effects of complement activation have been observed in the developing fetus resulting in neurocognitive and psychiatric disorders [**Figure 5**; (110–112)]. Maternal hypertension or preeclampsia that is associated with increased complement activation also significantly increases the risk of mental disorders in the offspring (113).

C5a–C5aR1 signaling plays a functional role in mammalian neurogenesis (114). During mouse embryogenesis, C5a signaling increases proliferation of neural progenitor cells in the ventricular zone that are required for normal brain development. Importantly, inhibition of C5aR1 in human and mouse models reduced proliferation and symmetric division of apical neural progenitors, demonstrating the crucial role of C5a- C5aR1 signaling in brain development (114). Proper brain formation and establishment of neural circuits requires neuronal migration. During development, excitatory neurons arising from the ventricular zone undergo radial migration to reach their correct laminar position in the cortex (109). This process appears regulated at least in part by the Lectin pathway. Mice deficient in C3, Masp1, or Masp2 exhibit impaired radial migration resulting in improper positioning of neurons and disorganized cortical layers (107). Interestingly, polypeptides that mimic C3a or a dual C3aR/C5aR agonist partially rescued the deficits in migration in C3 or Masp2-deficient mice. This suggests that activation of the Lectin pathway leading to C3a/C5a generation and activation of both C3aR and C5aR are critical for radial neuronal migration and cortical development (109). However, the role of the complement system in particular C5a in neuroinflammation, and neurodegeneration has also been demonstrated (114). The abnormal fetal brain development observed in a mouse model of PTB characterized by increased levels of C5a suggests that

exposure to increased complement activation *in utero* can also disrupt fetal brain development (101).

The timing and the degree of the stimuli leading to complement activation during fetal brain development is crucial in distinguishing between protective and harmful effects. The blood–brain barrier (BBB) is instrumental in limiting passage of cells and large molecules from the blood into the brain (115). Brain endothelial cells are exposed to complement proteins from the circulation as well as local brain synthesis, and these complement proteins and activation products can result in increased permeability of the BBB (116). The BBB permeability increases after C5a signaling (117). Similarly, maternal overexpression of cytokines and complement activation products observed during complicated pregnancies may increase the permeability of the fetal BBB allowing the invasion of peripheral blood-derived inflammatory cells and molecules, including complement proteins, leading to fetal brain injury (118). In addition, complement split product C5a might induce microglial inflammatory polarization in the fetal and neonatal brain. Activation of microglia has been associated with neurogenic hypertension and behavioral abnormalities in the offspring (72, 119).

The autoimmune disorder antiphospholipid syndrome (APS) is characterized by vascular thrombosis and/or adverse pregnancy outcomes in combination with circulating antiphospholipid antibodies. Over the past two decades, a critical role for complement activation emerged in the pathogenesis of pregnancy complications in APS in women and animal models (76, 120–124). For example, increased fetal brain C3 deposition and robust maternal complement activation occurred in a mouse model of obstetric antiphospholipid syndrome (OAPS) induced by the passive transfer of human antiphospholipid antibodies into a pregnant mouse (123). Ultrasmall paramagnetic iron oxide (USPIO) particles conjugated to monoclonal antibodies against complement C3 split products (C3b, iC3b, and C3c) are a useful tool to detect complement activation *in vivo in utero* by magnetic resonance imaging (MRI) in mice (110). Using this non-invasive technique, increased fetal brain C3 deposition was observed in OAPS-mice and was associated with disruption of the cortical axonal cytoarchitecture as well as increased neurodegeneration (110). Interestingly, C3 deposition in fetal brains in OAPS was also associated with diminished levels of glucose, lactate, and choline derivatives; molecules involved in energy metabolism, membrane lipid function, and neuroprotection (125). Increased placental C3 was also detected in OAPS-mice, using MRI (110). These observations are in agreement with human studies showing complement deposition in the placentas of women with OAPS (121).

Treatment with complement inhibitor hydroxychloroquine (HCQ) protected fetal brain development and prevented fetal brain metabolic abnormalities in OAPS-mice (125). C3 deposition detected in the fetal brains in OAPS-mice was associated with anxiety-related behavior after birth. The decrease in open field and open arm activity in the elevated plus maze (duration and/or entries) observed in the offspring of the OAPS-mouse indicates an anxiety-related behavior. In this line, the complement system is linked to developmental brain disorders, resulting in neuropsychiatric disorders such as schizophrenia (126) and autism spectrum disorder (127).

Similar to the OAPS mice, the offspring from mice infected with malaria showed abnormal neurodevelopment and neurocognitive impairment characterized by abnormal learning and memory and depressive-like behavior compared to controls (110). Interestingly, if the C5aR was deleted in the fetuses or infected pregnant mice were treated with anti-C5 antibody, the neurocognitive impairments of malaria-exposed offspring were prevented. This is similar to results in the PTB-model (101, 111). These studies clearly demonstrate a role for complement component C5a in cortical brain injury and associated behavioral abnormalities observed in fetuses exposed to excessive intrauterine inflammation.

Maternal infections and other pregnancy complications are a major risk for the development of neonatal hypoxic ischemic (HI) encephalopathy, a major cause of neonatal mortality and morbidity (128) with limited clinical options for treatment. Animal studies indicate that deficiency of Properdin in the neonatal HI brain is neuroprotective (128), suggesting that Properdin could be a therapeutic target to limit neonatal brain injury.

Clearly normal fetal brain development requires a functioning complement system for neuronal cell migration and synaptic pruning. Thus, any manipulations of complement during pregnancy must consider the stage of fetal development *in utero* and track consequences to brain health in the offspring. Much has been learned about a common role for the complement system in neurodevelopmental disorders in pregnancy using two different mouse models of adverse pregnancy outcomes: obstetric antiphospholipid syndrome as well as placental malaria. This provides evidence that limiting excessive complement activation *in utero* due to multiple causes may be valuable in minimizing neurodevelopment and neurocognitive impairment in complicated pregnancies.

Heart Development

Congenital heart block (CHB) accounts for nearly 30% of all major congenital anomalies (129). Recent studies showed that mutations in the immune-related molecules mannan-binding lectin (MBL)-associated serine protease (MASP)-3 underlie the etiology of congenital heart block (130). MASP activation resulted in complement deposition and inflammatory cell infiltrates in hearts of neonates who died from CHB (131), suggesting that complement activation is a mediator in the fetal cardiac tissue damage. Autopsy specimens from babies with CHB showed deposition of immunoglobulin and complement components in all cardiac tissues, reinforcing the pathogenic effects of complement in CHB (132).

COMPLEMENT ACTIVATION DURING PREGNANCY AND PREECLAMPSIA: LONG TERM CONSEQUENCES FOR MOTHER AND CHILD

Both animal and human studies demonstrated the importance of complement activation in the pathogenesis of preeclampsia.

In humans, mutations in complement protein and complement regulatory protein genes lead to increased susceptibility to preeclampsia (133, 134) and others suggest that complement cleavage products C3a and Bb can serve as predictors of preeclampsia (94, 135). Preeclampsia has historically been considered a transient condition since acute maternal symptoms resolve with delivery of the placenta. However, preeclampsia is not an isolated disease of pregnancy but results in long-term renal and cardiovascular disease associated with a history of maternal preeclampsia (**Figure 5**). In 2011, the American Heart Association included preeclampsia as a gender-specific risk for cardiovascular disease (136). Offspring of pregnancies affected by preeclampsia also have an increased cardiovascular risk profile. Increased blood pressure and body mass index are evident in children and young adults born to pregnancies complicated by preeclampsia (137). Therefore, it is now well-accepted that preeclampsia is linked to an array of maternal morbidities that occur later in life as well as long term adverse health effects in the offspring. However, the mechanism behind the maternal and offspring health complications after a preeclamptic pregnancy remain unknown. It is possible that abnormal placentation during preeclampsia results in placental insufficiency leading to the release of vasoactive and proinflammatory molecules that compromise the maternal and fetal health after pregnancy (72).

Maternal and umbilical cord plasma C5a concentrations are significantly higher in a preeclamptic pregnancy than in normotensive pregnancy (138). That maternal and cord plasma C5a concentrations directly correlate suggests that C5a freely moves between the maternal and fetal circulation (138). In addition, C5a interaction with C5aR on trophoblasts releases anti-angiogenic factors that impair normal placentation leading to preeclampsia and the associated placental insufficiency (139). Finally, C5a levels in women with preeclampsia positively correlate with the maternal autoantibody to the angiotensin Type 1 receptor, a potential contributor to the pathogenesis of preeclampsia (140).

In the mouse, a paternal deficiency of C1q results in impaired trophoblast migration and abnormal placentation leading to onset of preeclampsia-like symptoms including endothelial dysfunction and hypertension in the mother, along with high levels of C5a (72, 141, 142). Offspring of these pregnancies also experienced health complications (**Figure 5**). Glomerular injury persisted after the preeclampsia-like pregnancy, leading to fibrosis. In addition, left ventricular remodeling with increased collagen deposition and MMP-9 expression and enlarged cardiomyocytes developed after the preeclampsia-like pregnancy (72). Hearts were characterized by increased left ventricular internal wall thickness and mass, increased end diastolic and end systolic volumes, and increased stroke volume. Placenta-derived bioactive and proinflammatory factors (endothelin-1, IL-6, and C5a) increased in maternal sera during and after a preeclamptic pregnancy. Offspring of preeclamptic mice developed endothelial dysfunction, hypertension, and indicators of metabolic disease (72). Pravastatin treatment normalized C5a values in preeclamptic-mice and normalized cardiovascular and metabolic function in both mothers and offspring. This suggests that C5a elevation in this preeclampsia model of placental insufficiency resulted in long-term, cardiovascular and metabolic effects in the mother and offspring. Interestingly, increased C5a also occurs clinically in mother and offspring during and after preeclampsia (137).

Mechanically-induced placental ischemia in the third trimester of pregnant rats results in increased complement activation and hypertension. Inhibiting complement activation attenuates the hypertension, suggesting that complement is important (143). Offspring of these pregnancies also exhibit fetal growth restriction, high blood pressure, and glucose intolerance (144, 145) as well as reduced pancreatic β cell area (146). A role for the complement system in these events in the offspring has not been investigated to date. However, given that decreased C3 is associated with reduced pancreatic beta cell area in the adult with the potential for increased risk of Type 2 diabetes, further investigation of complement involvement in pancreatic development is certainly warranted (27).

Growing evidence indicates the importance of passage of maternal-derived mediators across the placenta to the fetus. These mediators include complement activation products that freely cross the placenta and reach the fetus affecting its cardiovascular and nervous system (**Figure 5**). This is in agreement with the "developmental origins of adult disease" hypothesis, which proposes that disease risk as an adult is determined by prenatal exposures (147). We previously described how exposure *in utero* to placental pathogenic mediators such as C5a might affect fetal neurodevelopment leading to abnormal fetal brain cytoarchitecture and abnormal behavior in the offspring (110). Microglia, resident brain macrophage cells express C5a receptors and therefore maternal-derived C5a may activate the cells. Microglia activation increased in the neonatal brains in the mouse preeclampsia model (72). Activated microglia can lead to neurogenic hypertension in the offspring and cognitive disorders (119).

Preeclampsia affects multiple organ systems, including the maternal brain. While cerebrovascular dysfunction during preeclampsia, leading to cerebral edema, seizures and stroke has been extensively characterized, the potential long-term effects of preeclampsia on neurocognitive functions and behavior are not well-understood (148). Some women with a history of preeclampsia reported cognitive and emotional changes during the postpartum period and subsequent years (149). While objective data supporting these findings are very limited, it is tempting to speculate that in a similar manner to the fetal brain, placental-derived factors might reach the maternal brain, and exert harmful effects. This might be aided by the increased permeability of the maternal BBB caused by proinflammatory molecules released during preeclampsia and other pregnancy complications associated with an excessive proinflammatory response.

PERSPECTIVES FOR FUTURE RESEARCH

The continual challenge with evaluating the role of the immune system, including the complement system, in pregnancy is a determination of too much or too little. Insufficient complement

system involvement as well as too much complement system involvement can lead to pathophysiology. Thus, determining the proper balance in each organ or disease state is required. This balance point differs over the course of a pregnancy, as well as the developmental stage of the organism. In addition, the complexity increases in terms of deciding whether increased complement involvement is the cause or a consequence of the problem i.e., intended to clean up or resolve the pathophysiology. Manipulation of complement in the normal state may have adverse effects, whereas manipulation of complement in the diseased state may be beneficial.

Our review of complement in pregnancy has suggested some gaps in the literature in terms of pre-implantation, preterm birth, induction of labor, and adverse effects in offspring following a preeclamptic pregnancy. The investigation of the role of complement regulators at points of cell interactions in pre-implantation embryos could further illuminate the homeostatic function of complement in development in organ systems other than the brain. The positive effects of eculizumab in treatment of pregnant women lacking complement regulators suggest that controlled studies are needed to determine if eculizumab affects incidence of PTB in pregnancies of mothers with fully functional complement systems. In addition, pre-clinical studies suggest that evaluating properdin or other therapeutic modulators of complement may decrease PTB and the ensuing developmental side effects in offspring. Additional studies with larger sample sizes are needed to examine variants of complement inhibitors as well as to determine the risk factors of adverse pregnancy outcomes. Further studies investigating interactions of the complement system and specific microbiome components which negatively influence a successful pregnancy are also needed. Studies assessing the role of surfactant proteins SP-A and SP-D in induction of labor make it clear that the role for another collectin, mannan-binding lectin (MBL) needs to be investigated since it also reaches a maximum concentration at term. In the absence of intra-amniotic infection, preterm parturition increases plasma concentrations of complement fragment Bb. Interestingly, this activation does not occur in spontaneous labor at term suggesting that the mechanisms leading to preterm and term labor fundamentally differ with regards to a role for complement activation. This is consistent with mouse studies of PTB which could be used to clarify the distinct mechanisms. Of course, complement effects *in utero* can have major developmental effects after birth in the offspring as clearly indicated by studies of brain development. These types of studies need to be extended to pathophysiology and incidence of other disorders associated with preterm birth, preeclampsia, intrauterine growth restriction such as hypertension, metabolic disorders, obesity, and Type 2 diabetes. Given that decreased C3 is associated with reduced pancreatic beta cell area in the adult with the potential for increased risk of Type 2 diabetes, further investigation of complement involvement in pancreatic development is certainly warranted (27), as is the connection between complement and obesity which increases risk for multiple adverse pregnancy outcomes.

CONCLUSION

While the role of complement in the pathogenesis of pregnancy complications such as miscarriage, preeclampsia and preterm birth has been supported by substantial evidence, new studies demonstrate that the complement system is also involved in the early steps of pregnancy such as conception and embryo implantation. Even more, the effects of complement activation seem to go beyond pregnancy and have long term effects. Exposure to excessive complement activation during pregnancy clearly has deleterious effects after pregnancy, both on the health of the mother as well as the offspring. Although additional studies are required, modulation of complement may have important ramifications, both beneficial and harmful, from pre-implantation through the lifetime of the offspring.

AUTHOR CONTRIBUTIONS

GG, JR, JL, and SF wrote the review article. GG prepared the figures. All authors reviewed the manuscript and approved the submitted version.

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REVIEW
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Mechanisms of Key Innate Immune Cells in Early- and Late-Onset Preeclampsia

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Preeclampsia is a complex cardiovascular disorder of pregnancy with underlying multifactorial pathogeneses; however, its etiology is not fully understood. It is characterized by the new onset of maternal hypertension after 20 weeks of gestation, accompanied by proteinuria, maternal organ damage, and/or uteroplacental dysfunction. Preeclampsia can be subdivided into early- and late-onset phenotypes (EOPE and LOPE), diagnosed before 34 weeks or from 34 weeks of gestation, respectively. Impaired placental development in early pregnancy and subsequent growth restriction is often associated with EOPE, while LOPE is associated with maternal endothelial dysfunction. The innate immune system plays an essential role in normal progression of physiological pregnancy and fetal development. However, inappropriate or excessive activation of this system can lead to placental dysfunction or poor maternal vascular adaptation and contribute to the development of preeclampsia. This review aims to comprehensively outline the mechanisms of key innate immune cells including macrophages, neutrophils, natural killer (NK) cells, and innate B1 cells, in normal physiological pregnancy, EOPE and LOPE. The roles of the complement system, syncytiotrophoblast extracellular vesicles and mesenchymal stem cells (MSCs) are also discussed in the context of innate immune system regulation and preeclampsia. The outlined molecular mechanisms, which represent potential therapeutic targets, and associated emerging treatments, are evaluated as treatments for preeclampsia. Therefore, by addressing the current understanding of innate immunity in the pathogenesis of EOPE and LOPE, this review will contribute to the body of research that could lead to the development of better diagnosis, prevention, and treatment strategies. Importantly, it will delineate the differences in the mechanisms of the innate immune system in two different types of preeclampsia, which is necessary for a more personalized approach to the monitoring and treatment of affected women.

Keywords: immune cells, pregnancy, late-onset preeclampsia, early-onset preeclampsia, preeclampsia, inflammation, innate immunity

INTRODUCTION

Preeclampsia accounts for over 70,000 maternal and 500,000 fetal/neonatal deaths annually, with maternal deaths being highest in developing countries (1, 2). The exact etiology of preeclampsia is unknown, however, endothelial dysfunction, inappropriate angiogenesis, inadequate trophoblast invasion and spiral uterine artery remodeling, have all been identified as key contributors (3–6). Adequate remodeling of spiral uterine arteries into dilated, elastic, and low-resistance blood vessels enables unlimited supplies of oxygen and nutrients to the fetus. This requires appropriate invasion by extravillous trophoblasts and replacement of maternal endothelial cells (7). Inappropriate activation of the innate immune system and subsequent inflammation, however, can lead to placental dysfunction or poor maternal vascular adaptation and contribute to the development of preeclampsia (8). In this review, we will outline mechanisms of key innate immune cells implicated in the development of preeclampsia and differentiate how these mechanisms are affected in two phenotypes of preeclampsia, early-onset preeclampsia (EOPE) and late-onset preeclampsia (LOPE). The 2018 recommendations from The International Society for the Study of Hypertension in Pregnancy (ISSHP) define preeclampsia as *de-novo* hypertension (systolic blood pressure > 140 mmHg and diastolic blood pressure > 90 mmHg) after 20 weeks of gestation, accompanied by one or more of the following features: proteinuria (>300 mg/day), maternal organ dysfunction (including hepatic, renal, neurological), or hematological involvement such as thrombocytopenia, and/or uteroplacental dysfunction, such as fetal growth restriction and/or abnormal Doppler ultrasound findings of uteroplacental blood flow (1, 2, 9). Preeclampsia with severe features is defined as cases with blood pressure values ≥160/110 mmHg, accompanied by significant proteinuria (≥300 mg of protein/day), or pulmonary edema, cerebrovascular and/or liver function deterioration or thrombocytopenia (10). EOPE is diagnosed before 34 weeks of gestation whereas LOPE is diagnosed from 34 weeks of gestation (2).

INCIDENCE AND TREATMENT OF PREECLAMPSIA

A systematic review of the incidence of hypertensive disorders of pregnancy, including 39 million women from 40 countries, found that preeclampsia affects ∼4.6% of all deliveries globally (11). Another review reported that preeclampsia complicates 2 to 8% of pregnancies (12). The reasons for differences in the incidence of preeclampsia among different countries, regions or hospitals include inconsistencies in the diagnostic criteria, difficulty in diagnosing preeclampsia, as well as differences in maternal age and nulliparity, access to prenatal care and education, and regional prevalence of other risk factors (2, 11, 13). Women who have chronic hypertension, autoimmune disorders, kidney disease, pre-gestational diabetes, maternal body mass index (BMI) > 30 kg/m^2 and a family or personal history of preeclampsia, are at higher risk of developing preeclampsia; older age (>40 years) is also associated with increased risk of preeclampsia (1). Treatment of preeclampsia can be divided into expectant care and interventionist care (14). Expectant care involves a balance of stabilizing the mother's condition and delaying delivery as far as the maternal condition allows, to reduce the mortality and morbidity associated with premature birth. Interventionist care involves early delivery to minimize serious maternal and fetal complications. Expectant care provides relief from symptoms, such as reducing blood pressure with antihypertensive therapy and the use of magnesium sulfate as anticonvulsant therapy (2, 15, 16). Evidence suggests that there is no clear difference between an expectant or interventionist care approach for preeclampsia with severe features (14). Without a clear contraindication, delaying delivery for as long as possible can improve outcomes for the fetus (17). Studies investigating the prophylactic use of aspirin in high-risk pregnancies have reported conflicting findings (9, 18). A meta-analysis including 18,907 women concluded that when taken before 16 weeks of gestation at a daily dose of ≥100 mg, aspirin could reduce the risk of preterm preeclampsia diagnosed before 37 weeks of gestation (19). As such, high-risk patients must be identified early in pregnancy for any beneficial effects to be observed (16). Calcium supplementation for women with low calcium diets may lead to a reduction in the severity of symptoms associated with preeclampsia and minimize the risk of preterm birth (2, 18). In the case of diabetic pregnancies, women who were given metformin with and without insulin treatment had a lower incidence of preeclampsia (20).

SIMILARITIES AND DIFFERENCES BETWEEN EARLY-ONSET AND LATE-ONSET PREECLAMPSIA

Gestational age has been identified as the most important clinical variable in predicting both maternal and perinatal outcomes (21). This led to stratification of preeclampsia into two phenotypes, EOPE and LOPE (1, 2, 22). LOPE accounts for the majority of preeclampsia cases, comprising ∼80 to 95% of all preeclampsia cases worldwide (23). EOPE, although less common, is associated with higher rates of neonatal mortality and a greater degree of maternal morbidity compared to LOPE (3). As a result, EOPE has attracted greater interest and more studies have focused on elucidating the mechanisms underlying this disease phenotype (23), leading to implementation of preventative treatments (e.g., aspirin) and predictive biomarkers more suited for EOPE than LOPE. LOPE, nevertheless, is also a serious condition, associated with a high prevalence of eclampsia and HELLP (hemolysis, elevated liver enzymes, and low platelets) syndrome, which are two life-threatening complications (24). Further studies are needed to address this gap in research. Preeclampsia has been described as a two-stage disease, with initial deficient remodeling of the uterine spiral arteries leading to a stage of maternal systemic inflammation and vascular dysfunction (25). This model is more representative of EOPE. Impaired placental development in early pregnancy and subsequent growth restriction is often

associated with EOPE, while LOPE is likely associated with maternal endothelial dysfunction (26). Both phenotypes exhibit an increased inflammatory response that leads to adverse maternal and fetal complications. Syncytiotrophoblast stress and placental hypoxia are implicated as the main cause of excessive systemic vascular inflammation. In EOPE, this is triggered by dysfunctional perfusion of the placenta. In the case of LOPE, syncytiotrophoblast stress likely occurs as a result of compression of placental terminal villi, as the placenta outgrows the space within the uterine cavity, which can also lead to uteroplacental malperfusion (24, 27).

Timely detection of preeclampsia is complicated by the fact that the disease is usually asymptomatic in its early stages (2). Close antenatal monitoring, especially during the third trimester, can be crucial in preventing maternal and fetal complications. Detection using angiogenesis-related biomarkers such as the ratio of soluble fms-like tyrosine kinase-1 (sFlt-1) and placental growth factor (PIGF), as well as Doppler ultrasound assessment, can be useful in detecting EOPE, and to a lesser extent LOPE. Recently, other angiogenesis-related biomarkers, FKBPL and CD44, were also implicated in prediction and diagnosis of preeclampsia, particularly LOPE (28). Further research is needed to elucidate the pathogenic mechanisms and develop diagnostic biomarkers for LOPE early in pregnancy, allowing interventions to begin before clinical features manifest.

COMPLICATIONS ASSOCIATED WITH PREECLAMPSIA

Women with a history of preeclampsia, in addition to short-term complications, have a higher risk of subsequent cardiovascular and metabolic disorders, especially following EOPE (29). A meta-analysis including datasets from 3,488,160 women found that women with previous preeclampsia were twice as likely to develop ischemic heart disease compared to normotensive pregnancies (30). It is not clear whether this increased risk of subsequent cardiovascular disease is caused by underlying maternal risk factors, which are exacerbated by preeclampsia, or if this increased risk is a consequence of preeclampsia (2). Potential overlapping mechanisms between preeclampsia and cardiovascular disease including hypertension and/or heart failure with preserved ejection fraction were recently identified using a bioinformatics "*in silico*" approach (31, 32).

Untreated preeclampsia, regardless of the phenotype, can result in severe complications including liver rupture, cerebral hemorrhage, myocardial infarction, stroke, acute respiratory distress syndrome, pulmonary edema, kidney failure, and abruptio placentae (1, 9, 16). Delivery of the baby, even if it is preterm, minimizes the risk of developing these maternal and fetal complications, including fetal growth restriction and fetal loss. Premature birth, nevertheless, is also associated with a number of neonatal complications such as respiratory distress syndrome, intraventricular hemorrhage, and necrotizing enterocolitis (14). While there are a multitude of factors that contribute to the pathogenesis and onset of preeclampsia, in recent years, it has been highlighted that an overactive maternal immune system can play a critical role in preeclampsia development.

INNATE IMMUNE SYSTEM IN HEALTHY PREGNANCY AND PREECLAMPSIA

The maternal innate immune system throughout the entire gestation period plays an important role in ensuring protection from pathogens, while concurrently inducing tolerance to the semi-allogeneic developing fetus and placental development. As outlined in **Figure 1**, this is achieved through a delicate balance of various cell functions and interactions between the innate immune system cells and other placental/uterine cells in a timely manner (33, 34). Unfortunately, this is not always the case and due to various factors, the aforementioned balance is disrupted by maladaptation of certain immune cells during gestation, which is demonstrated in **Figure 2**. In physiological pregnancies, decidual macrophages found in proximity to spiral uterine arteries help prepare these for remodeling via secretion of angiogenic molecules (35–37). Macrophages also phagocytize apoptotic cells during tissue remodeling, preventing the release of self-antigens or paternal alloantigens, which could trigger a maternal immunological response (38). There are two phenotypes of macrophages, M1 or classically activated macrophages, and M2 or alternatively activated macrophages. M1 macrophages are involved in phagocytosis, and are micro-biocidal and pro-inflammatory. M2 macrophages are immunomodulatory and responsible for inducing maternal tolerance, resolving inflammation, and are involved in tissue remodeling and cell proliferation (39, 40). Therefore, in normal physiological pregnancy, macrophages favor the M2 phenotype, whereas in preeclampsia, this balance is shifted toward the M1 phenotype (41). M1 cells secrete soluble fms-like tyrosine kinase-1 (sFlt-1), an anti-angiogenic molecule that is associated with impaired angiogenesis in preeclampsia (42). Consequently, the transition of macrophage phenotype from M2 to M1 is indicative of a pro-inflammatory response as observed in preeclampsia.

IMMUNOMODULATION BY INNATE IMMUNE CELLS

Innate immune cells, while assisting in the initial stages of pregnancy, also exhibit immunomodulatory characteristics targeted at immunological responses toward the fetus. Mast cells have demonstrated such characteristics through the release of histamine and stimulation of the G protein-coupled receptors ($H_{1-4}R$) (43). Activation of the H_4R appears to lead to proliferation of regulatory T cells (Tregs), and H_2R support angiogenesis at the fetal-maternal interface (43, 44). Tregs subsequently act to prevent lymphocytes from attacking the fetus. There is limited evidence regarding the behavior of mast cells during preeclampsia and their contribution to its onset. There are some reports that they accumulate at higher density adjacent to

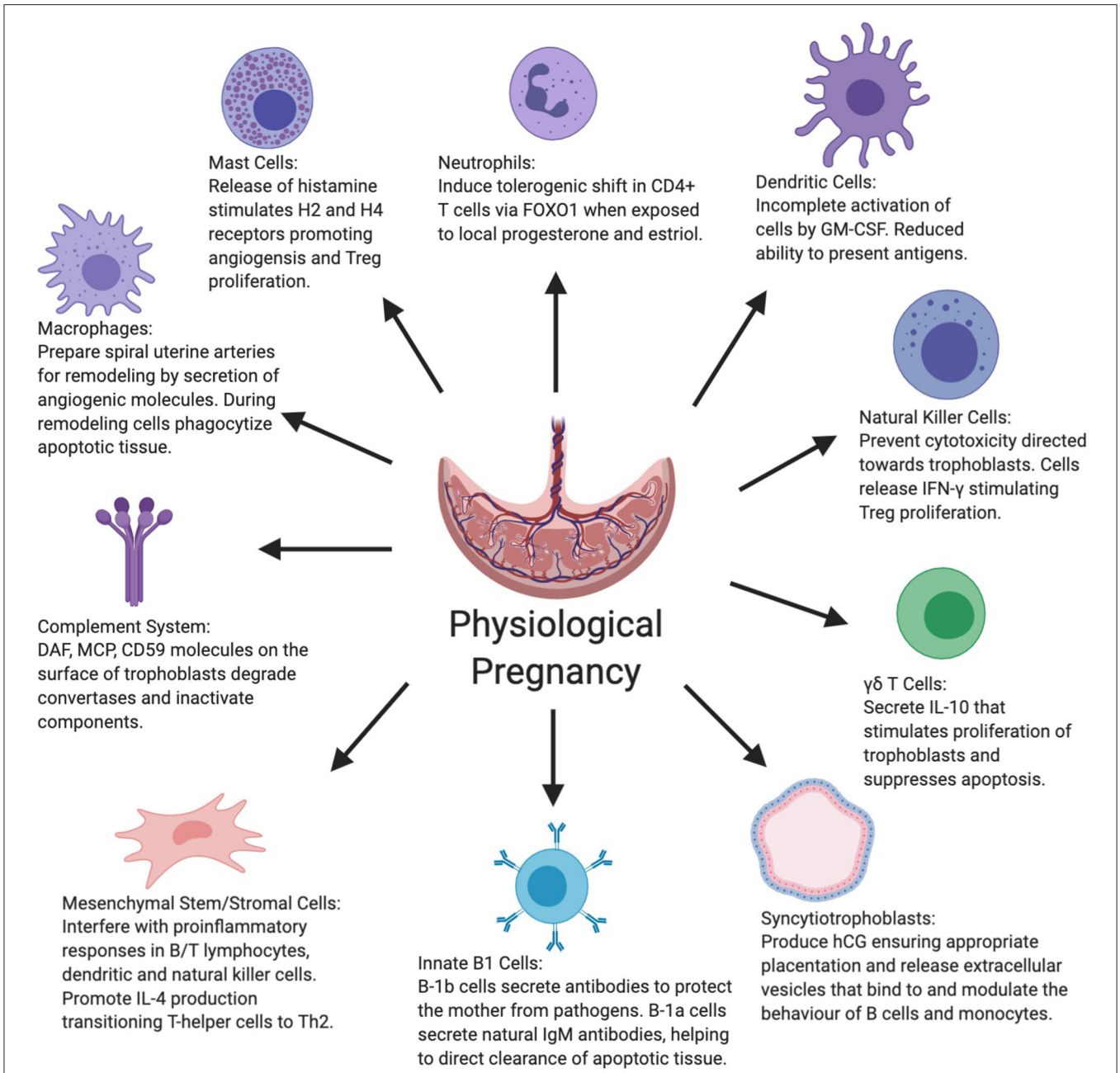

FIGURE 1 | Figure summarizes the various roles displayed by the innate and other key immune system cells as well as syncytiotrophoblasts and mesenchymal stem cells in normal physiological pregnancy. CD59+, Cluster of differentiation 59+; DAF, Decay-accelerating factor; FOXO1, Forkhead box protein-1; GM-CSF, Granulocyte-macrophage colony-stimulating factor; hCG, Human chorionic gonadotropin; IFN-γ, Interferon-gamma; IL-4, Interleukin-4; IL-10, Interleukin-10; MCP, Membrane cofactor protein.

spiral uterine arteries during preeclampsia and undergo intensive degranulation, leading to a release of large concentrations of histamines (45). The high histamine concentrations in the circulation stimulate pro-inflammatory responses from both the innate and adaptive immune system, leading to the secretion of pro-inflammatory cytokines and molecules, contributing to the increase in blood pressure, typical of preeclampsia (46).

THE ROLE OF NEUTROPHILS IN MATERNAL TOLERANCE DURING PREGNANCY

Neutrophils, also as part of a normal physiological pregnancy, are recruited to the developing placenta via the chemokine, IL-8 (47). Following the recruitment of circulating neutrophils

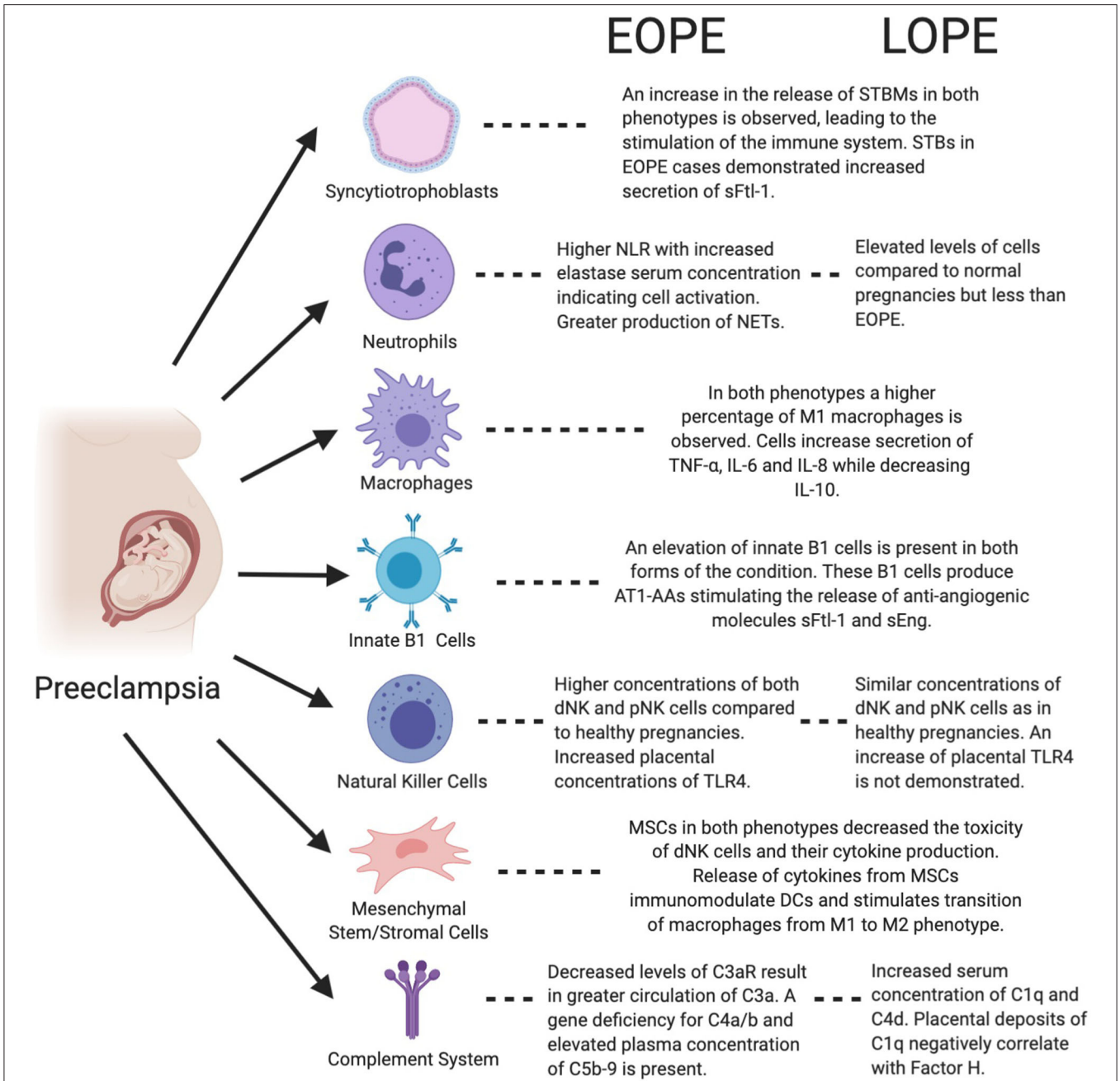

FIGURE 2 | Figure highlights the functions of both immune and placental cells in the context of preeclampsia and both of its phenotypes, EOPE and LOPE. AT1-AA, Angiotensin II type 1 receptor agonistic autoantibody; DC, Dendritic cell; dNK, Decidual natural killer; IL-6, Interleukin-6; IL-8, Interleukin-8; IL-10, Interleukin-10; MSC, Mesenchymal stem/stromal cell; NET, Neutrophil extracellular trap; pNK, Peripheral natural killer; NLR, Neutrophil to lymphocyte ratio; sEng, Soluble Endoglin; sFtl-1, Soluble fms-like tyrosine kinase-1; STB, Syncytiotrophoblast; STBM, syncytiotrophoblast micro-particles; TLR4, Toll-like receptor 4; TNF-α, Tumor necrosis factor-α.

to the placenta and under the influence of progesterone and estriol, $CD4^+$ T cells undergo immunomodulation via transfer of the forkhead box protein 1 (FOXO1) from neutrophils (48). These neutrophil-induced T (niT) cells, in addition to establishing maternal tolerance of the fetus, secrete IL-10, IL-17, and vascular endothelial growth factor (VEGF) promoting angiogenic processes (48). Also in normal physiological pregnancy, the natural cytotoxicity receptors (NCRs) on maternal decidual natural killer (NK) cells are inactivated to ensure maternal tolerance. This is not the case in preeclampsia due to abnormalities in the NCR structure, which increases their synthesis and expression on NK cells, thus affecting maternal tolerance to the fetus (49). Overactivation of NCRs on NK cells leads to discharge of

pro-inflammatory cytokines, thus contributing to weakened immunological tolerance potentially endangering the fetus (50).

INACTIVATION OF INNATE IMMUNE CELLS IN PREGNANCY

Whereas the aforementioned cells either assist in required gestational processes or exhibit immunoregulatory roles, other innate immune cells become partially or fully inactivated during pregnancy. NK cells within the uterus and decidua, for example, maintain their immunological function against infections without compromising the fetus. This is achieved by inhibiting NK cell-mediated cytotoxicity toward trophoblasts by interfering with their degranulation process (51). NK cells additionally release IFN-γ, which stimulates decidual $CD14^+$ myelomonocytic cells to induce Treg proliferation via transforming growth factor-beta (TGF-β) (52). The incomplete activation of DCs by granulocyte-macrophage colony-stimulating factor (GM-CSF) similarly leads to a reduction in the capacity of these cells to adequately present antigens (53). This effect consequently limits the development and activation of T cells, increasing maternal tolerance toward the fetus (54). DCs located proximally to the placenta remain inactivated or immature during normal pregnancy; however, these cells become inappropriately stimulated during preeclampsia. Although GM-CSF initially acts as a regulator of DCs, in higher concentrations, GM-CSF along with lnc-DC (long non-coding RNA expressed in DCs), induces DC maturation (55, 56). Once mature, DCs can more efficiently present antigens leading to an increase in the proliferation of Th1/Th17 cells (56, 57). The Th1/Th17 cells consequently stimulate pro-inflammatory responses, which significantly reduce maternal tolerance.

B1 CELL ACTIVATION IN HEALTHY PREGNANCY AND PREECLAMPSIA

B1 cells, despite originating from the lymphoid group of immune cells, also belong to the innate immune system and are important in the initiation and maintenance of a healthy pregnancy. The B1 cell population is subdivided into two groups, B-1a and B-1b, each exhibiting unique roles throughout gestation (58). B-1b cells produce typical antibodies in response to antigen identification, providing protection against invading pathogens (58). On the other hand, B-1a cells secrete "natural IgM antibodies" with low-affinity, poly-reactivity, and self-reactivity, regardless of antigenic stimuli (58, 59). These natural antibodies assist in the clearance of apoptotic tissue cell bodies, affecting the immune response during tissue remodeling (59). B1 innate cells, similar to other immune cells, are irregularly activated in preeclampsia. Notably, B-1a cells are stimulated to produce angiotensin II type 1 receptor agonistic autoantibodies (AT1-AA) in preeclampsia, which does not occur in normal physiological pregnancies (60). These antibodies, as the name suggests, act as agonists and induce signaling pathways, leading to the vasoconstriction of blood vessels and the secretion of aldosterone, which stimulates the renin-angiotensin system and increases blood pressure (61).

INFLUENCE OF THE COMPLEMENT SYSTEM

The capacity of the complement system during healthy physiological pregnancy is also modulated to prevent its activation, which could endanger the fetus. Regulatory proteins including decay-accelerating factor (DAF), membrane cofactor protein (MCP), and CD59, found on the surface membrane of trophoblasts, help to degrade convertases within the complement system inducing components of this system into their inactive forms by way of cleavage (62). Due to the widespread dysregulation of the maternal immune system during preeclampsia, the complement system becomes overstimulated as part of a compensatory mechanism. Over-activation of both the classical and lectin complement pathways leads to greater terminal activation, causing inflammation and the recruitment of large numbers of phagocytes to the origin of the stimuli (63). This subsequently contributes to the onset of maternal hypertension and organ damage (63).

SYNCYTIOTROPHOBLAST CELLS AND THEIR SECRETED EXTRACELLULAR VESICLES

The blastocyst, in anticipation of implantation, initiates secretion of human chorionic gonadotropin (hCG) before its synthesis is superseded by syncytiotrophoblast cells (STBs) (7). The continued secretion of hCG by STBs ensures appropriate invasion of trophoblast cells into the endometrium. Upon shedding of the STB layer, extracellular vesicles (EVs) are released from apoptotic STBs, which bind to monocytes and B cells (64). EVs encompass three main vesicle types: exosomes, micro-particles/micro-vesicles and apoptotic bodies (65–67). These EVs, upon binding, induce a shift in the cytokine secretion profile of the neighboring cells, causing the release of anti-inflammatory cytokines (64). On the other hand, in preeclampsia when placental ischemia and hypoxia are present, a greater number of the STB cells undergo apoptosis (68). There is subsequently an increase in secreted EVs into the maternal circulation, overwhelming the body's ability to adequately scavenge and clear them effectively (68, 69). These vesicles then act as antigenic stimuli for components of the immune system leading to unintended endothelial injury, inflammation, and hyper-coagulation (68).

IMMUNOMODULATION BY MESENCHYMAL STEM/STROMAL CELLS

Emerging evidence suggest that mesenchymal stem/stromal cells (MSCs) also have important immunomodulatory roles in pregnancy. Facilitated by paracrine signaling, MSCs target B/T lymphocytes, DCs, and NK cells and interfere with their pro-inflammatory responses. Simultaneously, MSCs stimulate the shift of T-helper cells from a Th1 to Th2 phenotype by promoting IL-4 production and inhibiting IFN-γ production, thus heightening maternal immunological tolerance of the fetus.

Furthermore, MSCs have also been shown to have a key pro-angiogenic role in pregnancy (70). In preeclampsia, the function of MSCs is likely impaired due to exposure to increased numbers of reactive oxygen species (ROS) (70, 71). The presence of aldehyde dehydrogenases (ALDH) during normal pregnancy assists in detoxification from ROS, thus providing a degree of protection against oxidative damage (71). However, due to an unknown mechanism, levels of ALDH in preeclampsia are decreased, exposing the MSCs to oxidative stress with resulting damage and reduced functional ability to modulate other immune cells (71).

In summary, immune cells are critical in the progression of normal physiological pregnancy, both in terms of maternal tolerance and placental development; the roles of key innate and other immunogenic cells in normal physiological pregnancy are depicted in **Figure 1**. The vast majority of immune cells and other associated placental/uterine cells become over-activated or dysregulated in preeclampsia, contributing to the overall symptoms and features of the condition, including hypertension and organ damage.

MACROPHAGE PHENOTYPE PLASTICITY IN PREECLAMPSIA

Macrophages can alter their phenotypic profiles in response to a variety of environmental factors (39). M1 macrophages develop in response to exposure to Th1 cytokines such as IFN-γ, tumor necrosis factor (TNF)-α and lipopolysaccharide (LPS) (72, 73). The development of M2 macrophages is favored in the presence of TGF-β, IL-4, IL-10, and IL-13. Inflammatory cytokines, TNF-α, IL-6, and IL-8, are significantly increased in preeclampsia, and IL-10 is significantly decreased compared to normal pregnancy, therefore promoting the M1 phenotype (74, 75). Decidual macrophages comprise 20% of the immune cells present within the placenta (76). Placental decidual macrophages in normal pregnancy are mainly of the M2 subset and can be found near spiral uterine arteries (40). They have an important role in preparing spiral uterine arteries for remodeling by trophoblasts, as well as phagocytozing pro-inflammatory substances formed during the process of remodeling. The predominance of the M1 decidual macrophage phenotype is conducive to the release of substances such as TNF- α, IFN-y and nitric oxide (NO), which inhibit trophoblast invasion and spiral uterine artery remodeling (39, 72, 76). Various studies have reported increased levels of decidual macrophages in preeclampsia (55, 77, 78). Other studies have reported a reduction in macrophages in the placental decidua in preeclampsia, possibly due to reduced monocyte migration to the decidua or lack of differentiation into macrophages (79, 80). Chitinase-3-like protein 1 (CHI3L1), also known as YKL-40, is indicative of the number of macrophages, and it has been shown to be present in significantly lower levels in women who developed EOPE compared to normal pregnancy (81). These conflicting findings may be a result of different macrophage cell markers, methods employed, use of tissue samples from different preeclampsia phenotypes and different sections of the placenta being studied, considering that decidual macrophages reside predominantly around spiral uterine arteries (82).

MARKERS OF SYSTEMIC INFLAMMATION: NEUTROPHIL EXTRACELLULAR TRAPS AND THE NEUTROPHIL-LYMPHOCYTE RATIO

Neutrophils are likely the main class of leukocytes that cause the majority of vascular cell dysfunction in women with preeclampsia (83). Neutrophil activation may occur from exposure to oxidized lipids secreted by the placenta as a consequence of placental damage. Activated neutrophils infiltrate the maternal systemic vasculature and release substances such as ROS, TNF-α and myeloperoxidase (MPO), causing endothelial dysfunction (74, 83). MPO has been associated with hypertension, and elevation in TNF-α is recognized as a driving inflammatory mechanisms to preeclampsia (84, 85).

Neutrophil numbers in the maternal systemic circulation and within the decidua steadily increase in pregnancy throughout gestation, yet this increase is further amplified in preeclampsia (4, 74, 86–88). Elevation in neutrophil count has been noted in both EOPE and LOPE compared to normal pregnancy, with a greater elevation present in EOPE (26). This surge in neutrophils may be an adverse reaction to the interaction between the maternal immune system and micro-debris originating from the placenta (89). Although the number of neutrophil granulocytes increases, the phagocytic function of these cells reportedly decreases in pregnancy, particularly in preeclampsia (88). Plasma elastase, a marker of neutrophil activation, has been noted to be elevated in preeclampsia when compared to normal pregnancy (90). A small sample cohort study reported a significant increase in plasma elastase in EOPE compared to normotensive controls (3). Neutrophil extracellular traps (NETs) have been found in the intervillous spaces of placentae in women with preeclampsia (25). The formation of these web-like chromatin structures is induced by STB microparticles (STBMs) released from the placenta and ROS (91). Neutrophils, in addition to causing inflammation, represent the first wave of leukocytes responding to inflammation (5, 83). NETs are abundant within sites of inflammation causing endothelial damage as demonstrated in cases of sepsis, and may also cause damage to villous trophoblast cells in preeclampsia (92, 93). The presence of NETs in the maternal circulation during pregnancy can contribute to thrombotic events, inflammation, and ultimately, fetal death (94). The neutrophil-to-lymphocyte ratio (NLR), is a measure of systemic inflammation, and has demonstrated prognostic value in several cardiovascular diseases, including preeclampsia (4). Both normal pregnancy and preeclampsia present with an increased NLR compared to non-pregnant controls (95). Several studies, however, have reported the NLR to be significantly higher in women with preeclampsia compared to normotensive controls (6, 16, 96, 97). A retrospective case-control study conducted with 186 patients found the NLR to be highest in EOPE, and LOPE demonstrating higher NLRs compared to normotensive controls (26). A change

in the NLR can be noted at 16–18 weeks of gestation, and thus has a potential as an inexpensive biomarker for the early detection, monitoring and prompt intervention particularly for EOPE (97).

NATURAL KILLER CELLS IN PREECLAMPSIA

As discussed previously, in normal pregnancy the appropriate remodeling of spiral uterine arteries into low resistance and high capacity vessels coordinated with appropriate trophoblast invasion is pivotal. When these two processes are not well-coordinated, the consequent insufficient blood flow leads to a series of events, which ultimately result in the development of preeclampsia. This suggests that interactions at the maternal-fetal interface in early gestation are important for determining the course of pregnancy. In order to preserve an adequate immuno-tolerant environment, DCs and T-lymphocytes have limited access to the decidua during pregnancy (98). NK cells, in fact, represent 70% of the immune cells in the decidua (99, 100). These decidual NK (dNK) cells are a distinct population from peripheral NK (pNK), both phenotypically and functionally. Unlike pNK, the dNK subpopulation has a $CD56^{+}/CD16^{-}$ phenotype (101) and demonstrates a lower cytotoxic potential and higher cytokine secretory profile (102). Decidual NK cells, by secreting VEGF and PlGF, stimulate spiral uterine artery remodeling, a process crucial for successful establishment of the placenta and the feto-maternal interface in pregnancy (103, 104). The lack of dNK cells has been shown to lead to lower fertility and higher fetal resorption (105). NK cells, on the other hand, are recruited by the innate immune system in response to inadequate trophoblast invasion or insufficient spiral uterine artery remodeling, processes observed in preeclampsia. There are some inconsistencies among studies, however, with respect to the number of these cells present in preeclampsia compared to normotensive pregnancy. While some studies have reported significantly lower numbers of NK $CD56^{+}$ cells within the decidua in preeclampsia (77, 106), other reports have indicated the opposite trend (107, 108). The heterogeneity among studies and the differences in patient characteristics offer possible explanations for these discrepancies. A recent study demonstrated that the increases in both dNK and pNK cells were higher in EOPE compared to LOPE (108).

TOLL-LIKE RECEPTORS IN PREECLAMPSIA

Toll-like receptors (TLRs) represent a family of transmembrane signaling receptors found on all innate immune cells. Ten different TLRs have been identified in humans based on their cellular localization and respective ligands (109). All 10 TLRs activate nuclear factor κB- (NF-κB) dependent and NF-κB-independent pathways to generate cytokines and chemokines (109). TLR expressions vary throughout pregnancy. Trophoblast expression of TLRs changes throughout gestation, with TLR2-4 being highly expressed during the first trimester and TLR1-10 in the third trimester (110–114). TLRs activate inflammatory responses by recognizing damage-associated molecular patterns (DAMPs) released following tissue injury, as well as pathogen-associated molecular patterns (PAMPs) specific to microbial components (115–117). Continuous signaling from DAMPs due to persistent cell death and remodeling of spiral uterine arteries leads to over-activation of TLRs. Excessive TLR activity may contribute to the pro-inflammatory effects and hypertension observed in preeclampsia. Studies report that overstimulation of these receptors due to either viral or bacterial infections may lead to adverse pregnancy outcomes including preeclampsia (114, 118). Upon trophoblast TLR-3 and TLR-4 activation by microbial byproducts, chemokine secretion initiates the innate immune response and the decidua becomes infiltrated with pNK cells and macrophages (113). TLR4 activation by bacterial LPS, in addition, inhibits trophoblast migration (119), while TLR3 activation by poly I:C, a double-stranded RNA (dsRNA) viral mimetic, increases inflammation and results in the development of preeclampsia-like symptoms in pregnant rats (120). Increased immunoreactivity of the TLR4 protein in placentae from complicated pregnancies suggests that their role in the activation of the innate immune system is in response to the presence of infectious agents (112). It has been recently shown that the expression of TLR4 in placentae from women with EOPE was higher than TLR4 expression in women with LOPE (121). It is possible that this upregulation is part of a compensatory mechanism in preeclampsia, given that higher expression of TLR4 has been described in human term placentae compared to first trimester (122). Activation of TLR3 in pregnant mice increased systolic blood pressure and endothelial damage, both of which were further exacerbated in the absence of IL-10 (123). Moreover, dsRNA and single-stranded RNA (ssRNA) were shown to upregulate expressions of TLR3, TLR7, and TLR8 in mouse placentae. This caused pregnancy-dependent hypertension, endothelial dysfunction, and placental inflammation (124). Women with preeclampsia displayed activation of the aforementioned TLRs; however the association between severity of the disease and activation of TLRs was not confirmed (124). Increased expression of TLR9 in the placentae and peripheral blood mononuclear cells from women with preeclampsia compared to normotensive controls has also been described (125, 126). A study by He et al. showed that when mice were treated with a TLR9 agonist, they developed preeclampsia-like symptoms. This preeclampsia murine model also showed that with exogenous overexpression of TLR9, the levels of sFlt-1 increased while VEGF was downregulated. This suggests that TLR9 is capable of suppressing angiogenesis (127) and that aberrantly activated ligand binding to different TLRs may significantly influence pregnancy outcomes. In a relatively recent study, it was demonstrated that inhibition of TLR activation and thus inhibition of downstream signaling, could not prevent embryo resorption in the absence of dNK cells (105, 128). Differential expressions of TLRs throughout pregnancy and in preeclampsia, suggest that these receptors might represent potential therapeutic targets.

THE ROLE OF INNATE B1 CELLS IN PREECLAMPSIA

As indicated above, there are two different subsets of B1 cells. B1a cells are $CD5^+$ and produce "natural antibodies," which are polyreactive, low-affinity and self-reactive antibodies. On the other hand, B1b cells are $CD5^-$ and produce adaptive antibodies when exposed to antigens (58). It is, however, the role of B1a cells that is more closely associated with adverse pregnancy outcomes. Namely, the proportion of B1a cells decreases throughout gestation likely as a protective mechanism against poly-reactive antibodies produced by B1 these cells, which recognize and target a variety of antigens including fetal antigens (129). Their role in preeclampsia has not fully been investigated. However, there are studies emerging regarding their association with hypertensive disorders in pregnancy. The number of peripheral blood B1a cells in women with preeclampsia is significantly increased compared to normal pregnant women (60), however no difference in their number between severe and mild preeclampsia has been observed (130). In addition to the well-established Th1/Th2/Th17-Treg paradigm of the pathogenesis of preeclampsia [as reviewed in (131)], the role of B1 cells is likely linked to stimulation of $CD4^+$ T cells and their differentiation into Th17 effector cells (132). It has also been demonstrated that B1a cells can produce agonistic autoantibodies to AT1-AA in pregnancy, which can lead to the development of preeclampsia (133). High affinity binding of AT1-AA to receptors within the placenta leads to increased secretion of anti-angiogenic factors (sFlt-1 and Endoglin), both of which are associated with the onset of preeclampsia (134–136). These autoantibodies appear to correlate with severity of preeclampsia (137). The depletion of B-cells in an animal model of preeclampsia resulted in a decrease in the level of AT1-AA and a reduction in preeclampsia symptoms (138). Natural antibodies secreted by B1a cells are mostly IgM antibodies and are important in clearing and neutralizing pro-inflammatory targets (139). Although the specific roles of B1 cells have not been elucidated yet, their numbers were not significantly increased following placental ischemia (140). Substantial depletion of B cells by the monoclonal anti-human CD20 antibody, rituximab, interestingly did not have a significant effect on the hypertensive response in the RUPP model (140).

In summary, only a limited number of studies have assessed the role of innate B1 cells in preeclampsia. Further research is needed to evaluate the association of innate B1 cells with hypertensive disorders in pregnancy, as well as their role and pathogenic mechanisms in EOPE vs. LOPE.

EMERGING ROLE OF γδ T CELLS IN PREECLAMPSIA

Within the decidua, γδ T cells despite originating from the lymphoid lineage facilitate proliferation of trophoblast cells while concurrently suppressing their apoptosis through the secretion of IL-10 (141, 142). This ensures adequate migration and invasion of trophoblast cells leading to appropriate placental development. The role of γδ T cells has not yet been determined in preeclampsia, but increases in the production of pro-inflammatory stimuli, interferon (IFN)-γ & IL-17, by γδ T cells have been reported in women with idiopathic recurrent pregnancy loss (143). Furthermore, in mice, a competitive antagonist binding of the histocompatibility complex (MHC) class II found on the surface of γδ T cells, resulted in the reduction of their immunological capabilities (144). The γδ T cell "knockout mice" displayed a resistance to developing preeclampsia-like features, implying that these cells could have a role in the pathogenesis of the condition (144). In the same study, preeclamptic placentae demonstrated significantly increased levels of γδ T cells (144).

THE DYSREGULATION AND OVER-ACTIVATION OF THE COMPLEMENT SYSTEM DURING PREECLAMPSIA

The distribution and activity of the complement system's components vary between EOPE and LOPE, likely stemming from their different underlying pathogeneses. Dysfunction related to the complement system in EOPE has been correlated with single nucleotide polymorphisms (SNPs) as demonstrated by Wu et al. (145). More specifically, C6 (rs7444800, rs4957381) and MASP1 (rs1108450, rs3774282, rs698106) polymorphisms were shown to correspond independently to a risk of EOPE and severe preeclampsia (145). Another modification to the complement system that is unique to EOPE is the reduction in the placental concentrations of complement 3a receptor (C3aR) mRNA and protein (146). These reductions lead to an increase in the plasma concentration of C3a, the ligand for this receptor (146). Lokki et al. expanded upon these findings and compared the activation of the complement pathways in EOPE vs. normal pregnancies. In their cohort study of 22 women, those with EOPE displayed higher placental deposition of C1q, specifically proximal to areas of fibrinoid necrosis (147). They demonstrated that 43% of EOPE cases had a gene deficiency for C4a/b, a deficiency known to also be implicated in certain autoimmune disorders (147). Finally, Lokki et al. noted that areas of C3b deposition were positively correlated with C1q and negatively with Factor H, a regulatory factor of the alternative pathway (147). The over-activation of the complement system in EOPE is reinforced by the rise in the plasma concentration of C5b-9, which is indicative of terminal activation (148). C-reactive proteins of the system, specifically, C3a, have also been found circulating in high concentration within the amniotic fluid in EOPE (149).

LOPE shares many characteristics with EOPE in dysregulation of the complement system, with some key distinctions. As in EOPE, the MASP1 gene has been shown to display SNPs, however, in LOPE the variants indicated are rs1357134 and rs698090 (145). The aforementioned variations in the genes are completely different from the ones detected in EOPE cases and are specifically correlated with LOPE (145). Examining the sera of both EOPE and LOPE, severe preeclampsia cases revealed some degree of activation of the complement system, as demonstrated by Jia et al. (150). Serum levels of C1q, Factor H, C3 and C4

significantly decreased, while the Bb concentration increased in the presence of either EOPE or LOPE compared to their respective controls (150). Despite this, the concentrations of the C-reactive proteins observed in LOPE were not significantly different than in the EOPE cohort (150). Nevertheless, another recent study by He et al. using similar sample size, characterized the components of the complement system using plasma samples from 30 EOPE and 30 LOPE patients with severe preeclampsia. The results obtained contradicted Jia's investigation, showing elevated Bb, C3a, C5a, and MAC in both EOPE and LOPE, whereas LOPE was specifically associated with elevated C1q and C4d compared to normotensive controls (151). Lokki et al. built upon this data, by inspecting the dissimilarities of C1q deposition in the STB layer of the placenta of LOPE patients. This investigation revealed that the C1q deposits negatively correlated with Factor H, characterizing a shift toward activation within the complement system (147).

SYNCYTIOTROPHOBLASTS PLAY IMPORTANT ROLE IN PREECLAMPSIA

STBs form the feto-maternal placental barrier, which separates the fetal and maternal circulations (65). The STB-containing layer, as described above, is shed into the maternal circulation by the placenta during normal pregnancy, releasing STBMs (152, 153). STBMs levels were increased in EOPE compared to matched normal pregnancies, whereas no change was observed between LOPE and normal pregnancy samples (154). This increase in STMBs potentially contributes to endothelial dysfunction and systemic inflammation (155). Another study confirmed no significant difference between levels of EVs from various cells including STBs, in normal pregnancy compared to LOPE (156). Further studies are needed to determine whether this shedding is potentially more prominent in EOPE compared to LOPE. STBM shedding has been linked to increased levels of active tissue factor, leading to enhanced aggregation of platelets (157). This is evident in EOPE with severe features, but not observed in LOPE, which supports evidence suggesting two distinct phenotypic pathogeneses. Further studies are needed to explore if higher levels of STBMs in EOPE are due to their prevalence being greater in early gestation, independent of the presence of preeclampsia (153). STBMs act as ligands for receptors, growth and coagulation factors, and RNA molecules, and have an important role in cell-cell communication (65). STBMs bind TLRs and activate monocytes, DCs, NK cells, and neutrophils. The subsequent release of various inflammatory cytokines and superoxide radicals contributes to the systemic inflammation associated with preeclampsia (74, 88, 94, 158).

The release of sFlt-1 from STBs exerts indirect anti-angiogenic effects by competitively blocking binding of VEGF and PIGF to their respective receptors (158, 159). Levels of sFlt-1 are increased in preeclampsia and can be used as a biomarker of STB stress associated with EOPE (67, 158). LOPE does not present with this early pathology, with studies reporting changes in angiogenic biomarkers near term, observing similar plasma concentrations in both normal pregnancies and LOPE, thus not providing reliable detection of LOPE (160). Contrary to findings describing the prominent role of STBMs, it has been suggested that soluble factors directly released from STBs mediate endothelial dysfunction in preeclampsia rather than EVs (161).

MSC REGULATION OF INNATE IMMUNE SYSTEM RESPONSE IS IMPAIRED IN PREECLAMPSIA

Increased attention has been directed toward investigating the role of MSCs and their immunomodulatory capacity during pregnancy and its complications. As their potential therapeutic role in preeclampsia has been discussed elsewhere (70, 162, 163), here we discuss their contribution to irregular innate immune system signaling in preeclampsia. MSCs are found in many tissues, such as bone marrow, and adipose, decidual and fetal tissue (164–166). Adipose-derived MSCs have demonstrated impaired function associated with senescence in women with preeclampsia (167). Decidual MSCs mediate appropriate placentation and ensure immune tolerance to the semi-allograft fetus (168, 169). These decidual MSCs have the ability to decrease NK cell cytotoxicity and cytokine production (170). This may potentiate the transition of peripheral into decidual NK cells, a process critical for adequate decidual function. Decidual MSCs in addition regulate dNK through their intracellular cytokine expression profile, including TNF-α and IL-4 and via the interaction between collagen and LAIR-1 (171). Bone marrow-derived MSCs are also capable of modulating NK cells by inhibiting their proliferation, cytokine secretion, and cytotoxicity against HLA-class I- expressing targets, either via soluble factors or via cell-to-cell specific interactions (172, 173). A study by Aggarwal and Pittenger showed that immunosuppressive MSC features are associated with the inhibition of TNF-α and IFN-γ, and the secretion of prostaglandin E_2 (PGE2) (174, 175). Notably, it has been previously suggested that the lack of this prostaglandin in preeclampsia leads to a decrease in both renal blood flow and sodium excretion (176). The immunomodulatory interactions between MSCs and NK cells, along with existing studies, provide promising results that strengthen the potential immunomodulatory effects of MSCs. Although MSC are considered privileged immune cells, they can be recognized and eliminated by activated NK cells (172).

Human placental MSCs also have an immunoregulatory effect on macrophage differentiation, favoring the expression of the M2-immunosuppressive phenotype (177). This immunoregulatory effect may be mediated by soluble molecules acting partially via glucocorticoid and progesterone receptors. MSC treatment decreases IL-6 and TNF-α, while increasing anti-inflammatory cytokine, IL-10 (178). A previous study has suggested that PGE2 plays an important role in the immunoregulatory effects of MSC, indicating that M2 macrophage polarization is initiated via the COX-2-PGE2 pathway (178, 179). MSCs are the most widely used stem cell-based therapies due to their beneficial immunomodulation, anti-oxidant, pro-angiogenic, and regenerative therapeutic effects. Their therapeutic potential for the prevention and treatment of

TABLE 1 | Therapeutic strategies targeting aberrant innate immune system mechanisms implicated in preeclampsia.

Innate immunity target	Treatment	Mechanism	Safety in pregnancy	References
Macrophages	Salidroside (SLDS) is a phenylpropanoid glycoside extracted from the root of *Rhodiola rosea* L	Reduction in M1 macrophage/microglia polarization and an increase in M2 macrophage/microglia polarization in mice	Unknown	(73, 181)
Macrophages	Macrophages transplantation	Increase in M2-polarized macrophages	Risk for fetal and maternal micro-chimerism	(182)
Neutrophils	Maternal corticosteroid administration-Betamethasone	Reversal of delayed neutrophil apoptosis (returning the normal rate of spontaneous neutrophil apoptosis)	Betamethasone acetate Category C (TGA) Betamethasone dipropionate Category B1 (TGA)	(90)
STBM	Neprilysin (NEP) inhibitors Racecadotril (Hidrasec®)	Inhibition of STBM released, promoting vasodilatation, and natriuresis	Category B1 (FASS)	(183, 184)
Maternal microbiome	Probiotic-rich food Milk-based probiotics e.g., *Lactobacillus acidophilus* and *Lactobacillus rhamnosus*	Consumption of probiotic-rich food during pregnancy has been associated with lower rates of preterm birth and preeclampsia Probiotics have been implicated in the modification of placental trophoblast inflammation, systemic inflammation, and blood pressure, all features of preeclampsia *Lactobacillus* could be associated with lower risk of preeclampsia in primiparous women Overstimulation of the innate immune system due to dysbiosis of the maternal microbiome has been linked to preeclampsia	Generally recognized as safe (GRAS) by FDA	(185–188)
IL-10	Recombinant Human Interleukin-10	Increased anti-inflammatory capacity	Recombinant IL-10 reverses hypoxia-induced effects in pregnant mice No significant effect on fetal development in mice	(189–191)
TNFα	Infliximab	TNFα antagonist Anti-inflammatory effects	Category B (FDA) No increases in miscarriage, structural neonatal malformations or prematurity were observed compared with non-exposed pregnancies	(85, 192)
Complement system	Ravulizumab (Ultomiris®)	Inhibit cleavage of C5 into C5a and C5b	Category B2 (FASS)	(193)
TLR9	TLR9 antagonist Low-dose naltrexone (LDN)	Reduced inflammatory activity (studied in Crohn's disease)	Category B3 (FASS)	(194–196)
TLR2 & TLR4	Sparstolonin B (SsnB) derived from the Chinese herb *Spaganium stoloniferum*	Blocks TLR2- and TLR4-mediated NFκB activation in mouse macrophages induced by LPS and Pam3CSK4	Anti-angiogenic and anti-estrogen toxicity effects in pregnant rodents	(197)
TLR4	Ibudilast	Upregulation of anti-inflammatory cytokines (IL-10, IL-4) Antagonism of TLR4	Not tested in pregnant women	(198) www.clinicaltrials.gov (NCT01389193)
TLR9	TLR9 inhibitory oligodinucleotide (ODN2088)	Antagonism of TLR9 associated with reduction in systolic blood pressure	No adverse effects were observed in mice receiving this treatment in a model of type 1 diabetes mellitus ODN2088-treated mice gave birth to healthy pups	(199–201)
TLR4	Berberine- isoquinoline alkaloid mainly extracted from *Rhizoma Coptidis*	LPS antagonist Inhibition of LPS/TLR4 signaling	Berberine can cause or worsen jaundice in newborn infants and could lead to kernicterus	(202–206)
TLR4/NF-κB pathway	Parthenolide- Feverfew (*Tanacetum parthenium* L.)	Inhibition of the TLR4/NF-κB pathway	Not safe in pregnancy Feverfew (Tanacetum parthenium L.) shows potential emmenagogue activity and induces abortion	(202, 207, 208)

(Continued)

TABLE 1 | Continued

Innate immunity target	Treatment	Mechanism	Safety in pregnancy	References
IL-1 beta	Canakinumab	Antibody targeting IL-1β Suppression of the innate immune response and systemic anti-inflammatory effects	Category B1 (FASS)	(209)
MSC	MSC-derived EVs	MSC-derived EVs containing molecular cargo and functional mitochondria metabolically reprogram macrophages M1 pro-inflammatory phenotype toward M2 anti-inflammatory phenotype	Unknown/no major adverse effects were reported in preclinical studies with pregnant rodents	(210) (Reviewed in (70)
MSC	PLacental eXpanded (PLX-PAD) cells	Suppress TLR-induced inflammation. Release anti-inflammatory cytokines (IL-15 & GM-CSF) and growth factors (EGF & VEGF-A)	No detrimental effects on fetal development of mice pups	(162)

The Swedish classification system (Farmaceutiska Specialiteter i Sverige (FASS), American Food and Drug Administration (FDA) and Australian Therapeutic Goods Administration (TGA) were used to determine the safety profile of drugs used during pregnancy. FASS reports on medications on the European market and reflects international text book recommendations (211).

Category A—safe in pregnancy; Category B1, B2, B3—unknown risk in pregnancy or based on animal studies/Categories B (C and D)—unsafe in pregnancy; Category C—possible harmful effects on the human fetus or neonate without causing malformations. The "probably safe" group include FASS and Australian categories A, B1, and B2 and FDA categories A and B; the "potentially risky" group include FASS and Australian categories B3, C, and D, Australian category X, and FDA categories C, D, and X.

preeclampsia is emerging from a number of pre-clinical studies, which show the ability of MSCs and their associated EVs to abrogate symptoms and features of preeclampsia (reviewed in (70). Their relevance specifically to EOPE and LOPE needs to be elucidated further.

THERAPEUTIC STRATEGIES FOR TARGETING INNATE IMMUNE SYSTEM ABERRANT MECHANISMS AS POTENTIAL TREATMENTS FOR PREECLAMPSIA

Finding potential novel treatments for preeclampsia is an area of unmet clinical need and is inherently challenging. Significant knowledge gaps exist surrounding the safety, effectiveness and long-term effects of drugs for the use in pregnancy (180). Clinical trials investigating therapeutics that could be potentially repurposed for preeclampsia often have pregnancy as an exclusion criterion because of possible teratogenic risks or other harmful effects to the fetus (**Table 1**). Consequently, phase 2 or 3 trial data in pregnancy are generally lacking, making it difficult to inform novel treatment strategies. Physiological changes occur in nearly all organs during pregnancy and the pharmacokinetics and pharmacodynamics of drugs are often significantly altered, although the specific changes are mostly undetermined (212). New micro-physiological systems technology such as "Organ on a chip" models may in the future be used to help fill these gaps in knowledge (213).

Dysregulation of TLRs and detection of host-derived DAMPs contribute to the pathogenesis of preeclampsia, as described above (214). Novel TLR antagonists, especially inhibitors of TLR4 and TLR9, have potential as exciting new therapeutic agents for inflammatory disorders. The anti-inflammatory properties of TLR antagonists have been explored in numerous clinical trials for diseases such as systemic lupus erythematosus, infection-associated sepsis and vascular disorders such as hypertension (194), yet it is unknown if these agents are safe to use in pregnancy. This is a research area that therefore warrants further investigation perhaps in pre-clinical models of preeclampsia. Many of the aforementioned immune cells have similar unexplored potential and are presented in **Table 1**. The understanding of the role of the innate immune system in the multifactorial pathogenesis of preeclampsia has been significantly advanced. This progress makes novel therapeutic strategies for targeting aberrant mechanisms within the innate immune system possible as potential treatments for preeclampsia. To support this advancement, greater research capacity and robust and safe clinical trials with pregnant women are needed, with particular focus on delineating differences in EOPE and LOPE management. Anti-inflammatory and immunomodulatory drugs used for other diseases may not be appropriate and safe to use in preeclampsia. It is important to rule out drugs that are not suitable for repurposing in order to streamline future research strategies to focus on more viable alternatives.

DISCUSSION

There is a plethora of evidence supporting the role of the maternal innate immune system in the pathogenesis of preeclampsia. Mechanisms of irregular signaling and function of the innate immune cells could be explored as potential biomarkers or therapeutic targets in preeclampsia. Moreover, these cells appear to play different roles in the two phenotypes of preeclampsia, EOPE and LOPE, which could lead to better risk stratification and personalized management of preeclampsia. Developing reliable predictive and diagnostic biomarkers especially for LOPE has been challenging given that preeclampsia is a multifactorial disease with a poorly understood pathogenesis (215). As depicted in this review, there are a number of different cell types, both from the innate immune system and other supportive systems such as MSCs and STBs, which if exhibiting irregular signaling, can lead to the development of EOPE or LOPE, or both (**Figure 2**).

While in some cases quantifying a particular cell types could be utilized as a biomarker of the disease, such as the number of innate B1 cells or NK cells (both pNK and dNK), the mechanisms involved are often diverse and therefore a panel of biomarkers would be necessary to accurately predict or diagnose preeclampsia. Given that the two phenotypes of preeclampsia are often not considered and distinguished in research, it is encouraging that in terms of the innate immune system, there is important evidence emerging regarding the influence of the innate immune system in both EOPE and LOPE. For example, both dNK and pNK cells, as well as TLR4, are likely increased in EOPE, whereas in LOPE there does not appear to be a difference in these factors compared to healthy pregnancy. Another frequently observed difference between EOPE and LOPE is the proliferation of neutrophils and neutrophil associated processes, with increases in NLR, elastase and NETs being much higher in EOPE than in LOPE. Macrophages and innate B1 cells, on the other hand, do not seem to be dysregulated differently between EOPE and LOPE.

Despite the emergence of novel research highlighting the differences in the behavior of certain innate immune cells in terms of the pathogenesis of EOPE and LOPE, it is important to acknowledge that given the complexity of this condition, there are often inter-personal variations in both the mechanisms and symptoms of the disease. Consequently, these factors impose further difficulties in monitoring, diagnosis, and treatment of preeclampsia. In light of this, it is not surprising that there is a lack of effective treatment strategies for this devastating pregnancy condition. A holistic approach to disease monitoring is necessary to identify women at high risk of developing preeclampsia in conjunction with determining a panel of biomarkers representative of the multifactorial nature and different phenotypes of this disease.

Our evaluation of the existing literature describing interactions between maternal innate immune cells and cells of placental/uterine origin identified a number of limitations in the field. As the heterogeneous nature of preeclampsia has only been recently classified, there is a delay in current research, with a limited number of studies fully examining the interplay among innate immune cells and components of the placenta in the context of both EOPE and LOPE. Certain cell types, nevertheless, have been well-characterized for these two phenotypes of preeclampsia. Evidence is lacking, however, for other cell types of the innate immune system regarding their involvement in the pathogenesis of preeclampsia regardless of the phenotype. These include eosinophils, basophils, mast cells, DCs, and Langerhans cells. It is possible that some of these do not play an important role in the development of preeclampsia. However, given the key roles of mast cells and DCs in pregnancy (**Figure 1**) and some evidence of their roles in the placental bed in preeclampsia, albeit with conflicting results (40), it is likely that these cells could influence preeclampsia monitoring and treatment in the future. A portion of the reviewed literature did examine the various cell types discussed above, however, evidence was provided regarding their behavior in cases of mild and severe preeclampsia, rather than EOPE and LOPE. As a consequence, while there is currently some literature reporting on the behavior of innate immune cell types in preeclampsia, more substantial evidence is required to accurately distinguish immune cell behaviors in both phenotypes of the condition.

Carrying out research with vulnerable groups such as pregnant women is inherently challenging and results in certain limitations. Adherence to stringent ethical considerations and difficulty in obtaining early placental tissue reduces the ability of an investigation to fully elucidate the roles that the immune cells may play in the pathogenesis of preeclampsia. Recent developments in a number of microfluidics or 3D multicellular platforms may greatly increase our understanding of the cellular and molecular mechanisms of the innate immune system associated with inadequate remodeling of spiral uterine arteries or placental development/growth relevant to preeclampsia. The DAX-1™ chip manufactured by AIM Biotechnology has been demonstrated to successfully and accurately recapitulate human tumor immune microenvironments (216). Utilizing this microfluidics platform, investigators were able to examine cell type dependent interactions and provide a novel insight into the tumor immune responses (216). Utilization of these or similar platforms might be able to reproduce the multicellular autocrine and paracrine conditions of preeclampsia, and the behavior of innate immune cells within the microenvironment could be further studied. Thus, researchers can circumnavigate the hurdles of collecting early pregnancy placental tissue while still producing accurate and relevant data. Replicating an EOPE or LOPE environment will be challenging given the distinct and overlapping features of these two phenotypes of preeclampsia. Nevertheless, additional benefits of the microfluidics platforms include the ability to track molecular changes in real-time and the potential to test emerging drug treatments.

CONCLUSION

Components of the innate immune system are fully or partially inactivated, or experience a tolerogenic shift in

their immunological function throughout gestation. This, in conjunction with the ability of certain placental cells to modulate the immune system, confers a level of protection to the developing fetus against detrimental immunological responses. This delicate balance is disrupted in preeclampsia, leading to the inappropriate over-activation of these immune cells, causing disruption of appropriate placentation and contributing to the development of this hypertensive condition with end-organ damage. Although the dysfunction of these cells is observed in LOPE, the imbalance appears to be most pronounced in EOPE. While existing literature provides some evidence regarding the roles of the innate immune cells, including NK cells and neutrophils in EOPE and LOPE, further investigation specifically in the context of both phenotypes of preeclampsia, is required to address knowledge gaps. This could lead to the identification of specific disease mechanisms, which could be explored as new diagnostic biomarkers or treatment targets, hence improving the management of preeclampsia and identifying potential emerging treatments for both EOPE and LOPE.

AUTHOR CONTRIBUTIONS

IA, SS, and DP carried out literature search and created a draft of the manuscript. LM, VG, and TS conceptualized the topic, supervised, and revised the draft. All authors approved the manuscript.

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Differential Response of Gestational Tissues to TLR3 Viral Priming Prior to Exposure to Bacterial TLR2 and TLR2/6 Agonists

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Background: Infection/inflammation is an important causal factor in spontaneous preterm birth (sPTB). Most mechanistic studies have concentrated on the role of bacteria, with limited focus on the role of viruses in sPTB. Murine studies support a potential multi-pathogen aetiology in which a double or sequential hit of both viral and bacterial pathogens leads to a higher risk preterm labour. This study aimed to determine the effect of viral priming on bacterial induced inflammation in human *in vitro* models of ascending and haematogenous infection.

Methods: Vaginal epithelial cells, and primary amnion epithelial cells and myocytes were used to represent cell targets of ascending infection while interactions between peripheral blood mononuclear cells (PBMCs) and placental explants were used to model systemic infection. To model the effect of viral priming upon the subsequent response to bacterial stimuli, each cell type was stimulated first with a TLR3 viral agonist, and then with either a TLR2 or TLR2/6 agonist, and responses compared to those of each agonist alone. Immunoblotting was used to detect cellular NF-κB, AP-1, and IRF-3 activation. Cellular TLR3, TLR2, and TLR6 mRNA was quantified by RT-qPCR. Immunoassays were used to measure supernatant cytokine, chemokine and PGE2 concentrations.

Results: TLR3 ("viral") priming prior to TLR2/6 agonist ("bacterial") exposure augmented the pro-inflammatory, pro-labour response in VECs, AECs, myocytes and PBMCs when compared to the effects of agonists alone. In contrast, enhanced anti-inflammatory cytokine production (IL-10) was observed in placental explants. Culturing placental explants in conditioned media derived from PBMCs primed with a TLR3 agonist enhanced TLR2/6 agonist stimulated production of IL-6 and IL-8, suggesting a differential response by the placenta to systemic inflammation compared to direct infection as a result of haematogenous spread. TLR3 agonism generally caused increased mRNA expression of TLR3 and TLR2 but not TLR6.

Conclusion: This study provides human *in vitro* evidence that viral infection may increase the susceptibility of women to bacterial-induced sPTB. Improved understanding of interactions between viral and bacterial components of the maternal microbiome and host immune response may offer new therapeutic options, such as antivirals for the prevention of PTB.

Keywords: toll like receptor, viral priming, preterm labour, pregnancy, inflammation

INTRODUCTION

Preterm birth (PTB) occurs in 5–18% of pregnancies worldwide, with rates varying depending on geography, ethnicity, and lifestyle factors (1). PTB causes approximately 1 million neonatal deaths per year (1), and is the leading cause of mortality in children under the age of five (2). The associated neonatal morbidity leads to a global social and financial burden, with the estimated annual cost of PTB being $26 billion in the USA alone in 2007 (3). While many risk factors for PTB have been identified, the underlying aetiology and biological mechanisms are poorly understood. The lack of progress in the development of new therapeutic strategies to prevent PTB is partly due to the multiple aetiological factors that drive it. Between 30–35% of PTBs are medically indicated. The remaining PTBs result from spontaneous preterm labour (sPTL) and/or preterm premature rupture of membranes (PPROM) (4). Of the women who experience PPROM or sPTL, it is widely accepted that both systemic and local infection/inflammation are major causal factors, especially in early PTB (5–7).

The maternal fetal interface may come into contact with pathogens ascending from the lower reproductive tract or haematogenously spread as part of a systemic illness. Maternal illnesses such as pyelonephritis (8), appendicitis (9), and pneumonia (10), are associated with spontaneous preterm labour (sPTL), and many animal models have demonstrated that systemic delivery of pathogens will induce PTL (11, 12). In women, systemic infection by *Listeria monocytogenes* has been reported to cause sPTL in approximately a third of infected women (13). Hematogenous spread of organisms to the placenta has been postulated to explain the association between periodontal disease and PTL, with oral cavity microorganisms being isolated from amniotic fluid (AF) as a result of transplacental passage (14).

Using standard culture techniques, as few as 1% of women are found to have bacteria in their amniotic fluid at term prior to the onset of labour (15). However, microbial invasion of the amniotic cavity (MIAC) occurs in as many as 50% of women with an open cervix (16), and 32% of women with PPROM (17), placing them at significantly increased risk of preterm delivery (18, 19). The microbes commonly detected in these studies include *Ureaplasma urealyticum,* Group B *streptococcus*, *Mycoplasma hominis*, and *Gardnerella vaginalis*, and *Lactobacillus* species, which are all commonly found in the vagina. Several studies using bacterial DNA sequencing approaches have confirmed the importance of vaginal microbial composition in the risk of PPROM and PTL (20–23).

There is substantial evidence to support a role for inflammation of the fetal membranes, myometrium and cervix in the mechanisms of parturition, contributing to membrane rupture, uterine contractility and cervical ripening in both term and sPTL (24). Inflammation involves recruitment of leukocytes and the production of cytokines, chemokines, and prostaglandins. An augmented inflammatory response is seen in cases of PTL, with higher concentrations of Interleukin (IL)-6, IL-1β, and IL-8 in amnion, choriodecidua and in placenta compared to term labour (25). Furthermore, high concentrations of IL-6 in cervicovaginal fluid associates with an increased risk of preterm delivery (26). In microbial driven inflammation, these responses are initiated upon recognition of unique molecular structures found on the microorganisms by pattern recognition receptors (PRR) such as Toll-like receptors (TLRs). Once bound by their respective ligands, TLRs in the lower reproductive tract and placenta trigger downstream signalling cascades via the inflammatory transcription factors NF-κB and AP-1 (27). These transcription factors play a key role in regulating the expression of pro-labour (matrix metalloproteinases (MMPs), COX-2 and prostaglandins) and pro-inflammatory mediators (IL-1β, TNF-α, IL-8, and IL-6) (28–31). In turn, these mediators lead to cervical remodelling, membrane rupture, and uterine contractility.

Toll like receptors (TLRs) are transmembrane proteins with extracellular domains containing highly conserved leucine-rich repeat motifs. TLR1, 2, 4, 5, and 6 recognize bacterial pathogen associated molecular patterns (PAMPs), whereas TLR3, 7, 8, and 9 mediate viral recognition. Transcripts from all ten TLRs are detected in placental explants, decidua and amnion epithelial cells (AECs) (32–34), and various studies have reported on their expression in vaginal and cervical cells, as well as uterine smooth muscle cells, as reviewed by Nasu and Narahara (35). Support for the functional role of TLRs in microbial driven sPTL comes from their increased expression at the maternal-fetal interface in women with chorioamnionitis (36, 37), and the ability of TLR agonists to induce PTB in animal models (38, 39). Gram-positive bacterial products are the main ligands of the TLR2 receptor, including Group B *streptococcus* (*S. agalactiae*) and *Ureaplasma urealyticum*, which are both associated with sPTL (40). Intraperitoneal administration of Lipoteichoic acid (LTA) or Peptidoglycan (PGN) induces sPTL in the mouse (41). TLR4 recognizes lipopolysacharride (LPS) motifs found on most Gram-negative bacteria, such as *Escherichia coli* and *Mycoplasma hominis*, both of which are associated with sPTL. LPS is a potent inducer of sPTL in the mouse, an effect which is mitigated in TLR-4 mutant mice, supporting the key functional role of TLR4 (42).

In contrast to the causal link between bacterial infection and sPTL, the role of viruses is less well established. However, HIV, hepatitis B, and RSV infection during pregnancy are associated with an increased risk of PTB (43–45). Histological chorioamnionitis has been reported to be more common in preterm placentas positive for adenovirus compared to both adenovirus negative preterm placentas and adenovirus positive term placentas (46). However, the most compelling evidence for the role of viruses in inducing sPTB is the ability of viral TLR agonists to induce sPTB in animal models. A dose dependent increase in preterm delivery rates is seen in mice treated with the TLR3 agonist poly I:C, an effect which is mitigated in TLR3 knockout mice (47).

Mouse models have demonstrated a synergistic effect of bacterial and viral TLR agonists in induction of inflammation and sPTL (39, 48–50). These studies lend support to the concept of multi-pathogen induced preterm labour, and the possibility that viruses can increase susceptibility to bacterial induced preterm labour. To explore whether this concept might apply in humans, we tested the "double-hit" hypothesis in *in vitro* human cell models of ascending infection and haematogenous infection. Vaginal epithelial cells lines (VECs), primary amnion epithelial cells (AECs) and primary myocytes (ascending infection model), or peripheral blood mononuclear cells and placental explants (haematogenous model) were primed with the TLR3 agonist poly I:C prior to treatment with the TLR2 agonist heat-killed *Listeria Monocytogenes* (HKLM) or TLR2/6 agonist FSL-1 to determine their effect on pro-inflammatory and pro-labour mediators.

MATERIALS AND METHODS

Ethics Statement

Placenta and myometrial biopsies were collected in accordance with Ethical Approval from Hammersmith, Queen Charlotte's & Chelsea Hospitals Research Ethics Committee (Ref 2002/628) and Riverside Research Ethics Committee (Ref 3358), respectively. Peripheral blood collection was approved by the South East London Ethics Committee (Ref 10/H0805/54). Informed written consent was obtained from all participants prior to obtaining the samples.

Reagents

The agonists for TLR2 (heat-killed *Listeria monocytogenes*, HKLM, Cat tlr1-hklm), and TLR2/6 (FSL-1, Pam2CGDPKHPKSF, Cat tlr1-fsl) were purchased from Invivogen, (Toulouse, France), the TLR3 agonist polyinosinic-polycytidylic acid, Poly I:C, Cat P9582) was purchased from Sigma-Aldrich (Gillingham, UK). Total RNA was extracted using TRIzol™ (Invitrogen Life Technologies) or the RNeasy Micro Kit (Qiagen). All other reagents for cDNA synthesis and quantitative PCR were purchased from Sigma-Aldrich (Gillingham, UK). All primers were from Thermo-Scientific (Waltham, MA). **Table 1** shows sequences used for amplification of target genes. Antibodies to detect the phosphorylated p-65 NF-κB subunit Cat #3031 (AB_330559), phosphorylated c-Jun subunit Cat #9164 (AB_330892), phosphorylated IRF3 Cat #29047 (AB_2773013), and ß-actin Cat #A5441 (AB_476744) were purchased from Cell Signalling Technology (Beverly, MA) and Sigma-Aldrich (Gillingham, UK), respectively. The Meso Scale Discovery Immunoassay was used to detect a panel of 9 cytokines and chemokines MSD (Rockville, MD). The Prostaglandin E2 parameter assay kit (KGE004B) was purchased from R&D Systems (Minneapolis, MN).

TABLE 1 | Primers sequences for RT-QPCR.

Genes	Forward primer	Reverse primer
TLR2	TGCTGCCATTCTCATTCTTCTG	AGGTCTTGGTGTTCATTATCTTCC
TLR3	CCTGGTTTGTTAATTGGATTAACGA	TGAGGTGGAGTGTTGCAAAGG
TLR6	GCCACCATGCTGGTGTTGGCT	CGCCGAGTCTGGGTCCACTG
ß-actin	AGGCATCCTCACCCTGAAGTA	CACACGCAGCTCATTGTAGA

TABLE 2 | Concentration and duration of TLR agonist treatments.

Cell/Tissue type	TLR3 agonist Poly I:C	TLR2 agonist HKLM	TLR 2/6 agonist FSL-1
VECs	25 μg/ml for 6 h	10^5 cells/ml for 24 h	0.01 μg/ml for 24 h
AECs	25 μg/ml for 12 h	10^5 cells/ml for 24 h	0.01 μg/ml for 24 h
Myocytes	25 μg/ml for 12 h	10^5 cells/ml for 24 h	0.1 μg/ml for 24 h
PBMCs	5 μg/ml for 30 min	10^8 cells/ml for 18 h	0.1 μg/ml for 18 h
Placental explants	25 μg/ml for 24 h	10^8 cells/ml for 24 h	0.1 μg/ml for 24 h

In-vitro Cell and Tissue Explant Culture and Treatment Protocols

The vaginal epithelial cell line VK2/E6E7 was purchased from American Type Culture Collection (ATCC® CRL2616™). All samples of placenta, myometrial biopsies, and peripheral blood were collected at the time of planned pre-labour caesarean section from healthy women at term. The indications for caesarean delivery were either previous caesarean section or breech presentation.

Preparation of Vaginal Epithelial Cells (VECs)

Cells from ATCC were stored in liquid nitrogen until the time of cell culture. Cells were thawed for 1 min at 37°C in a water bath and immediately transferred into pre-warmed Dulbecco's Modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS). The suspension was centrifuged at 125 × *g* for 5 min to pellet the cells and resuspended in keratinocyte serum free media (KSFM) supplemented with bovine pituitary extract (BPE), epidermal growth factor (EGF) and calcium chloride ($CaCl_2$). Cells were grown in T25 culture flasks at 37°C, 5% CO_2 until confluent prior to treatment with viral and bacterial agonists (**Table 2**). Cells were grown to a maximum of passage seven and all experiments were repeated a minimum of three times.

Preparation of Amnion Epithelial Cells (AECs)

AECs were processed as described previously (51). Placenta was collected within 1 h of delivery and processed immediately. The amnion layer was separated from the choriodecidua, cut

into strips and washed in phosphate buffer saline (PBS) then incubated in pre-warmed 0.5 mM of EDTA-PBS for 15 min at room temperature. The strips were then rinsed in PBS and further incubated for 50 min at 37°C in 60 mls of pre-warmed dispase (Gibco) (2 g/L in PBS) to digest the intracellular matrix. The strips were shaken vigorously in pre-warmed DMEM containing 10% FCS, 2 mM/L L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin for 4 min to isolate the AECs from the intracellular matrix. Tissue strips were then removed, and the solution was centrifuged at 2,000 rpm for 10 min at room temperature to pellet the cells. Pelleted cells were resuspended in pre-warmed medium and grown in plates at 5% CO_2 until confluent and ready for TLR agonist treatment. Once cells reached 80% confluence, they were serum starved in DMEM containing 1% FCS, 2 M/L L-glutamine, 100 U/ml penicillin and 100 μg/ml in preparation for TLR agonist treatment. A minimum of three and maximum of six biological replicates were used for each experiment.

Preparation of Myocytes

Myometrial biopsies were taken from the upper margin of the lower segment incision and prepared as previously described (52). The biopsies were mechanically dissected and myocytes were isolated by incubating the dissected tissue in 10 mg of collagenase 1 A, 10 g collagenase X and 200 mg of bovine serum albumin (BSA) in 30 mls of 1:1 DMEM and F-12 HAM for 45 min at 37°C. DMEM containing 10% FCS, was added to inactivate the enzymes and the suspension was filtered through a 100 μm cell strainer then centrifuged at 3,000 rpm for 5 min. Pelleted cells were resuspended in (DMEM) containing 10% FCS, 2 mM/L L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin and cultured in T25 culture flasks until confluent. Cells were used for experiments up until passage 6. Prior to TLR agonist treatment, cells were serum starved in DMEM containing 1% FCS, 2 mM/L L-glutamine, 100 U/ml penicillin and 100 μg/ml. Three to six biological replicates were used for each experiment.

Preparation of Peripheral Blood Mononuclear Cells (PBMCs)

PBMCs were isolated as described previously (53). Blood was processed within 30 mis of collection and was diluted 1:1 ratio with PBS and layered on top of Ficoll-Paque^{TM} PLUS (GE Healthcare, Uppsala, Sweden) and centrifuged at 400 × *g* for 40 min at room temperature. The cloudy halo formed in the middle containing PBMC was extracted and washed twice with PBS for 10 min at 400 × *g*. The pelleted cells were resuspended in complete RPMI 1640 culture medium (Invitrogen Life Technologies, Grand Island, NY) containing 10% FCS, 2 mM/L L-glutamine, 100 U/ml penicillin and 100 μg/ml of streptomycin and cultured in 24 well plates at a concentration of 1.7×10^5 /ml.

Preparation of Placental Explants

Placentas were collected and processed immediately after delivery for explant studies and processed as previously described (54). Several 2 cm^3 tissue samples were cut, washed in PBS to remove excess blood, and further dissected to samples of 20–30 mg weight. Samples were incubated in 24 well plates containing RPMI 1640 culture medium supplemented with 10% FCS, 2 mM/L L-glutamine, 100 U/ml Penicillin and 100 μg/ml Streptomycin in 8% oxygen and 5% CO_2. Medium was changed every 48 h and explants were serum starved on day 4 prior to TLR agonist treatment on day 5 to allow for syncytiotrophoblast regeneration.

Treatment Protocols

a) To examine the activation of NF-κB (p-p65), AP-1 (p-c-Jun) and IRF3 (p-IRF3) proteins, VECs, AECs, myocytes, PBMCs and placental explants were treated with poly I:C over a timecourse of 30 min, 1, 4, 6, 12, and 24 h.
b) To determine whether TLR3 receptor priming led to an augmented pro-inflammatory and pro-labour response in cells then exposed to TLR2 or TLR2/6 agonist, cells were treated with either vehicle control or poly I:C (TLR3 agonist). Following a wash out, cells were then treated with either vehicle control, HKLM (TLR2 agonist) or FSL (TLR 2/6 agonist). Fold changes of cytokine, chemokine and prostaglandin E2 (PGE2) were compared to baseline concentrations, and comparisons between cells treated with vehicle alone, poly I:C alone, HKLM or FSL-1 alone, and priming with poly I:C prior to HKLM or FSL-1 were made.
c) To determine the effect of poly I:C on TLR2, 3, and 6 mRNA expression level, each cell type and explant were treated with poly I:C for up to 24 h. mRNA expression was examined at 0, 30 min, 1 h, 4 h, 6 h,12 h, and 24 h.

Western Blot Analysis

NF-κB, AP-1, and IRF-3 activation were analysed in all cell types; VEC, AECs, myocytes, PBMCs and placental explants after poly I:C stimulation by western blotting. Cells and tissues were lysed with whole cell lysis buffer (Cell Signalling) containing 5 μl/mL of protease inhibitor cocktail (Sigma Aldich), 5 μl/mL of phosphatase inhibitor cocktail (Sigma Aldich) and 1 mM of phenylmethylsulfonyl fluoride (PMSF). Lysed cells were incubated on ice for 5 min and centrifuged for 10 min at 14,000 × *g* at 4°C. The supernatant was collected, and protein concentration was determined using Bradford method with BSA as the standard. SDS Page gel electrophoresis was run and the protein was transferred onto polyvinylidene difluoride (PVDF) membranes (300 mA, 1.5 h, 4°C) and blocked in 5% milk in 1× tris-buffered saline with tween (TBS-T) for 1 h at room temperature. The PVDF membrane was incubated either with phosphorylated p65 rabbit polyclonal antibody (Cell Signalling, #3031) for NF-κB, phosphorylated c-Jun rabbit polyclonal antibody (Cell Signalling, #9164) for AP-1, phosphorylated IRF3 rabbit monoclonal antibody (Cell Signalling, #29047) or anti ß-actin mouse monoclonal antibody (Sigma Aldrich, A5441) overnight at 4°C. The blots were washed 3 times with 1× TBS-T before incubated with diluted horseradish-peroxidase-conjugated secondary antibodies, 1:2,000 p-p65, p-c-Jun and p-IRF3, Cell Signalling, and 1:25,000 for ß-actin, Santa Cruz]) for 1 h at room temperature. Blots were then washed 3 times with 1x TBS-T and proteins were detected using Clarity ECL Western

(BioRad) or Luminata Forte (Millipore) blotting substrate of horseradish peroxidase.

mRNA Expression of Toll Like Receptors 2, 3, and 6

Total RNA was extracted from VECs, AECs, myocytes and placental explants, using TRIzol™ (Invitrogen, CA, USA), whereas PBMCs RNA was extracted using the RNeasy Micro Kit (Qiagen) according to the manufacturer's instructions. Total RNA was synthesised into cDNA as previously described (51). Relative quantification of *TLR2, 3,* and *6* gene expression was performed using real- time PCR performed on an Applied Biosystems StepOne Real-Time PCR system using SYBR® Green Master Mix (Applied Biosystems, Foster City, CA). Primers sequences were examined with BLAST software against the National Center for Biotechnology Information database and are summarised in **Table 1**. Water non-template controls were used, and correctly sized amplified products were confirmed using gel electrophoresis. The comparative C_T method ($\Delta\Delta C_T$) was used for relative quantification, and results were calculated

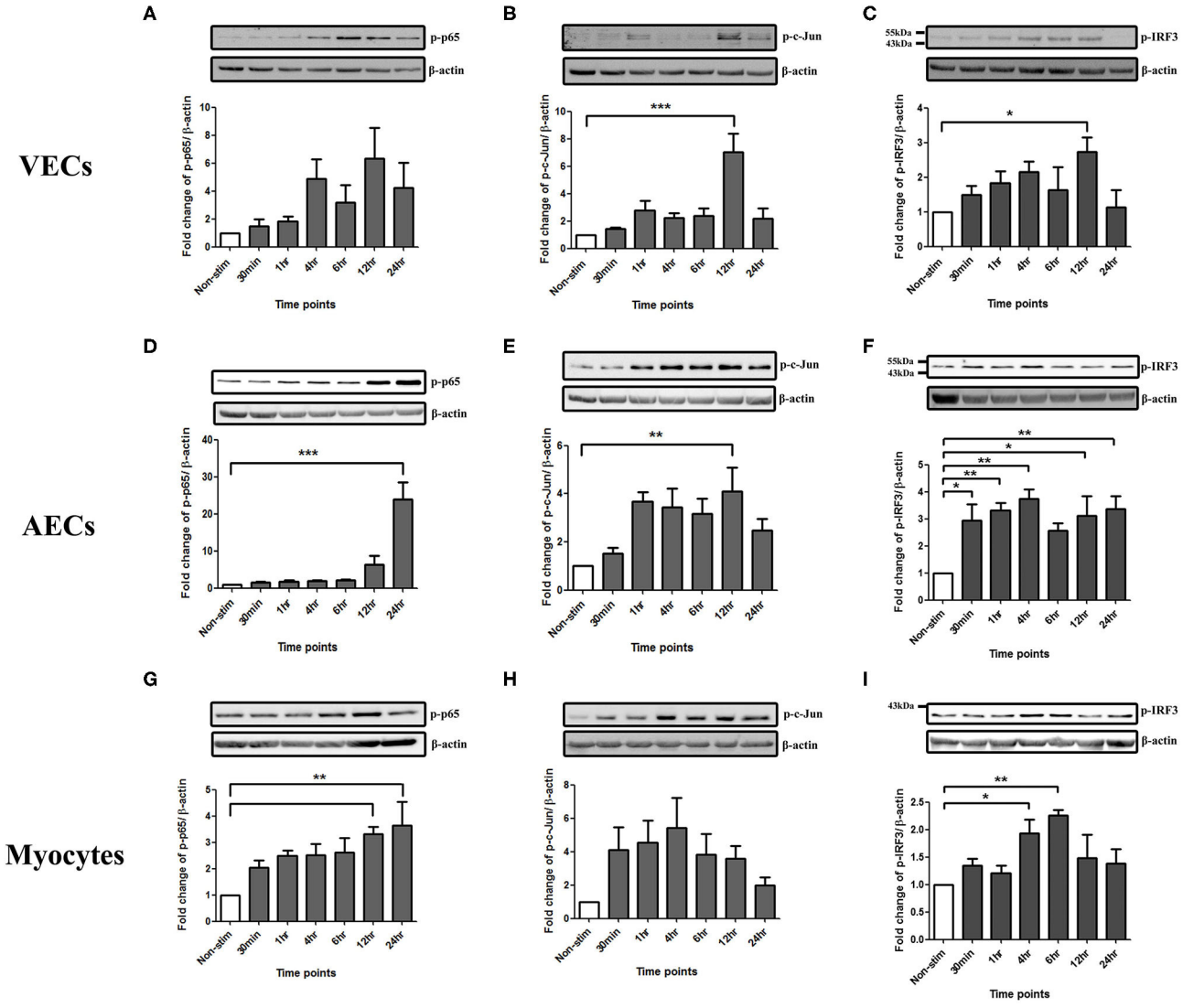

FIGURE 1 | Activation of NF-κB, AP-1, and IRF in VECs, AECs, and myocytes. VECs, AECs, and myocytes were treated with poly I:C for 30 min, 1, 4, 6, 12, and 24 h and phospho-p-65, phospho-c-Jun and phospho-IRF3 were examined. In VECs, no statistical significance was observed with p-p65 expression **(A)**, however, p-c-Jun **(B)**, and p-IRF3 **(C)** increased significantly at 12 h ($p < 0.001$ and $p < 0.05$, respectively) with 25 μg/ml. An increase in p-p65 ($p < 0.001$) **(D)** and p-c-Jun ($p < 0.01$) **(E)** at 24 and 12 h, respectively, was observed in AECs. 25 μg/ml. An earlier significant increase of p-IRF3 activation also was seen at 30 min ($p < 0.05$), 1, 4 ($p < 0.01$), 12 ($p < 0.05$), and 24 h ($p < 0.01$) **(F)**. In myocytes, p-p65 increased significantly at 12 and 24 h ($p < 0.01$) **(G)** whereas a non-significant increase was observed in p-c-Jun at 30 min, 1, 2, 6, and 12 h **(H)** with 10 μg/ml. Significant increase was also observed in p-IRF3 at 4 ($p < 0.05$) and 6 h ($p < 0.01$) **(I)** with 25 μg/ml. For statistical analysis, One-way ANOVA was used for normally distributed data and Kruskal-Wallis was used for data not normally distributed with Dunnett's multiple comparison test $n = 3$–8. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

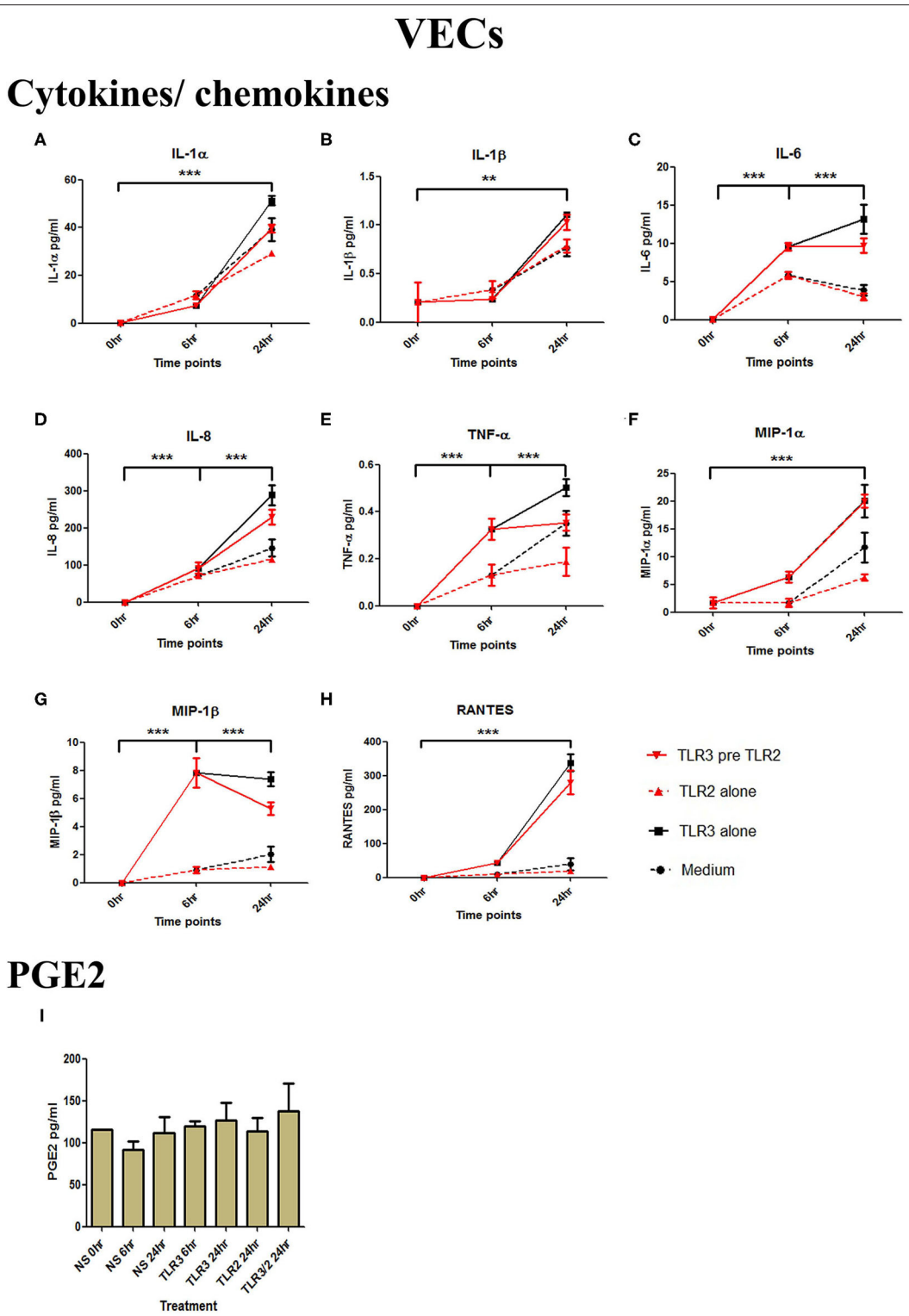

FIGURE 2 | The effect of TLR3 viral priming on TLR2 agonist induced pro-inflammatory and pro-labour mediators in vaginal epithelial cells. Monolayer VECs were primed with 25 μg/ml of poly I:C for 6 h prior to 10^5 cells/ml of HKLM for 24 h and the supernatant was collected to quantify the pro-inflammatory and pro-labour mediators. Poly I:C stimulation alone significantly increased the production of all cytokines and chemokines. TLR3 priming prior to HKLM stimulation showed no augmentation in IL-1α **(A)**, IL-1ß **(B)**, IL-6 **(C)**, IL-8 **(D)**, TNF-α **(E)**, MIP-1α **(F)**, MIP-1ß **(G)**, RANTES **(H)**, and PGE2 **(I)**. For statistical analysis Two-way ANOVA with Bonferroni's multiple comparison test was used. * = effect of TLR3 compared to 0 h. ** = $p < 0.01$, *** = $p < 0.001$.

relative to baseline gene expression and expression of the ß-actin housekeeping gene.

Quantification of Cytokines, Chemokines and PGE2

Supernatant from cultured cells and explants was used to analyse IL-6, IL-8, IL-10, IL-1α, IL-1ß, TNF-α, IL-4, MIP-1α, MIP-1ß, and RANTES production. Supernatant was collected at the following timepoints; baseline, following TLR3 treatment, and following TLR2 or TLR2/6 treatment. Supernatant was also analysed from single agonist treatment and from vehicle control samples. Quantification of the cytokines and chemokines were performed using the Meso Scale Discovery platform. U-Plex kits were used according to manufacturer's instructions, MSD (Meso Scale Diagnostics, Rockville, Maryland). Plates were read by the QuickPlex SQ 120 (Meso Scale Diagnostics).

Supernatant was also used to analyse PGE2 at the same timepoints. The PGE2 ELISA kit was used according to the manufacturer's guidelines (KGE004B, R&D Systems). Briefly supernatant was diluted 1:3 with the assay diluent and plated alongside the calibration curve standards (Range of 39–2,500 pg/ml). The optical density of each well and readings from the absorbance value of 450 nm was subtracted from 540 and 570 nm in order to obtain PGE2 concentrations.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism 8 (GraphPad Software, San Diego, CA). The statistical tests were selected depending on the number of groups analysed and the distribution of the data. One-way ANOVA was used when comparing more than two groups of one variable if data was normally distributed, or by using the Kruskal-Wallis for non-parametric distributions. A Two-way ANOVA was used when comparing data with more than one variable for both data found to be normally distributed, and for experiments in which experimental replicates were <5. The *Bonferroni* or *Dunnett's post hoc* tests were used as appropriate. A $p < 0.05$ was considered to indicate statistical significance.

RESULTS

TLR3 Induced Activation of NF-κB, AP-1, and IRF in VECs, AECs, and Myocytes

To study the effect of poly I:C on the activation of the transcription factors NF-κB, AP-1 and p-IRF3 in VECs, AECs and myocytes cells were stimulated with poly I:C for a total of 24 h, and cells harvested at appropriate intervals to determine phosphorylated transcription factor levels at 30 min, 1 h, 4 h, 6 h, 12 h, and 24 h. A significant increase in p-c-Jun and p-IRF-3 was seen in VECs by 12 h ($p < 0.001$ and $p < 0.05$, respectively) (**Figures 1B,C**), and a trend of increased p-p65 was seen, although this was not significant with 25 μg/ml (**Figure 1A**). All transcription factors were significantly activated in AECs with the earliest activation seen with p-IRF3 ($p < 0.01$) (**Figures 1D–F**) with 25 μg/ml of poly I:C. A significant increase in p-p65 by 12 h was seen myocytes treated with 10 μg/ml poly I:C, which was sustained until 24 h ($p < 0.01$) (**Figure 1G**), no significant change was seen in p-c-jun (**Figure 1H**), and early activation of p-IRF3 was seen at 4 h and 6 h with 25 μg/ml poly I:C ($p < 0.05$ and $p < 0.01$) (**Figure 1I**).

Model of Ascending Infection: TLR3 Agonist Viral Priming Augments TLR2/6 Agonist Induced Pro-inflammatory and Pro-labour Mediators in VECs, AECs, and Myocytes

In a model of ascending infection, VECs, AECs, and myocytes were treated with bacterial TLR agonists. Heat-killed *Listeria Monocytogenes* (HKLM) (10^5 cells/ml for 1 h) was used as a pure TLR2 agonist, and FSL-1 (0.01 μg/ml in VECs and AECs for 1 h or 0.01 μg/ml in myocytes for 4 h) was used as a TLR2/TLR6 agonist (**Supplementary Figure 1**, **Figure 2**). HKLM led to a significant increase in both phospho-p65 and p-c-Jun in all cell types. FSL-1 led to a significant increase in phospho-p65 and p-c-Jun in VECs and AECs, but only p-c-Jun in myocytes.

TLR3 Priming Prior to TLR2 Agonist Treatment in Model of Ascending Infection

VECs were treated with 25 μg/ml of poly I:C or vehicle for 6 h prior to treatment with 10^5 cells/ml of HKLM or vehicle. No significant changes in IL-1α or IL-1β were seen with any treatment condition (**Figures 3A,B**). Treatment with the TLR3 agonist alone significantly increased the production of pro-inflammatory cytokines IL-1α ($p < 0.001$), IL-1β ($p < 0.01$), IL-6, IL-8, TNF-α ($p < 0.001$), and the chemokines MIP-1α, MIP-1β, and RANTES ($p < 0.001$) (**Figures 2A–H**). HKLM treatment alone or with poly I:C priming did not change cytokine or chemokine concentrations. Similarly, there was no increase in PGE2 production in VECs following any of the treatment conditions (**Figure 2I**).

AECs were treated with 25 μg/ml of poly I:C or vehicle for 12 h prior to treatment with 10^5 cells/ml of HKLM or vehicle. Poly I:C led to a significant increase in IL-6 ($p < 0.001$), IL-8 ($p < 0.01$), TNF-α, MIP-1α, MIP-1β, and RANTES ($p < 0.01$), and IL-4 ($p < 0.05$) (**Figures 3C–I**). As with VECs, there was no increase following incubation with 10^5 cells/ml of HKLM or following sequential incubation with poly I:C then by HKLM. Furthermore, no increased production of PGE2 was seen with any treatment combination (**Figure 3J**).

Although poly I:C significantly increased the production of IL-1β, IL-6, IL-8, TNF-α, and RANTES ($p < 0.001$) in myocytes, there was no increase in cells treated with 10^8 cells/ml of HKLM alone, and no augmented production with combined TLR3/TLR2 treatment (**Figures 4A–E**). No increase in PGE2 occurred with either treatment combination in myocytes (**Figure 4F**).

TLR3 Priming Prior to TLR 2/6 Agonist Treatment in a Model of Ascending Infection

VECs were treated with 25 μg/ml of poly I:C or vehicle for 6 h prior to treatment with 0.01 μg/ml of FSL-1 or vehicle. The TLR3 agonist poly I:C alone significantly increased the production of IL-1α, IL-1β, IL-6, IL-8, TNF-α, and the chemokines MIP-1α, MIP-1β, and RANTES ($p < 0.001$) (**Figures 5A–H**). FSL-1 alone

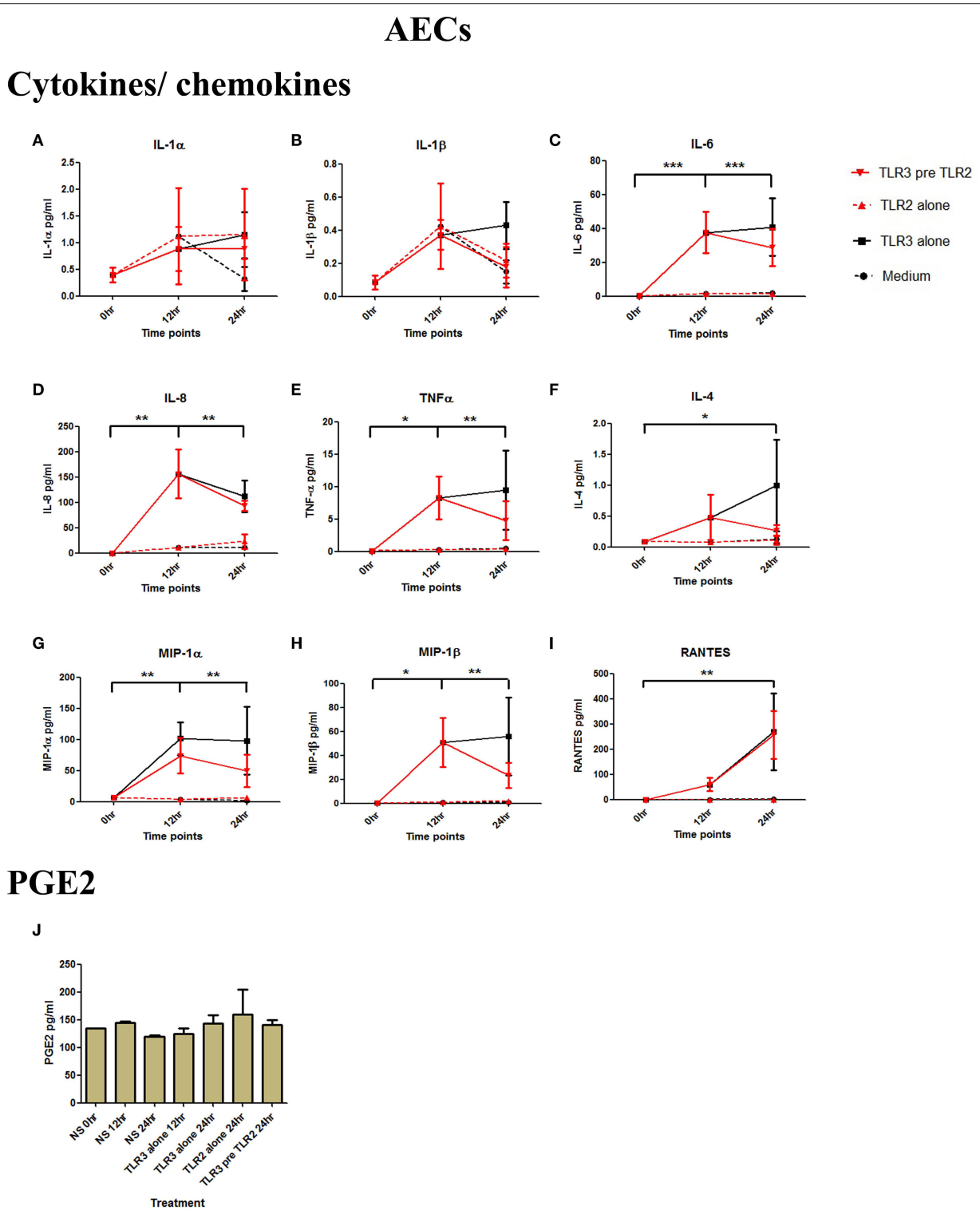

FIGURE 3 | The effect of TLR3 viral priming on TLR2 agonist induced pro-inflammatory and pro-labour mediators in amnion epithelial cells. Monolayer cells were primed with 25 μg/ml of poly I:C for 12 h prior to 10^5 cells/ml of HKLM for 24 h. Supernatant was collected to quantify the pro-inflammatory and pro-labour mediators. Poly I:C stimulation alone significantly increased the production of IL-6, IL-8, TNF-α, IL-4, MIP-1α, MIP-1ß, and RANTES. TLR3 priming prior to HKLM stimulation showed no augmentation in IL-1α **(A)**, IL-1ß **(B)**, IL-6 **(C)**, IL-8 **(D)**, TNF-α **(E)**, IL-4 **(F)**, MIP-1α **(G)**, MIP-1ß **(H)**, RANTES **(I)**, and PGE2 **(J)**. For statistical analysis Two-way ANOVA with Bonferroni's multiple comparison test was used. * = effect of TLR3 compared to 0 h. For statistical analysis Two-way ANOVA with Bonferroni's multiple comparison test was used. * = effect of TLR3 compared to 0 h. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

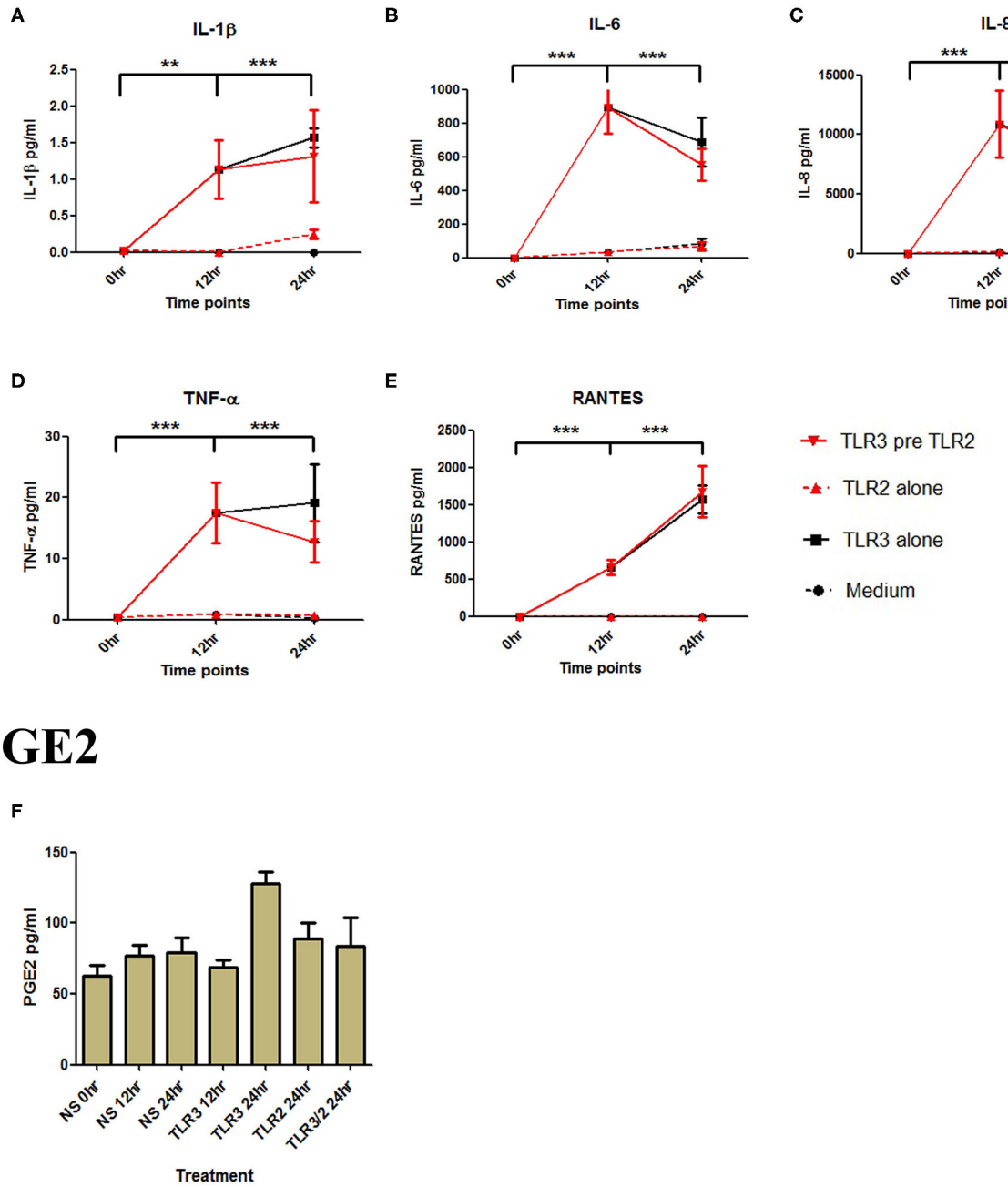

FIGURE 4 | The effect of TLR3 viral priming on TLR2 agonist induced pro-inflammatory and pro-labour mediators in myocytes. Monolayer cells were primed with 25 μg/ml of poly I:C for 12 h prior to 10^5 cells/ml of HKLM for 24 h. Supernatant was collected to quantify the pro-inflammatory and pro-labour mediators. Poly I:C stimulation alone significantly increased the production of IL-1ß, IL-6, IL-8, TNF-α, and RANTES. TLR3 priming prior to HKLM stimulation showed no augmentation in IL-1ß **(A)**, IL-6 **(B)**, IL-8 **(C)**, TNF-α **(D)**, RANTES **(E)**, and PGE2 **(F)**. For statistical analysis Two-way ANOVA with Bonferroni's multiple comparison test was used. * = effect of TLR3 compared to 0 h. ** = $p < 0.01$, *** = $p < 0.001$.

also lead to a significant increase in IL-6, IL-8, TNF-α, MIP-1α, and MIP1-β. However, a further augmented response was seen in cells pre-treated with poly I:C in the production of in IL-6 ($p < 0.001$), IL-8, TNF-α ($p < 0.01$), MIP-1α, and MIP-1β ($p < 0.001$) (**Figures 5C–G**). Additionally, an augmented response was seen in the production of PGE2 ($p < 0.001$) (**Figure 5I**).

Poly I:C (25 μg/ml, 12 h) treatment increased the production of IL-6 ($p < 0.001$), TNF-α ($p < 0.01$), MIP-1α, and RANTES ($p < 0.01$), and IL-4 ($p < 0.05$) in AECs, and FSL-1 (0.01 μg/ml) treatment alone also increased concentrations of IL-1β and IL-8 ($p < 0.05$) (**Figure 6**). However, where cells were primed with poly I:C prior to FSL-1, there was an increase in the concentrations of IL-6 ($p < 0.001$), MIP-1α, and MIP-1β ($p < 0.01$) and RANTES ($p < 0.001$). A synergistic increase in of PGE2 ($p < 0.001$), with TLR3/TLR2/6 treatment was also seen in AECs (**Figure 6J**). Similarly, in myocytes, a synergistic increase in IL-1α ($p < 0.05$), IL-1β, IL-6 ($p < 0.001$), IL-8 ($p < 0.01$), TNF-α, RANTES ($p < 0.001$) was seen with poly I:C priming prior to treatment with FSL-1 (0.1 μg/ml) (**Figures 7A–G**).

TLR3-Induced Activation of NF-κB, AP-1, and IRF in PBMCs and Placenta Explants

In placental explants, Poly I:C led to a significant increase in p-p65 at 4 h and p-IRF3 at 30 min ($p < 0.01$ and $p < 0.001$), with no significant increase in p-c-Jun observed (**Figures 8A–C**, **Supplementary Figure 3**). in PBMCs, A significant increase in p-p65 was seen with poly I:C incubation at 30 min and 1 h ($p < 0.05$) (**Figure 8D**). However, p-c-Jun or p-IRF3 levels were too low for detection in PBMCs.

A Model of Haematogenous Spread of Infection: Placental Explants Show an Enhanced Anti-inflammatory Response With TLR3 Agonist Viral Priming Prior to TLR2 and TLR2/6 Agonist Treatment

To mimic the effect of haematogenous infection, PBMCs and placental explants were incubated with the same TLR agonists used in the model of ascending infection above. HKLM led to an increase in NF-κB activation in PBMCs and p-c-Jun in placental explants, however no significant increase was seen with FSL-1 treatment (**Supplementary Figures 1, 3**).

In PBMCs, HKLM significantly increased the production of IL-1α, IL-1β, IL-6, IL-8, TNF-α, and the chemokines MIP-1α, MIP-1β and RANTES, but poly I:C only increased the production of RANTES (**Figures 7A–H**). However, priming with poly I:C prior to HKLM treatment led to a synergistic increase in IL-1α ($p < 0.001$), IL-1β ($p < 0.01$), IL-6 ($p < 0.001$), IL-8 ($p < 0.05$), MIP-1α ($p < 0.01$), and MIP-1β ($p < 0.05$) and PGE2 ($p < 0.001$) (**Figures 9A–I**). FSL-1 treatment alone did not change cytokine concentrations in PBMCs apart from RANTES, which increased in response to both FSL-1 and poly I:C treatment alone ($p < 0.05$ and $p < 0.01$, respectively; **Figure 10**). However, a synergistic effect was seen on pro-inflammatory and pro-labour mediator production with poly I:C priming prior FSL-1 treatment in PBMCs including IL-1β, IL-6 ($p < 0.05$), IL-8, MIP-1α, and MIP-1β and PGE2, but not IL-1α (**Figures 10A–I**).

Poly I:C treatment of placental explants led to a significant increase in the production of IL-1α, IL-1β, IL-6, IL-8, TNF-α, IL-4, and the chemokines MIP-1α, MIP-1β ($p < 0.001$), and RANTES, and PGE2 ($p < 0.01$) (**Figures 11A–K**, **12A–K**). HKLM incubation alone led to the significant increase of all inflammatory mediators except for RANTES (**Figure 11I**). However, with priming, an augmented response was only seen in the production of IL-10 ($p < 0.01$), MIP-1α ($p < 0.01$), and RANTES ($p < 0.001$). Similarly, although FSL-1 incubation alone increased the production of IL-1α, IL-1β, IL-6, IL-8, TNF-α, IL-4, and the chemokines MIP-1α, MIP-1β, and PGE2, priming did not lead to a further augmentation in pro-inflammatory/pro-labour mediators, but instead led to a significant increase in IL-10 ($p < 0.001$) and RANTES ($p < 0.01$) (**Figure 12**).

PBMC-Derived Inflammatory Mediators From TLR3 Primed and TLR2/6 Treated Cells Induce an Augmented Pro-inflammatory Response in Placental Explants

The anti-inflammatory response of placental explants to TLR2/6 with TLR3 priming led us to test the immunomodulatory effect of PBMC conditioned media on the placental explants as a model systemic inflammation. Cytokine concentrations were measured from explant cultures treated with the TLR agonists (pink histograms) or PBMC derived supernatant (light blue histograms), or from placental explants cultured in PBMCs conditioned media (dark blue histograms) (**Figure 13**). Incubation of placental explants with conditioned medium from PBMCs treated with poly I:C and HKLM led to a synergistic increase in IL-6 ($p < 0.05$) and IL-8 ($p < 0.05$), but not IL-1β (**Figures 13A–C**). However, no synergistic increase was seen in cytokine production from placental explants grown in conditioned medium from PBMCs treated with poly I:C and FSL-1 (**Figures 13D–F**).

The TLR3 Agonist Poly I:C Increases the Expression of Both TLR3 and TLR2 Receptors, but Not of the TLR6 Receptor

TLR3 and TLR2 mRNA expression were significantly increased after poly I:C stimulation in VECs ($p < 0.05$ at 6 h, $p < 0.01$ at 12 h), AECs ($p < 0.001$ at 12 h), and myocytes (TLR3; $p < 0.01$ at 12 h and TLR2; $p < 0.001$ at 12 h) (**Figures 14A–I**). In PBMC, only TLR3 mRNA ($p < 0.01$ at 6 h) was significantly increased, although a non-significant increase was seen in TLR2 expression at 12 h (**Figures 14J,K**). No differences were seen in TLR6 expression in any of the cell types (**Figures 14C,F,I,L**). No changes in TLR3 or 6 expression were seen in placental explants, but there was a transient increase in TLR2 expression at 4 and 6 h ($p < 0.05$), which was lost by 12 h (**Figures 14M–O**).

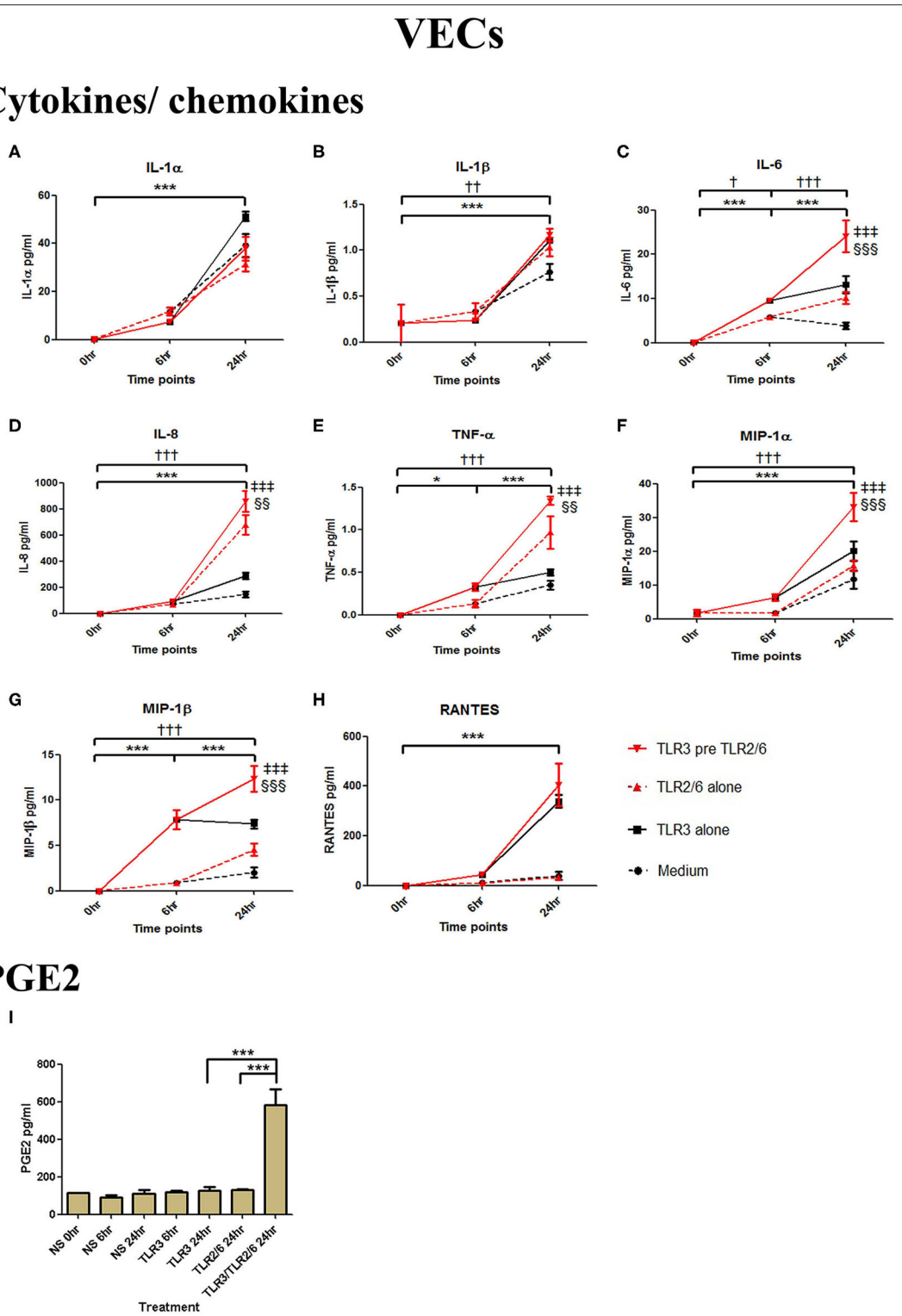

FIGURE 5 | The effect of TLR3 viral priming on TLR2/6 agonist induced pro-inflammatory and pro-labour mediators in vaginal epithelial cells. Monolayer VECs were primed with 25 μg/ml of poly I:C for 6 h prior to 0.01 μg/ml of FSL-1 for 24 h and the supernatant was collected to quantify the pro-inflammatory and pro-labour mediators. Poly I:C and FSL-1 stimulation alone significantly increased the production of the cytokines and chemokines. TLR3 priming prior to FSL-1 stimulation showed no augmentation in IL-1α **(A)** and IL-1ß **(B)**. However, significant increase was observed in IL-6 **(C)**, IL-8 **(D)**, TNF-α **(E)**, MIP-1α **(F)**, and MIP-1ß **(G)**. No significant increase was observed in RANTES **(H)** chemokine. Pro-labour PGE2 **(I)** showed significant augmentation compared to TLR3 and TLR2/6 agonist stimulation alone. For statistical analysis Two-way ANOVA with Bonferroni's multiple comparison test was used. * = effect of TLR3 compared to 0 h, † = effect of TLR2/6 compared to 0 h, ‡ = effect of TLR3 pre TLR2/6 compared to TLR3 alone, § = effect of TLR3 pre TLR2/6 compared to TLR2/6 alone. †† = $p < 0.01$, ††† = $p < 0.001$, *** = $p < 0.001$, §§ = $p < 0.01$, §§§ = $p < 0.001$, ‡‡‡ = $p < 0.001$.

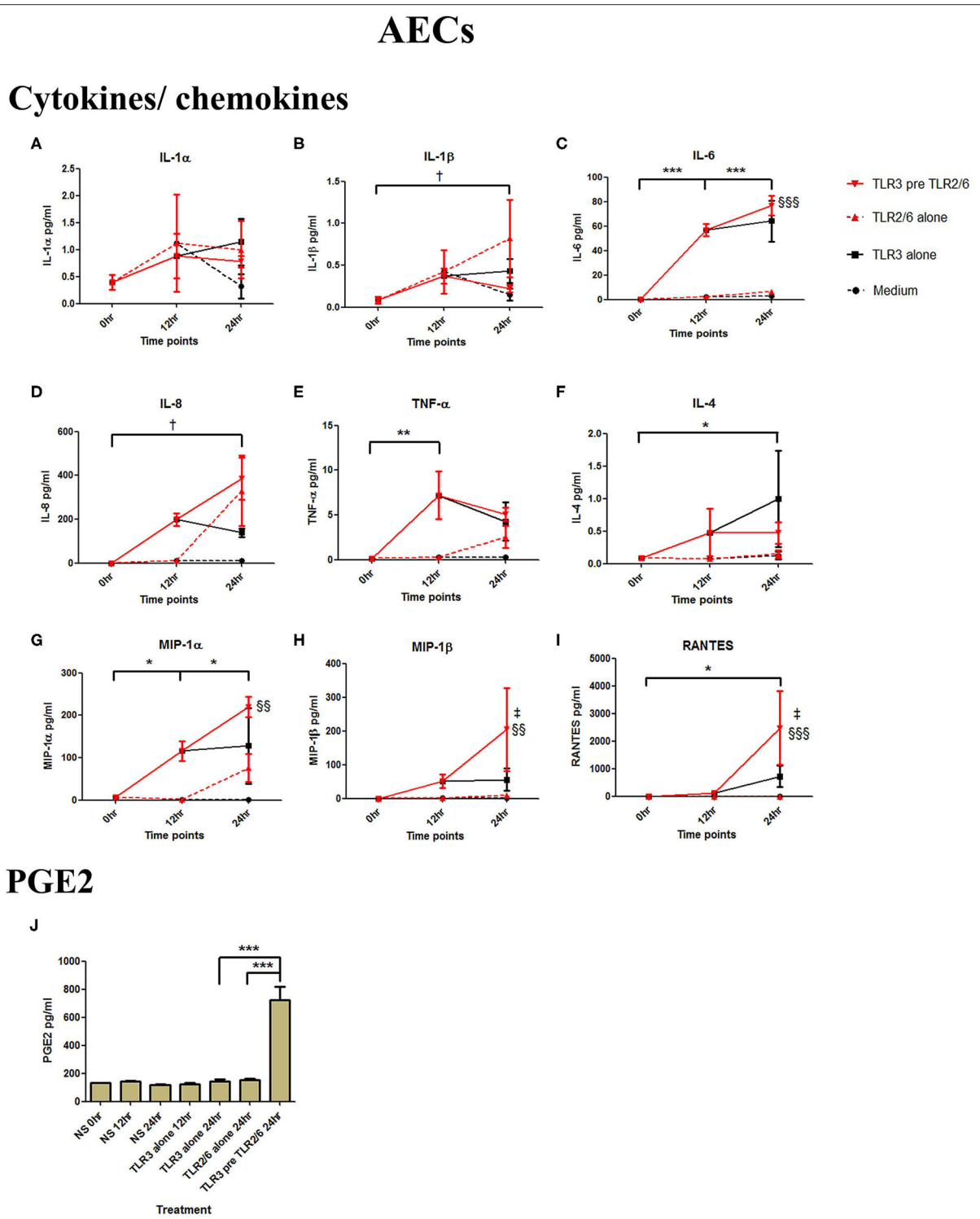

FIGURE 6 | The effect of TLR3 viral priming on TLR2/6 agonist induced pro-inflammatory and pro-labour mediators in amnion epithelial cells. Monolayer cells were primed with 25 μg/ml of poly I:C for 12 h prior to 0.01 μg/ml of FSL-1 for 24 h and the supernatant was collected to quantify the pro-inflammatory and pro-labour mediators. Poly I:C and FSL-1 stimulation alone significantly increased the production of the cytokines and chemokines. TLR3 priming prior to FSL-1 stimulation showed no augmentation in IL-1α **(A)** and IL-1ß **(B)**. However, significant increase was observed in IL-6 **(C)**. No augmentation was observed with priming in IL-8 **(D)**, TNF-α **(E)** or IL-4 **(F)**. Chemokines MIP-1α **(G)**, MIP-1ß **(H)** and RANTES **(I)** showed significant augmentation with priming. Pro-labour PGE2 **(J)** also showed significant augmentation compared to TLR3 and TLR2/6 agonist stimulation alone. For statistical analysis Two-way ANOVA with Bonferroni's multiple comparison test was used. * = effect of TLR3 compared to 0 h, † = effect of TLR2/6 compared to 0 h, ‡ = effect of TLR3 pre TLR2/6 compared to TLR3 alone, § = effect of TLR3 pre TLR2/6 compared to TLR2/6 alone. ** = $p < 0.01$, *** = $p < 0.001$, §§ = $p < 0.01$, §§§ = $p < 0.001$.

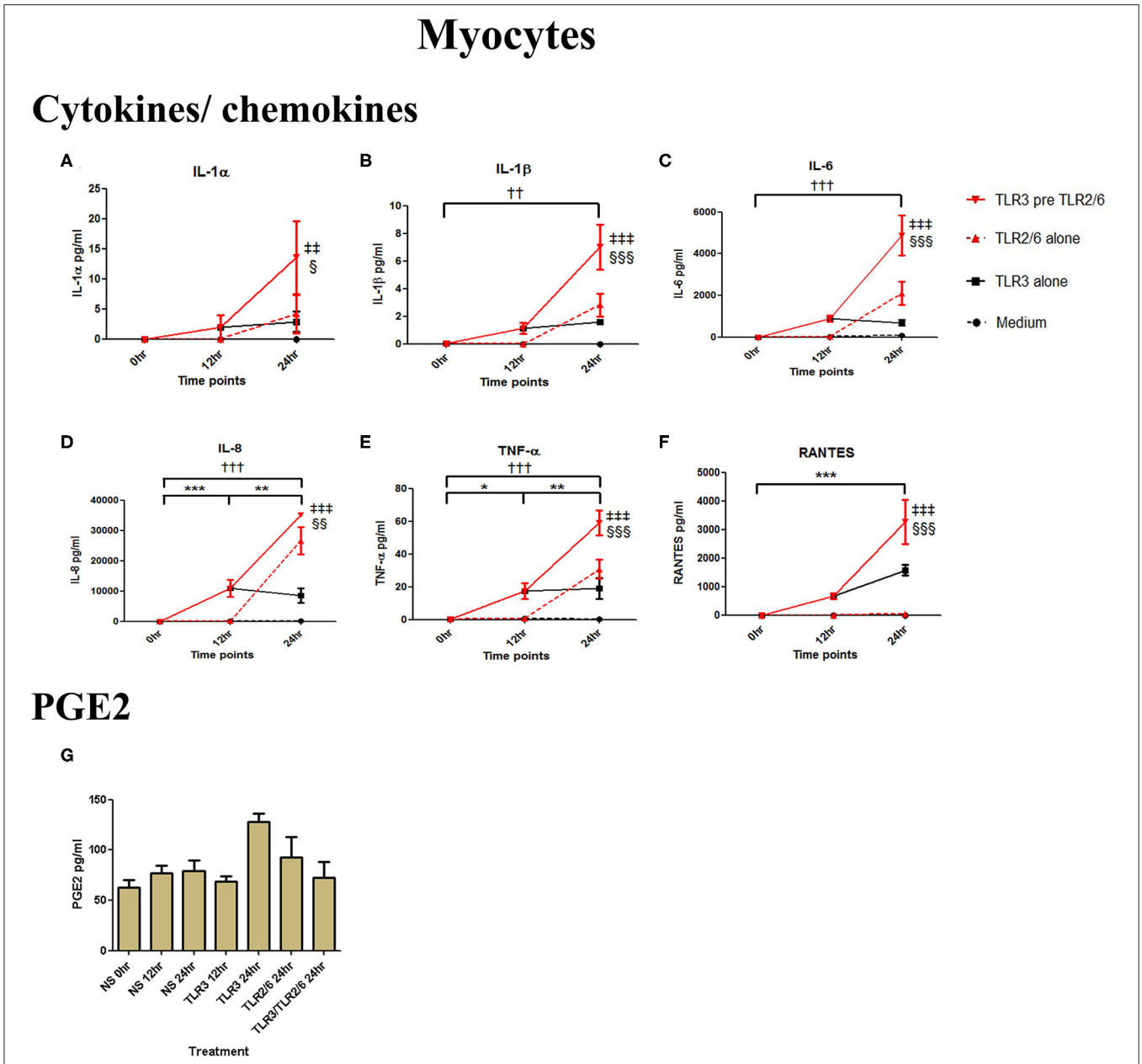

FIGURE 7 | The effect of TLR3 viral priming on TLR2/6 agonist induced pro-inflammatory and pro-labour mediators in myocytes. Monolayer cells were primed with 25 μg/ml of poly I:C for 12 h prior to 0.1 μg/ml of FSL-1 for 24 h. Supernatant was collected to quantify the pro-inflammatory and pro-labour mediators. Poly I:C and FSL-1 stimulation alone significantly increased the production of the cytokines and chemokines. TLR3 priming prior to FSL-1 stimulation showed significant augmentation in IL-1α **(A)**, IL-1ß **(B)**, IL-6 **(C)**, IL-8 **(D)**, TNF-α **(E)**, and RANTES **(F)**. However, no augmentation was observed in pro-labour PGE2 **(G)**. For statistical analysis Two-way ANOVA with Bonferroni's multiple comparison test was used. * = effect of TLR3 compared to 0 h, † = effect of TLR2/6 compared to 0 h, ‡ = effect of TLR3 pre TLR2/6 compared to TLR3 alone, § = effect of TLR3 pre TLR2/6 compared to TLR2/6 alone. †† = $p < 0.01$, ††† = $p < 0.001$, ** = $p < 0.01$, *** = $p < 0.001$, §§ = $p < 0.01$, §§§ = $p < 0.001$, ‡‡ = $p < 0.01$, ‡‡‡ = $p < 0.001$.

DISCUSSION

There is an established causal link between infection/inflammation and PTB (7) and growing evidence for a role for the vaginal microbiota in shaping PTB risk (20–23). Although certain bacterial species are associated with PPROM and sPTL, clinical use of antibiotics does not mitigate risk, and not all women with an adverse vaginal microbial composition deliver preterm. Other factors must therefore be involved, and one plausible contributor is clinical or subclinical viral infection. Animal studies have shown that viral infection modulates host immune response, increasing susceptibility to bacterial induced

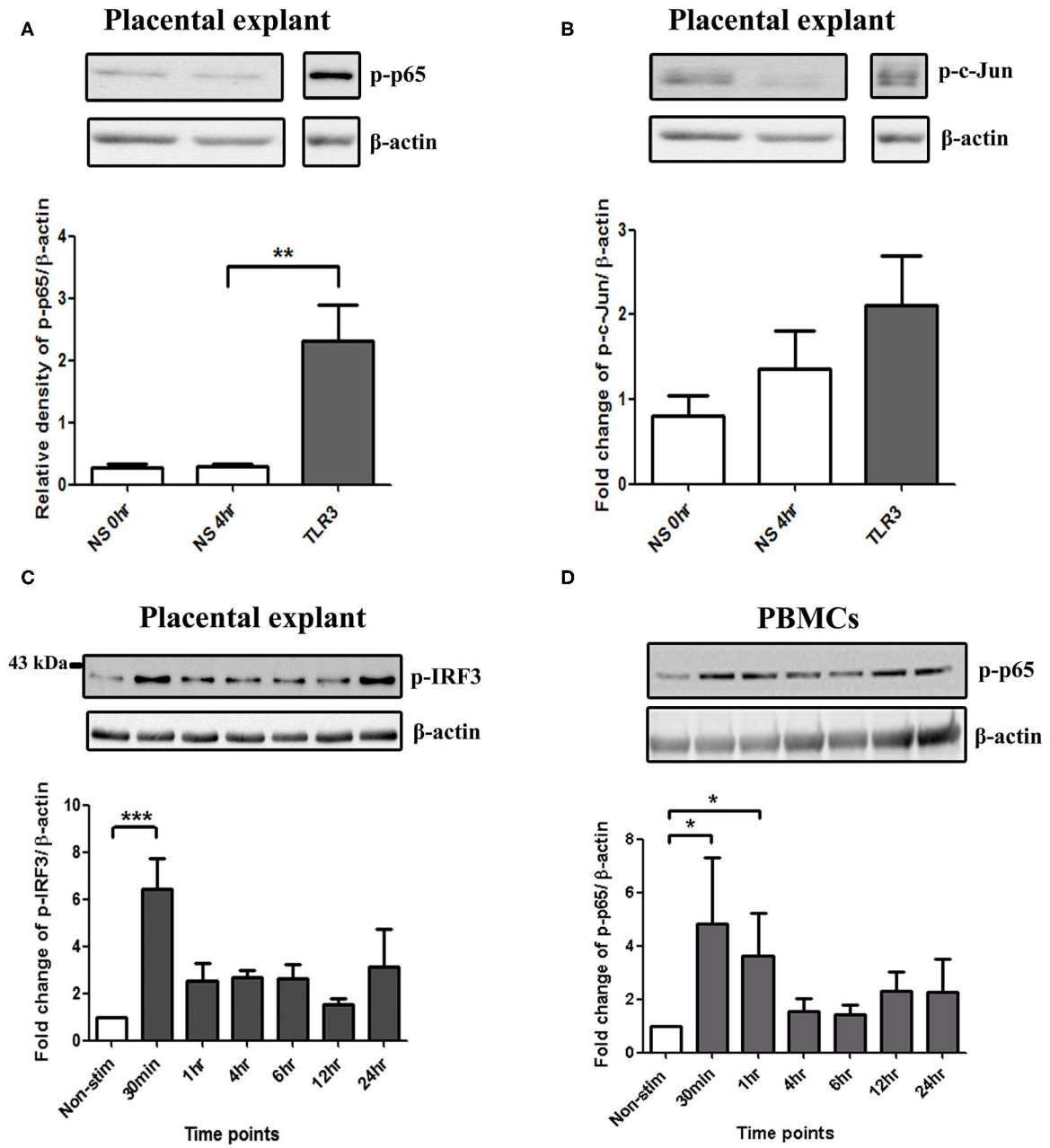

FIGURE 8 | Activation of NF-κB in PBMCs, and NF-κB, AP-1, and IRF in placental explants. An increase in p-p65 was seen after 4 h of treatment with 25 μg/ml poly I:C in placental explants ($p < 0.01$) **(A)** but no significant increase was observed in p-c-Jun **(B)**. Early activation of IRF3 at 30 min ($p < 0.001$) was seen in placental explants **(C)**. PBMCs were treated with 5 μg/ml of Poly I:C for 30 min, 1 h, 4 h, 6 h, 12 h, and 24 h. A significant increase in phospho-p-65 was seen by 30 min ($p < 0.05$) **(D)**. For statistical analysis, One-way ANOVA was used for normally distributed data and Kruskal-Wallis was used for data not normally distributed with Dunnett's multiple comparison test $n = 4$. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

sPTL. However, there are limited studies in the human examining this double hit effect. To address this, we considered two possible routes by which viral and bacterial stimuli might impact the maternal-fetal interface and myometrium. In this study, VECs, AECs and myocytes were used to represent the target cells in an *in vitro* model of ascending infection, and PBMCs and placental explants to represent the target cells in a model of systemic inflammation and haematogenous infection. We demonstrate that viral priming prior to incubation with bacterial products leads to a synergistic increase in pro-inflammatory and pro-labour mediators which is more pronounced in our model of ascending infection.

Systemic viral infections such as human immunodeficiency virus (HIV) (55), Hepatitis B (56) and influenza have all been

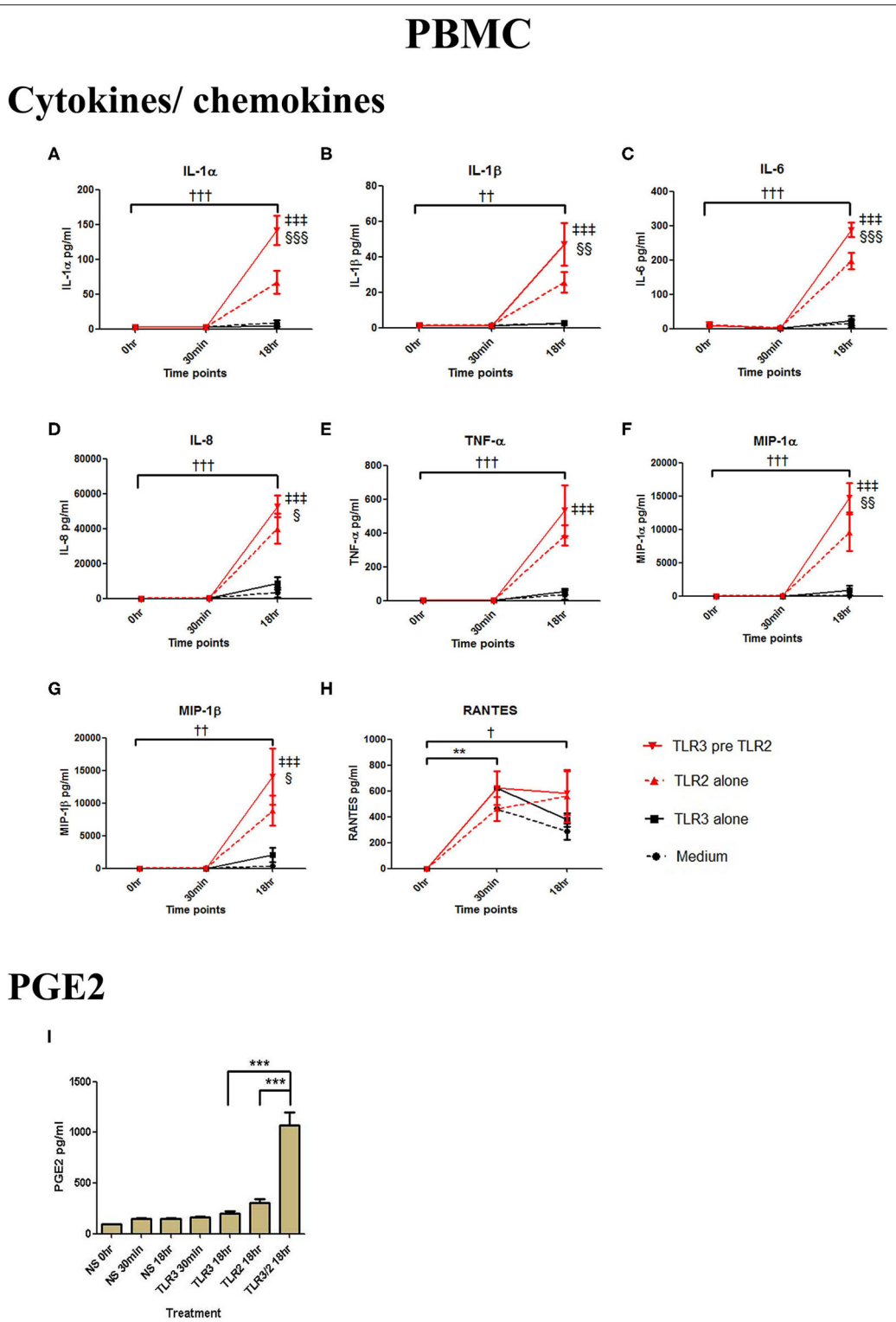

FIGURE 9 | The effect of TLR3 viral priming on TLR2 agonist induced pro-inflammatory and pro-labour mediators in PBMCs. PBMCs were primed with 5 μg/ml of poly I:C for 30 min prior to 10^8 cell/ml of HKLM for 18 h. Supernatant was collected to quantify the pro-inflammatory and pro-labour mediators. Poly I:C and HKLM stimulation alone significantly increased the production of the RANTES chemokines. TLR3 priming prior to HKLM stimulation showed a significant augmentation in IL-1α **(A)**, IL-1β **(B)**, IL-6 **(C),** and IL-8 **(D)**. No significant augmentation was observed in TNF-α. **(E)** Chemokines MIP-1α **(F)** and MIP-1β **(G)** showed significant augmentation but not RANTES **(H)**. Significant augmentation was also observed in pro-labour PGE2 **(I)**. For statistical analysis Two-way ANOVA with Bonferroni's multiple comparison test was used. * = effect of TLR3 compared to 0 h, † = effect of TLR2 compared to 0 h, ‡ = effect of TLR3 pre TLR2 compared to TLR3 alone, § = effect of TLR3 pre TLR2 compared to TLR2 alone. †† = $p < 0.01$, ††† = $p < 0.001$, ** = $p < 0.01$, *** = $p < 0.001$, §§ = $p < 0.01$, §§§ = $p < 0.001$, ‡‡ = $p < 0.01$, ‡‡‡ = $p < 0.001$.

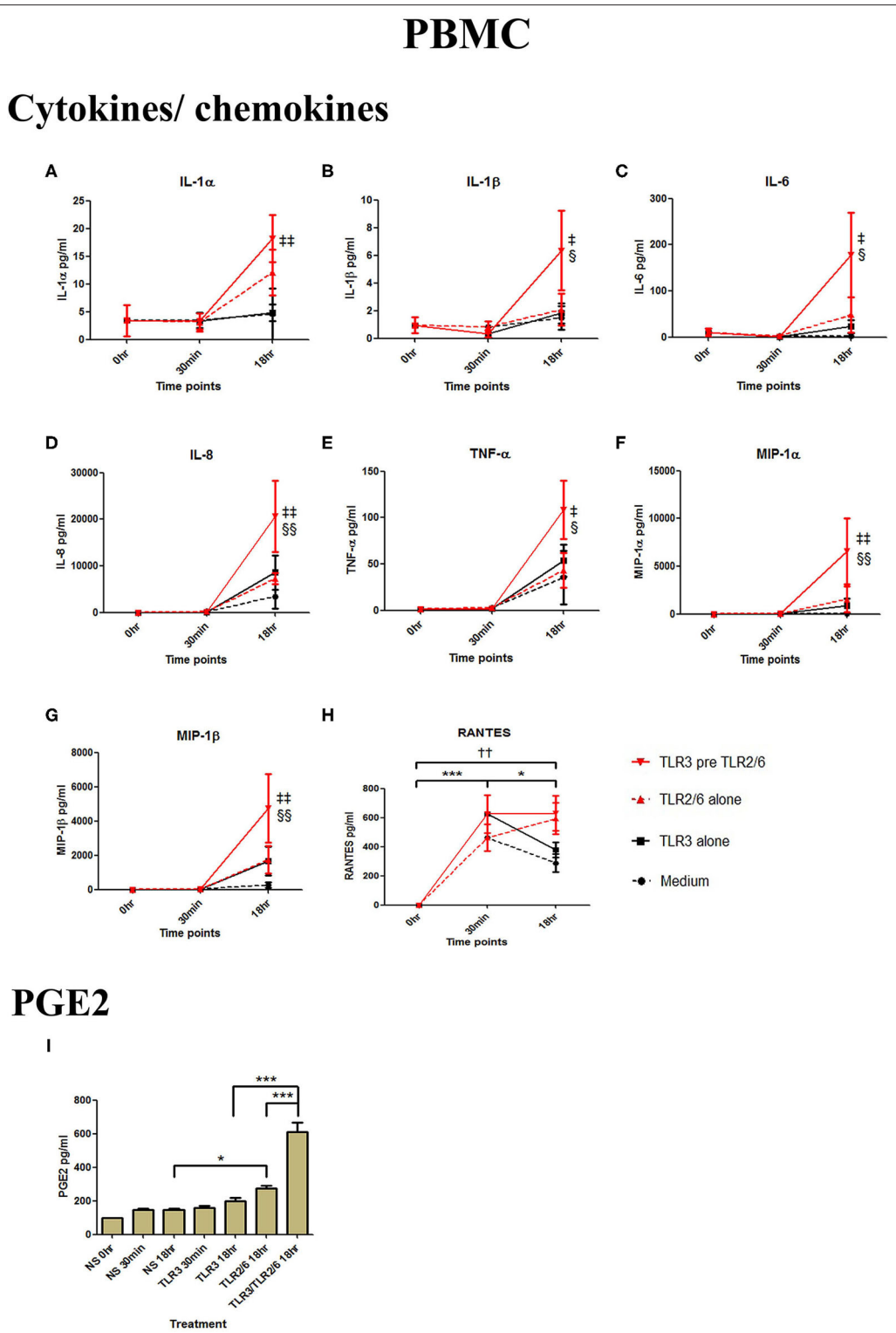

FIGURE 10 | The effect of TLR3 viral priming on TLR2/6 agonist induced pro-inflammatory and pro-labour mediators in PBMCs. PBMCs were primed with 5 μg/ml of poly I:C for 30 min prior to 0.1 μg/ml of FSL-1 for 18 h. Supernatant was collected to quantify the pro-inflammatory and pro-labour mediators. TLR3 and FSL-1 stimulation alone only significantly increased the production of RANTES. TLR3 priming prior to FSL-1 stimulation showed no augmentation on IL-1α but a significant augmentation in IL-1ß **(B)**, IL-6 **(C)** and IL-8 **(D)**, TNF-α **(E)**, MIP-1α **(F)**, and MIP-1ß **(G)**. However, no significant augmentation was observed in RANTES **(H)**. Significant augmentation was also observed in pro-labour PGE2 **(I)**. For statistical analysis Two-way ANOVA with Bonferroni's multiple comparison test was used. * = effect of TLR3 compared to 0 h, † = effect of TLR2/6 compared to 0 h, ‡ = effect of TLR3 pre TLR2/6 compared to TLR3 alone, § = effect of TLR3 pre TLR2/6 compared to TLR2/6 alone. †† = $p < 0.01$, * = $p < 0.05$, *** = $p < 0.001$, § = $p < 0.05$, §§ = $p < 0.01$, ‡ = $p < 0.05$, ‡‡ = $p < 0.01$.

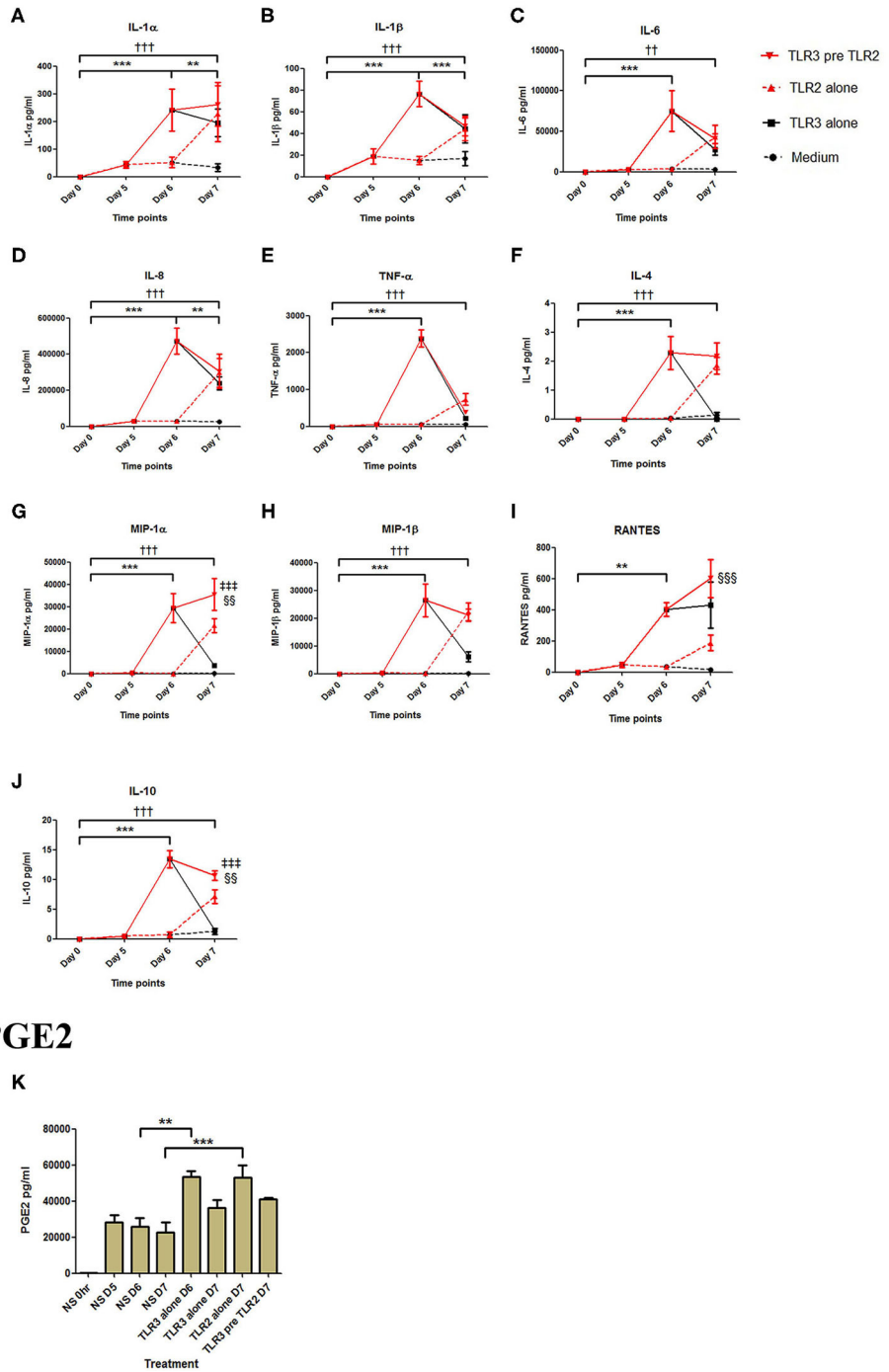

FIGURE 11 | The effect of TLR3 viral priming on TLR2 agonist induced pro-inflammatory and pro-labour mediators in placental explants. Explants were primed with 25 μg/ml of poly I:C for 24 h prior to 10^8 cells/ml of HKLM for 24 h. Supernatant was collected to quantify the pro-inflammatory and pro-labour mediators. Poly I:C and HKLM stimulation alone significantly increased the production of the cytokines, chemokines and PGE2. TLR3 priming prior to HKLM stimulation showed no augmentation in IL-1α **(A)**, IL-1ß **(B)**, IL-6 **(C)** IL-8 **(D)** TNF-α **(E)**, IL-4 **(F)** or MIP-1ß **(H)**. A significant augmentation was only observed in MIP-1α **(G)** RANTES **(I)** and the anti-inflammatory cytokine IL-10 **(J)**. No augmentation was observed in pro-labour PGE2 **(K)**. For statistical analysis Two-way ANOVA with Bonferroni's multiple comparison test was used. * = effect of TLR3 compared to 0 h, † = effect of TLR2 compared to 0 h, ‡ = effect of TLR3 pre TLR2 compared to TLR3 alone, § = effect of TLR3 pre TLR2 compared to TLR2 alone. ** = $p < 0.01$, *** = $p < 0.001$, †† = $p < 0.01$, ††† = $p < 0.001$, §§ = $p < 0.01$, §§§ = $p < 0.001$.

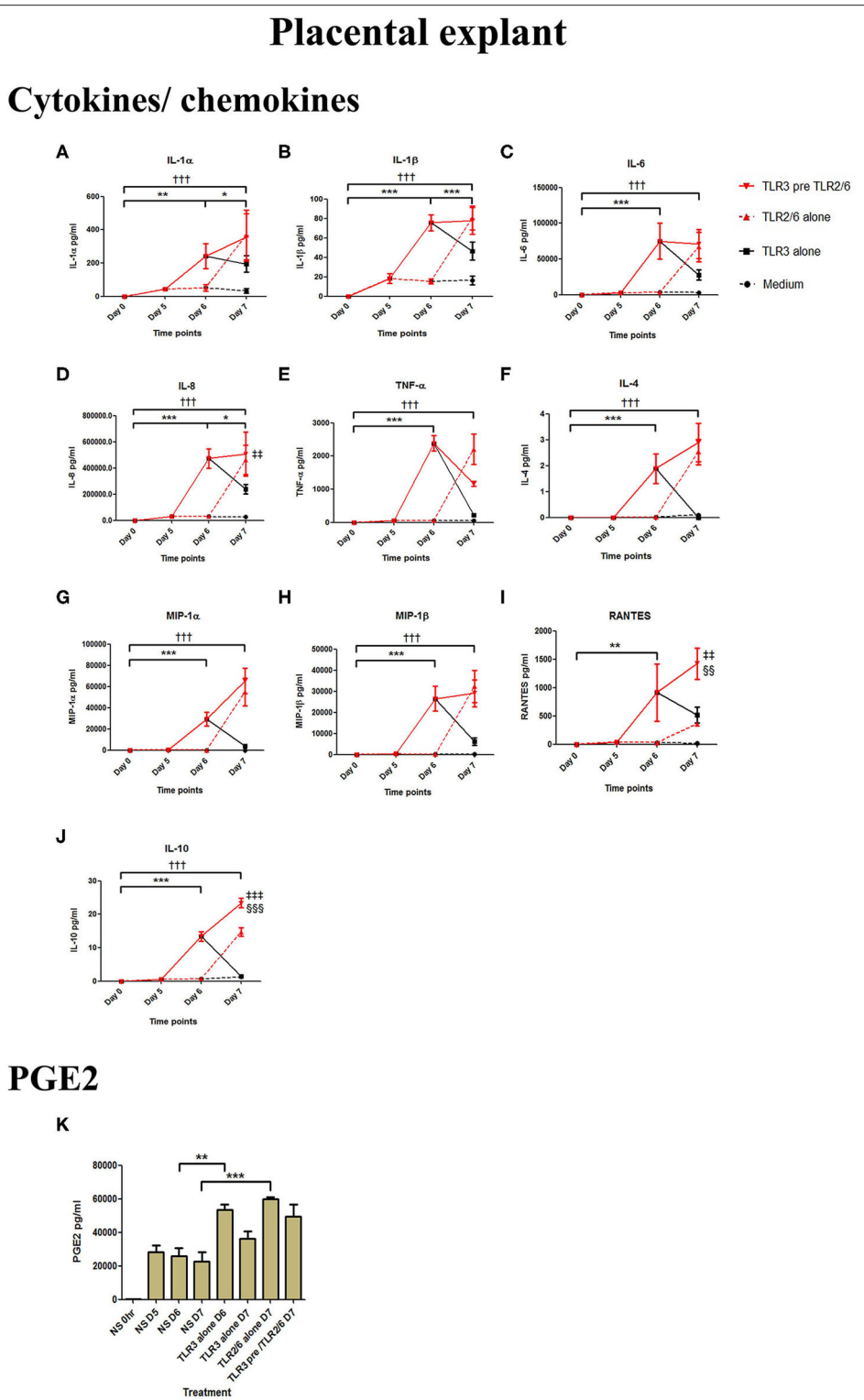

FIGURE 12 | The effect of TLR3 viral priming on TLR2/6 agonist induced pro-inflammatory and pro-labour mediators in placental explants. Explants were primed with 25 μg/ml of poly I:C for 24 h prior to 0.1 μg/ml of FSL-1 for 24 h. Supernatant was collected to quantify the pro-inflammatory and pro-labour mediators. Poly I:C and FSL-1 stimulation alone significantly increased the production of the cytokines, chemokines and PGE2. TLR3 priming prior to FSL-1 stimulation showed no augmentation in IL-1α **(A)**, IL-1ß **(B)**, IL-6 **(C)**, IL-8 **(D)**, TNF-α **(E)**, IL-4 **(F)**, MIP-1α **(G)**, MIP-1ß **(H)**. However, significant augmentation was observed in RANTES **(I)** and anti-inflammatory IL-10 **(J)**. No augmentation was observed in pro-labour PGE2 **(K)**. For statistical analysis Two-way ANOVA with Bonferroni's multiple comparison test was used. * = effect of TLR3 compared to 0 h, † = effect of TLR2/6 compared to 0 h, ‡ = effect of TLR3 pre TLR2/6 compared to TLR3 alone, § = effect of TLR3 pre TLR2/6 compared to TLR2/6 alone. ** = $p < 0.01$, *** = $p < 0.001$, ††† = $p < 0.001$, §§ = $p < 0.01$, §§§ = $p < 0.001$, ‡‡ = $p < 0.01$, ‡‡‡ = $p < 0.001$.

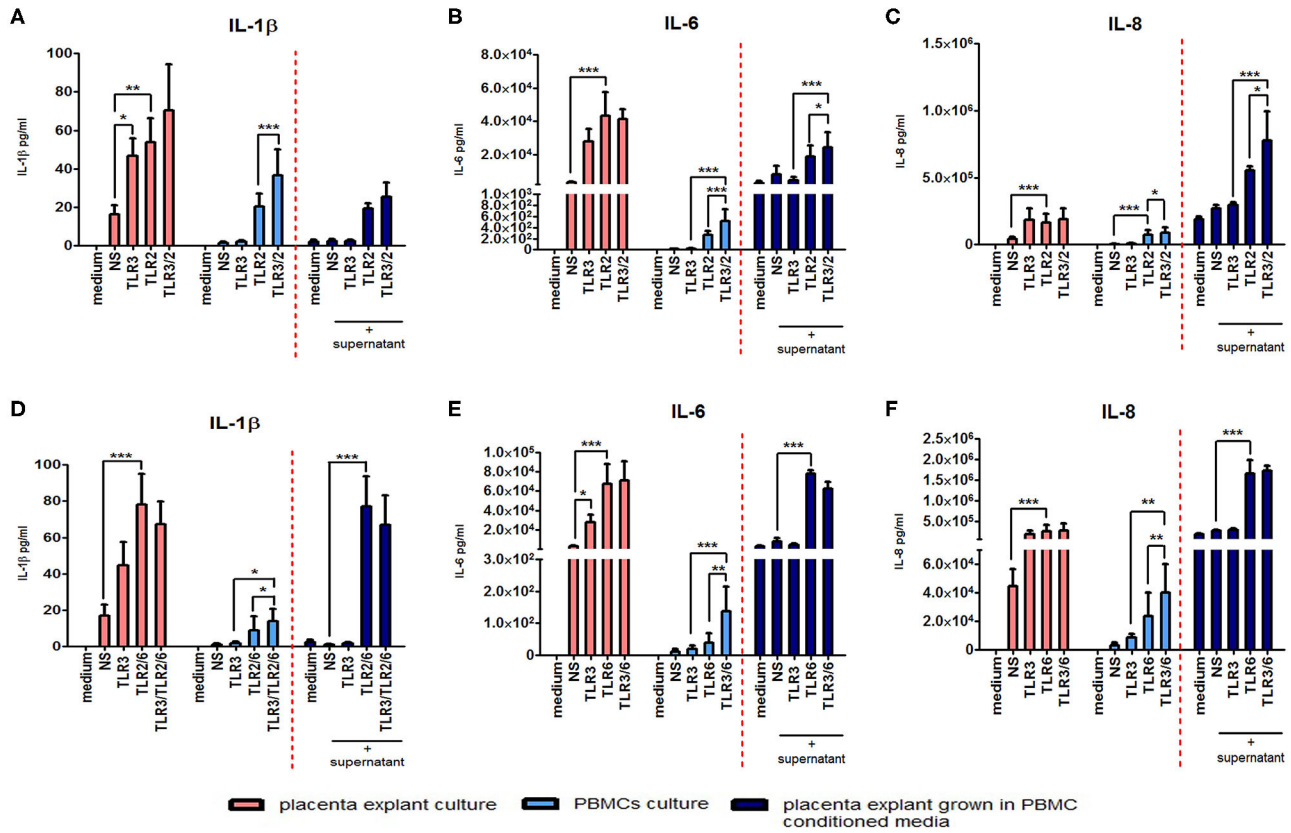

FIGURE 13 | The effect of co-culturing placental explants with PBMC conditioned media on IL-1ß, IL-6 and IL-8. Placental explants were cultured and treated with media derived from TLR3-primed or TLR2 or TLR2/6 or TLR3 agonist treated PBMCs for 24 h. Supernatant was collected and the level of pro-inflammatory cytokines IL-1ß, IL-6, and IL-8 production was determined using ELISA. Pink-coloured histograms demonstrate placental explant priming experiment results, showing no augmentation of the cytokine production with TLR3/TLR2 treatment **(A–C)** or TLR3/ and TLR2/6 treatment **(D–F)**. Light blue-coloured histograms IL concentrations from PBMCs following TLR3/TLR2 treatment **(A–C)** or TLR3/ and TLR2/6 treatment **(D–F)**. Cytokine concentrations from culturing placental explants in PBMC conditioned media is represented in the dark blue-coloured histograms for TLR3/TLR2 treatment **(A–C)** or TLR3/ and TLR2/6 treatment **(D–F)**. No augmentation was seen with IL-1ß **(A)** but a significant augmented production of IL-6 **(B)** and IL-8 **(C)** was observed compared to placental explants cultured in conditioned media from PBMCs-treated with TLR3 and TLR2 agonists alone. Treatment of placental explants with media derived from PBMCs treated with TLR3 and TLR2/6 agonist media showed no augmentation of IL-1ß **(D)**, IL-6 **(E)**, or IL-8 **(F)**. For statistical analysis, Two-way ANOVA with Bonferroni's multiple comparison test was used $N = 4$. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

linked to higher rates of sPTL. Insight from epidemiological studies following the H1N1 pandemic also reported on higher rates of sPTL in women with underlying health conditions (57), and lower rates of preterm delivery in women who had been vaccinated (58). In support of the role of haematogenous spread of viral infection in preterm birth, a study of 71 preterm and 122 full term placentas demonstrated a greater proportion of adenovirus positive placentas in preterm deliveries and in cases with histological evidence of chorioamnionitis (46). Although a significant number of viral taxa have been detected in amniotic fluid, only a few such as herpes simplex virus, adenovirus, enterovirus and cytomegalovirus have been linked to PTB (59). Viral infections of the lower reproductive tract can also predispose to PTB, with several studies implicating cervical human papillomavirus (HPV) (60, 61) and herpes simplex virus (HSV) (62, 63) in the aetiology of PTB.

Many animal studies have also provided evidence for a potential role for viruses in sPTB. Animal models investigating the role of single viral pathogens and PTB commonly use the TLR3 agonist, poly I:C. Koga et al. demonstrated a 100% sPTB rate in mice treated with intraperitoneal poly I:C and a 0% rate in TLR3 knockout mice (47). However, TLR9 activation from intraperitoneal injection of CpG oligodeoxynucleotide into IL-10 deficient mice has also been reported to cause a 100% sPTB rate (64). There is also evidence that viral infection may mediate local inflammation and pro-labour changes. HSV-2 causes histological evidence of collagen remodelling and increased hyaluronic acid synthesis leading to cervical ripening, and aberrant expression of oestrogen and progesterone receptors in the cervical epithelium (65).

In vitro studies also support a role for viruses in immune modulation in human gestational tissues. Murine herpes

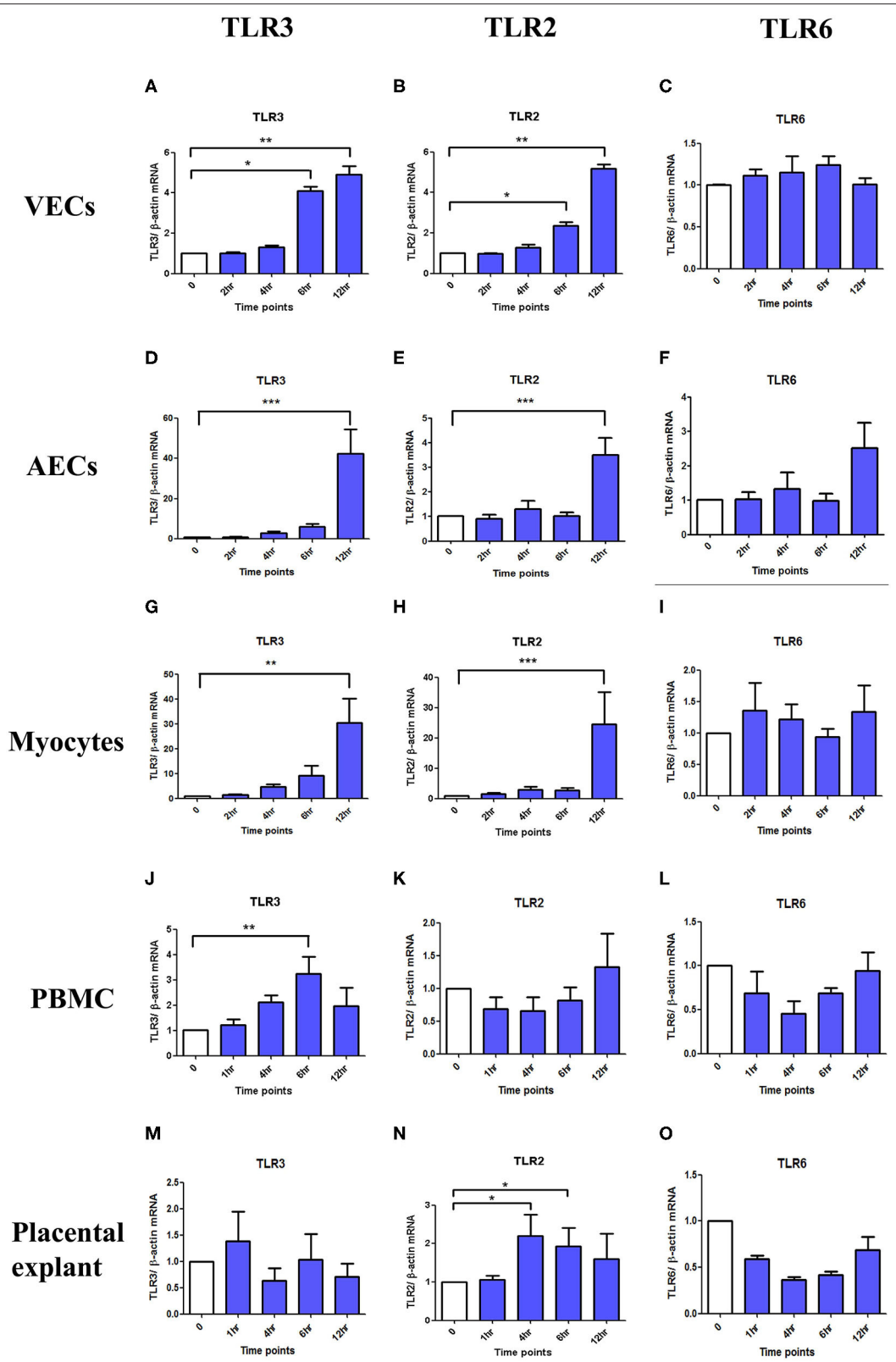

FIGURE 14 | The effect of TLR3, 2 and 6 mRNA expression with poly I:C stimulation in VECs, AECs, myocytes, PBMC and placental explants. VECs, AECs, myocytes, and placental explant were treated with 25 μg/ml while PBMCs were treated with 5 μg/ml of poly I:C. The level of the mRNA expression was determined

(Continued)

FIGURE 14 | after 2, 4, 6, and 12 h in VEC, amniocytes and myocytes while at 1, 4, 6, and 12 h in PBMC and placental explant using ß-actin as the loading control. Total RNA was extracted and RT-QPCR was performed to quantify TLR3, 2, and 6 mRNA level. In VEC, amniocytes and myocytes, TLR3 **(A,D,G)** and TLR2 **(B,E,H)** are significantly increased after poly I:C stimulation, however, no increased was observed in TLR6 **(C,F,I)**. In PBMC, only TLR3 is significantly increased **(J)** but a non-significant increase was observed with TLR2 **(K)** and TLR6 **(L)**. In contrast to PBMC, in placental explant, no significant increase was observed in TLR3 **(M)**, but a significant increase was observed in TLR2 **(N)**. No change was observed in TLR6 mRNA expression **(O)**. For statistical analysis Kruskal-Wallis with Dunnett's multiple comparison test was used. A minimum of $n = 3$ biological replicates were used. * $= p < 0.05$, ** $= p < 0.01$, *** $= p < 0.001$.

virus-68 (MHV-68) stimulation of human fetal membranes causes increased production of IL-1β, IL-6, IL-8, and IFN-γ (66). Amnion, choriodecidua and placental explants are highly responsive to both the TLR3 agonist poly I:C and the TLR agonist ssRNA40, each leading to increased release of IL-6 and IL-8 (67). Several studies have also demonstrated pro-inflammatory cytokine release by cultured myocytes, vaginal and cervical epithelial cells upon incubation with poly I:C (68–72). In our study, we confirmed that the TLR3 agonist poly I:C induces inflammation in VECs, myocytes, and placental explants, and demonstrate that the effect reported in fetal membranes is reproduced at the cellular level in AECs. TLR3 recognises both virus–derived double-stranded RNA (dsRNA) (73) and its synthetic analogue poly I:C. While TLR3 activation leads to a pro-inflammatory response, it also is capable of activating an anti-viral response through the production of the Type I, II, and III interferons (74–76) as observed in cases of hepatitis B and C viruses, herpesvirus, and rotavirus (77).

Unlike other TLR family members, TLR3 is not dependant on myeloid differentiation factor 8 (MyD88) as the signalling adaptor protein (78). TLR3 mediates transduction via the adaptor protein TICAM-1/TRIF (79, 80) activating transcription factors NF-κB, AP-1, and IRF3, leading to the induction of cytokine and chemokine production (73). As anticipated, we saw a cell type dependent increase in all three transcription factors when cells were treated with poly I:C and a significant increase in phospho-IRF3 was seen in all cell types of the model of ascending infection. Activation of NF-κB in the fundal myometrium (81) and cervix (82) is strongly associated with labour onset and NF-κB activation is observed in fetal membranes prior to labour (83). The promoter region of pro-inflammatory cytokines TNF-α, IL-8, IL-6, IL-1β, OTR (28), and those of COX-2, MMP-1, and MMP-9 genes all contain binding sites for NF-κB (84) lending support to its role in uterine contractility, membrane rupture and cervical modelling. Similarly, activation of AP-1 upregulates an array of pro-inflammatory and pro-labour genes as the promoter regions for IL-8 (31), COX-2 (29, 85), oxytocin receptor (OTR) (86, 87), Cx43 and MMP-9 all contain AP-1 binding sites (86, 88). We have previously shown that AP-1 activation is a key terminal mediator of inflammation induced PTL in the mouse (29). More recently, the transcription factor IRF3 has also been highlighted as potentially playing a role in PTB. A review of studies linking genetic polymorphisms with the risk of sPTL identified IRF-3 as a strong candidate transcription factor in the aetiology of sPTB (89). Taken together, our data demonstrate the capacity for signalling via TLR3 to activate the key transcription factors known to regulate pro-labour and pro-inflammatory mediators in a situation of ascending infection, systemic inflammation and haematogenous spread of infection induced PTL.

We next explored whether activation of TLR3 leads to augmentation of the bacterial product induced inflammation in VECs, AECs and myocytes in a model of ascending infection/inflammation. We found significant synergy between TLR3 and TLR2/6 agonists in inducing cytokine, chemokine and PGE2 production (**Figures 5–7, 10**). Murine studies clearly demonstrate increased rates of preterm labour in response to exposure to both viral and bacterial stimuli (47, 48, 50), however most models focus on the effect of systemic infection, with few models using vaginal or intrauterine routes for administration. Racicot et al. tested the effect of systemic viral infection on local vaginal infection and demonstrated that systemic administration of MHV-68 was required to facilitate ascending infection of *U. urealyticum* from the vagina to decidua in mice (90). However, in this study, MHV-68 led to a reduction in *U. urealyticum* induced cytokine and chemokine production and in TLR 2,3 and 4 mRNA expression. Racicot and colleagues later tested the effect of vaginal HSV-2 infection on vaginally administered *E. coli* induced PTB, and showed histological evidence of cervical remodelling and a significant increase in sPTB rates with dual treatment compared to single treatment alone (65).

As has previously been reported in a study using a 3D culture model of vaginal epithelial cells (91), we found that VECs produced high concentrations of pro-inflammatory cytokines including IL6 and IL-8 in response to poly I:C (TLR3 agonist) or FSL-1 (TLR2/6 agonist) alone (**Figure 5**). We also demonstrated a synergistic increase when cells were primed with poly I:C. Similarly, a synergistic increase in concentrations of the chemokines MIP-1α and MIP-β, and PGE2 was seen. However, neither HKLM (TLR2 agonist) alone or in combination with poly I:C led to increases in any of the mediators (**Figure 2**). TLR2 forms heterodimers with TLR1 and TLR6; TLR2/1 recognises lipopeptides from Gram-negative bacteria, whereas the TLR2/6 heterodimers recognise lipopeptides from Gram-positive bacteria. FSL-1, used in our study, elicits its response via the TLR2/TLR6 heterodimer. There is an association between Gram-positive bacteria colonisation, particularly Group B *Streptococcus,* and preterm birth (92), which may be explained exclusively through promotion of increases in pro-inflammatory cytokines, as demonstrated by *in vitro* studies of cultured vaginal and cervical epithelial cells (93). However, not all women with Group B *Streptococcus* deliver preterm, therefore other factors must contribute to the pathophysiology. High diversity and instability of the bacterial vaginal communities are associated with sPTB (94, 95). Consistent with our findings, recent data suggests having both high bacterial diversity and a high viral diversity in early pregnancy is associated with an even higher risk (96), supporting a mechanism for multi-pathogen induced preterm labour in some women.

As with VECs, we also saw an increase in cytokine, chemokine and PGE2 concentrations with poly I:C and FSL-1 alone, with a synergistic increase with priming in AECs (**Figure 6**). The predominant cytokine that was increased was IL-6, a marker of infection associated PTL (97), which has been previously shown to be significantly increased in amnion in women who deliver preterm compared to term (25). Poly I:C has been shown to increase TNF-α, MIP-1α and MIP-β production in fetal membranes by a combination of MyD88 and TRIF dependant and independent mechanisms (98). Although fetal membranes express TLR2, and have the capacity to respond to TLR2 activation, we saw no increase in cytokine or chemokine production in AECs with HKLM. In a study comparing FM explants responses to a panel of TLR agonists, when stimulated with the TLR2 agonist peptidoglycan (PDG), only the cytokine IL-8 was increased, and at lower concentrations when compared with the response to TLR4 and TLR5 agonists (99). PDG, like HKLM, does not require TLR1 or TLR6 to from heterodimers to achieve its effect. We acknowledge that examining primary AECs rather than fetal membrane explants may have limited the response due to the complex bidirectional communications that exist between amnion and the choriodecidua (100). However, by treating AECs, our aim was to mimic the intra-amniotic environment in response to the viral and bacterial components on the cell type that is in the closest proximity to the fetus.

Poly I:C has been shown in several studies to increase mRNA expression of cytokines in myocytes (68–70), consistent with our results. FSL-1 has been shown to be capable of inducing COX-2 mRNA expression and PGF2α production. Although we did not see NF-κB activation FSL-1 in myocytes, we did see a significant increase in p-c-Jun (**Supplementary Figures 1**, **2**). Moreover, we observed a synergistic effect in cytokine and chemokine production with TLR3 viral priming and TLR2/6 agonist stimulation (**Figure 7**). The implication of this is that IL-8 could serve to chemoattract leukocytes to the myometrium, which is known to occur at the time of term and preterm labour (101). Although there was not a significant increase in PGE2 by the myocytes *in vitro*, leukocytes infiltrating the myometrium *in vivo* would contribute to local prostaglandin synthesis, which in turn could trigger uterine contractility. Additionally, increased IL-6 production by myocytes could lead to the facilitation of uterine contractility since incubation of cultured myocytes with IL-6 increases oxytocin receptor (OTR) mRNA *in vitro* (102). The increase in TNF-α and IL-1β can lead to further activation of NF-κB which in turn leads to a positive feed forward loop further increasing the transcription of pro-labour and pro-inflammatory genes. Additionally, the chemokines macrophage inflammatory protein-1α (MIP-1α), MIP-1β, and RANTES attract immune effector cells which will also lead to amplification of the inflammatory response. Increased local concentrations of RANTES may also induce uterine contractility through induction of basophils to release histamines which are potent uterotonics (103, 104).

Although an augmented effect was seen with the TLR2/6 agonist, we did not see an augmented response to TLR3 priming with TLR2 agonist HKLM or with HKLM treatment alone (**Figure 4**). This was unexpected, since we had confirmed that HKLM at these concentrations activates both NF-κB and AP-1 (**Supplementary Figures 1, 2**), and murine studies have shown an increase in sPTB rate with combined TLR2 and 3 agonist treatment (50). It is possible that both agonists are needed to be administered together to achieve synergy, rather than being administered sequentially. It is also plausible that by using different TLR2 agonists, different downstream pathways will be activated, leading to differential effects in TLR2-TLR3 cross-talk. Nevertheless, our data suggests that the lower reproductive tract is more responsive to TLR2/6 activation than to TLR2 alone, and that is responses seen in animal models of systemic infection may not be directly comparable.

Most animal studies of the effect of multi-pathogen induced preterm labour have drawn on models of systemic infection, using intraperitoneal (i.p.) injections. Combined i.p. administration of the murine herpes virus MHV-68 and LPS leads to a 100% PTB rate, in contrast to a 0% PTB rate with MHV-68 alone, and 29% LPS alone (48). Similarly, i.p. of the TLR2 ligand peptidoglycan PGN and poly I:C leads to a 100% PTL, compared to 15% and 22% in Poly I:C or PGN alone, respectively (50). We compared the production of cytokines, chemokines and PGE2 in response to either TLR3, TLR2, or TLR2/6 agonists alone, with the response to TLR2 or TLR2/6 agonists following TLR3 priming in PBMCs as a model of systemic infection and inflammation, and in placental explants as a model of haematogenous spread of multi-pathogen infection. We selected PBMCs since lymphocytes and monocytes are key immune cells for regulating the response to viruses and bacteria. We used PBMC conditioned media to culture placental explants in order to reflect the high circulating volume through the placenta and to assess the effect of systemic inflammation on placental tissue.

PBMCs exposed to TLR3 viral priming showed augmented production of IL-1β, IL-6, IL-8, TNF-α, and PGE2 when incubated with both the TLR2 and TLR2/6 agonists (**Figure 10**). This is consistent with the reported synergy between the Pam_3CSK_4 TLR2 agonist and poly I:C seen in murine dendritic cells in pro-inflammatory cytokine production, NK cell derived IFN-γ production and increased T cell proliferation (105). However, in contrast, placental explants did not show an augmented pro-inflammatory cytokine response to dual treatment. To the contrary, a significant increase in IL-10 concentrations were seen with TLR3 agonist priming prior to stimulation with TLR2 and TLR2/6 agonists (**Figures 11**, **12**). This implies that the placenta can form a barrier to local multi-pathogen exposure. However, placental explants did elicit a pro-inflammatory response following single agonist (TLR2, TLR2/6, and TLR3) exposure. This data is consistent with that of several studies exploring the functional role of TLRs in human trophoblast and placental explants (106–109). However, we also report on an increase in the production of PGE2 following the exposure to each of the agonists, which could act locally on fetal membranes and myometrium leading to membrane rupture and uterine contractility. Furthermore, when placental explants were grown in PBMC conditioned media

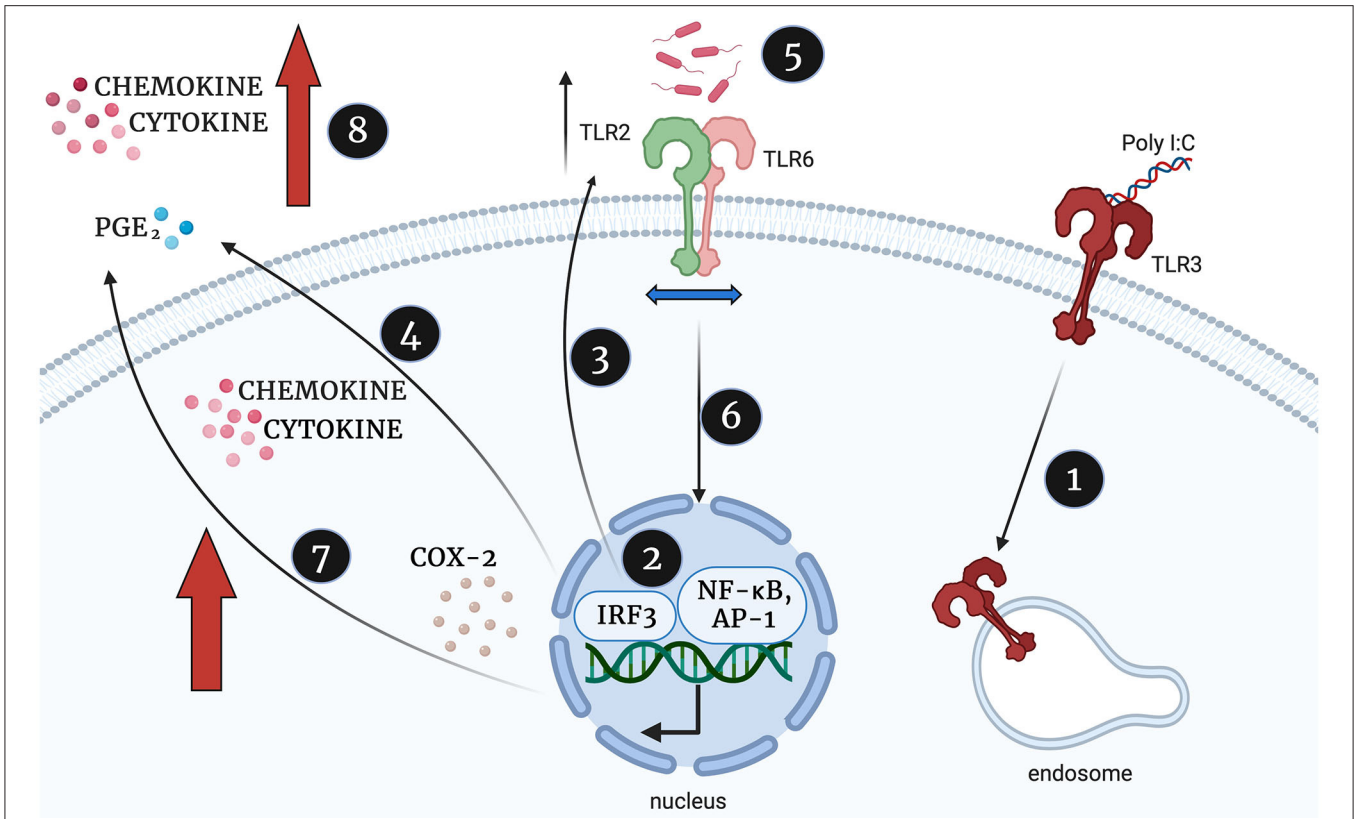

FIGURE 15 | Proposed mechanism of synergistic increase in cytokine, chemokine and PGE2 production in TLR3 primed and TLR2/6 treated cells, created with BioRender.com. Poly I:C activates TLR3 (1) which leads to TLR3 mediated activation of NF-κB, AP-1 and p-IRF3 (2). NF-κB, AP-1 and IRF-3 increases TLR2 expression (3) and stimulates the production of cytokines, COX-2 and PGE2, (4). The TLR2/6 agonist shows increased potency due to increased availability of TLR2 to dimerise with TLR6 (5), TLR2/6 leads to increased NF-κB activation (6) and augmented production of cytokines, COX-2 and PGE2 production (7). This leads to an uncontrolled positive feed forward loop and triggering uterine contractility, fetal membrane rupture and cervical dilation and ultimately a multi-pathogen induced preterm labour.

from priming experiments, an increase in IL-8 and IL-6 was seen with TLR3/TLR2 PBMC conditioned media (**Figure 13**). The clinical implication of this finding could relate to the well-recognised risk of neonatal brain injury that is associated with the presence of systemic maternal inflammation (110, 111).

Finally, we examined the effect of poly I:C on TLR3, TLR2, and TLR6 expression (**Figure 14**). Poly I:C induced the expression of both TLR3 and TLR2, except in placental explants, but no effect was seen on TLR6 expression. We conclude that TLR2 is likely to be the rate limiting step in TLR2/TLR6 dimer formation necessary for the synergism seen with poly I:C and FSL-1 incubation. We hypothesise that increasing TLR2 expression results in increased dimerization with TLR6. In contrast, the TLR2 agonist HKLM does not require heterodimerization exert activity, and our data would suggest that the degree of receptor expression of TLR2 does not influence its efficacy. To lend support to the increased synergy seen with the TLR2/6 agonist compared to the more pure TLR2 agonist, mice treated with lipoteichoic acid (TLR2/6 agonist) show higher rates of preterm birth compared with those treated with peptidoglycan (PGN) which requires only TLR2 dimerization to exert its effect (39). In addition, mice treated with Pam2Cys (reliant on TLR2/6 dimerization) showed higher preterm birth rates and excessive proinflammatory cytokine production compared with Pam3Cys (reliant on TLR2/1) (112). Future work on the effect of viral priming with TLR3 prior to TLR2 agonist treatment on the formation of TLR2/1 and TLR2/6 heterodimers would provide further mechanistic insight into the role of TLRs in multi-pathogen induced preterm labour.

In summary, and illustrated in **Figure 15**, we propose that viral stimulation of TLR3 leads to activation of NF-κB, AP-1, and p-IRF3, increased TLR2 expression and increased production of cytokines, COX-2 and PGE2. We hypothesise that this initial TLR3 priming increases the availability of TLR2 to dimerise with TLR6 leaving target cells more susceptible to bacterial sensing via TLR2/6. As a result, further activation of NF-κB, AP-1 and p-IRF3, leads to augmented production of cytokines, COX-2 and PGE2 sufficient to trigger uterine contractility, fetal membrane rupture and cervical dilation and ultimately a multi-pathogen induced preterm labour.

CONCLUSIONS

Although our study confirms that the placenta has the capacity to mount a pro-inflammatory response to single pathogen exposure, multi-pathogen exposure does not cause an augmented pro-inflammatory effect but does cause an increase in the anti-inflammatory cytokine IL-10. We hypothesise that this response is needed at the maternal-fetal interface to form a protective barrier for the fetus to allow time for the mother to recover from her systemic illness, whilst keeping the fetus *in utero*. This response is in contrast to the heightened pro-inflammatory and pro-labour mediator responses of VECs and AECs seen in our model of ascending multi-pathogen infection. This response is in keeping with clinical cases of chorioamnionitis secondary to ascending infection, where often the inflammatory response drives labour and delivery to expel the fetus to protect the mother. We conclude that viruses lead to modulation of TLR mediated cellular signalling responses that could increase susceptibility to bacterial induced preterm birth, and that this is likely to be more pronounced in cases of ascending rather than systemic infection. Future, *in vivo*, experimental medicine and clinical studies should explore the potential role of local or systemic viral infection in the aetiology of sPTL.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Hammersmith, Queen Charlotte's & Chelsea Hospitals Research Ethics Committee (Ref 2002/628) and Riverside Research Ethics Committee (Ref 3358) and South East London Ethics Committee (Ref 10/H0805/54). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ZR, LS, DM, PB, and MS were responsible for the conception and design of the study. ZR, RR, CR, EA, YL, SK, and LS conducted experiments and data analysis. All figures and tables were created by ZR. ZR drafted the manuscript and all authors critically reviewed it. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.01899/full#supplementary-material

Supplementary Figure 1 | Effect of HKLM (TLR2 agonist) and FSL-1 (TLR2/6 agonist) phospho-65 in VECs, AECs, myocytes and PBMCs. Cells were treated with TLR2 (HKLM) orTLR2/6 (FSL-1) agonists and protein was extracted to quantify the expression level of NF-κB activation via Western immunoblotting. VECs, AECs, and myocytes were treated with 10^5 cells/ml and 10^8 cells/ml of HKLM in PBMCs. VECs and AECs were treated with 0.01 μg/ml of FSL-1 and myocytes, PBMCs were treated with 0.1 μg/ml. Stimulation was performed for over 24 h and protein was extracted and quantified using immunoblotting for phosphorylated p-65. P-p65 increased significantly at 4, 6, 12, and 24 h with HKLM **(A)** and 30 min, and 1 h with FSL-1 in VECs **(B)**. In AECs, p-p65 increased significantly at 30 min, 1 and 4 h with HKLM **(C)** and 30 min and 1 h with FSL-1 **(D)**. Myocytes only showed a significant increase with HKLM at 1 h **(E)** while a non-significant increase was seen at 4 h with FSL-1 **(F)**. PBMCs also showed a significant increase at 24 h with HKLM **(G)** and a trend of increase with FSL-1 **(H)**. For statistical analysis One-way ANOVA was used for normally distributed data and Kruskal-Wallis was used for data not normally distributed with Dunnett's multiple comparison test $n = 3$–6.

Supplementary Figure 2 | Effect of HKLM (TLR2 agonist) and FSL-1 (TLR2/6 agonist) on p-c-Jun in VECs, AECs, myocytes, and PBMCs. Cells and placental explants were treated with TLR2 (HKLM) orTLR2/6 (FSL-1) agonists and protein was extracted to activation of AP-1 via Western immunoblotting. VECs, AECs, and myocytes were treated with 10^5 cells/ml and 10^8 cells/ml of HKLM in PBMCs and placental explants. VECs and AECs were treated with 0.01 μg/ml of FSL-1 and myocytes, PBMCs and placental explants were treated with 0.1 μg/ml. Stimulation was performed for over 24 h and protein was extracted and quantified using Western immunoblotting for p-c-Jun. P-c-Jun increased significantly at 30 min, 1 h, and 24 h with HKLM **(A)** and 30 min and 1 h with FSL-1 in VECs **(B)**. In AECs, p-c-Jun increased significantly at 30 min and 1 h with HKLM **(C)** and 30 min, 1, 4, and 6 h with FSL-1 **(D)**. Myocytes showed a significant increase with HKLM at 1 and 4 h **(E)** while a significant increase was seen at 4 h with FSL-1 **(F)**. For statistical analysis One-way ANOVA was used for normally distributed data and Kruskal-Wallis was used for data not normally distributed with Dunnett's multiple comparison test $n = 3$–6.

Supplementary Figure 3 | Original western blots that we used in the cut blots from **Figure 8**. Activation of NF-κB and AP-1 in placental explants. The blots were taken from a priming experiment of placental explants with 25 μg/ml of poly I:C for 4 h prior to 4 h stimulation with 10^8 cell/ml of TLR2 agonist HKLM, 0.1 μg/ml the TLR4 agonist LPS or 0.1 μg/ml of the TLR2/6 agonist FSL-1 with their respective non-stimulated controls. The red histograms in both graphs reflect the summary results shown in **Figure 8**. The immunoblot results were cut and presented in **Figure 8**.

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REVIEW
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The Complement System in the Pathophysiology of Pregnancy and in Systemic Autoimmune Rheumatic Diseases During Pregnancy

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The complement system plays a double role in pregnancy exerting both protective and damaging effects at placental level. Complement activation at fetal-maternal interface participates in protection against infectious agents and helps remove apoptotic and necrotic cells. Locally synthesized C1q contributes to the physiologic vascular remodeling of spiral arteries characterized by loss of smooth muscle cells and transformation into large dilated vessels. Complement activation triggered by the inflammatory process induced by embryo implantation can damage trophoblast and other decidual cells that may lead to pregnancy complications if the cells are not protected by the complement regulators CD55, CD46, and CD59 expressed on cell surface. However, uncontrolled complement activation induces placental alterations resulting in adverse pregnancy outcomes. This may occur in pathological conditions characterized by placental localization of complement fixing antibodies directed against beta2-glycoprotein 1, as in patients with anti-phospholipid syndrome, or circulating immune complexes deposited in placenta, as in patients with systemic lupus erythematosus. In other diseases, such as preeclampsia, the mechanism of complement activation responsible for complement deposits in placenta is unclear. Conflicting results have been reported on the relevance of complement assays as diagnostic and prognostic tools to assess complement involvement in pregnant patients with these disorders.

Keywords: complement, pregnancy, obstetric complications, anti-phospholipid syndrome, systemic lupus erythematosus

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INTRODUCTION

Motherhood has become a feasible option in recent years even for women with rheumatic diseases, thanks to the marked improvement in the diagnostic modalities and therapeutic approaches developed in the field of rheumatology. Clinicians devoted to the management of pregnant women with systemic autoimmune rheumatic diseases have accumulated a particular experience in systemic lupus erythematosus (SLE), which disproportionately affects women during childbearing age (1, 2). A unique scenario in the obstetric/rheumatologic field is provided by anti-phospholipid

syndrome (APS) that manifests with pregnancy complications and vascular thrombosis. Anti-phospholipid antibodies (aPL) interfere directly with pregnancy progression as documented by the ability of aPL administered to pregnant animals to reproduce the disease, offering an invaluable tool to investigate the pathogenic mechanisms implicated in obstetric complications (3).

Pregnancy has become a relatively frequent condition in SLE and APS women over the last decade and its incidence in patients with these diseases does not appear to be different from that of normal pregnant women. However, despite the progress made in recent years, pregnancies in these conditions are still burdened by a high rate of obstetric complications, mainly in terms of pre-eclampsia, preterm delivery, and intrauterine growth restriction (IUGR) (4) and a tight control is recommended for a positive pregnancy outcome (4, 5). Thus, surrogate biomarkers are highly needed in early gestation to identify women at risk of adverse pregnancy outcome and to monitor progression thanks to serial sampling. Similarly, a better elucidation of the pathogenic steps could lead to the development of more effective targeted therapeutic strategies. In this regard, the complement (C) system has attracted much attention as candidate pathogenic effector of autoimmune and non-autoimmune pregnancy complications and surrogate biomarker to stratify obstetric risk in the general population of pregnant women. Earlier notions on C levels refer to lupus pregnancies, which has become a topic of particular interest following the observation of an association between serum C3 and C4 levels and disease flares in non-gravid patients. *In vivo* APS models have progressively unraveled the importance of C in the pathogenesis of obstetric complications. It is important to emphasize that C is a complex system with a subtle balance between protective and damaging effects. This balance undergoes physiologic modifications during gestation, which may bias the accuracy of results. It is thus timely to review available evidence on the actual and potential relevance of C as pathogenic effector of pregnancy complications and biomarker of obstetric outcome in women with systemic autoimmune rheumatic conditions.

THE COMPLEMENT SYSTEM: A DOUBLE-EDGED SWORD

Complement is a humoral component of the innate immune system that contributes to host defense neutralizing infectious agents, removing immune complexes and clearing apoptotic and necrotic cells. The protective function is accomplished through the action of biologically active products that are released as a result of C activation and exert their effects by enhancing phagocytosis, causing cell cytotoxicity, and promoting inflammation (6). Furthermore, the C system plays an important role in bridging innate and adaptive immunity, as its activation is critical for the development of adaptive immunity (7, 8). C is a versatile system organized to provide protection from a variety of targets using different recognition molecules that sense danger signal coming from foreign agents and altered self and trigger the classical, lectin and alternative activation pathways (9) (**Figure 1**). All pathways converge at the level of C3 and proceed along a common terminal pathway leading to the release of the anaphylotoxins C3a and C5a, cell deposition of C3b and assembly of the terminal C complex. The complex inserts into the cell membrane as membrane attack complex (MAC) forming membrane pores that are responsible for cell lysis. Alternatively, the complex that fails to exert a cytotoxic effect accumulates in blood and extravascular fluids as soluble SC5b-9, which can trigger cytokine synthesis, stimulates inflammation, and induces vascular leakage (10, 11).

Although C is quite selective in focusing the defense activity on dangerous targets recognized by the initiators of the activation pathways, the effector molecules released during the activation process are unable to discriminate between self and non-self and may easily bind to bystander cells. This may happen in physiological conditions, as the C system usually operates at a steady state level of activation, and the split product C3b continuously formed in the circulation and in the extravascular fluid is deposited on the cell surface. As a result, normal cells and tissues are exposed to C attack that may be destructive under conditions of unrestricted C activation. Fortunately, the potential danger that may derive from an undesired C attack is prevented by the protective effect of C regulators and inhibitors present in the fluid phase and widely expressed also on the cell surface (12). These molecules act at various steps of the C sequence and control the function of the system in various ways preventing the assembly of C complexes, favoring their disassembly, and neutralizing the activity of the biologically active products. The membrane−bound regulatory proteins CD46, CD55, and CD59 play a particularly important role in cell protection and may be used by microorganisms and cancer cells to evade C attack. They are often present on the same cells and combine their efforts to control critical steps of C activation at the level of C3 convertases (CD55 and CD46) and MAC assembly (CD59).

The exquisite selectivity of the C system for dangerous targets can be circumvented by C-fixing autoantibodies that react with self-antigens expressed on normal cells and tissues and triggers C activation leading to cell death and tissue damage. However, it is important to emphasize that C activation does not necessarily result in tissue injury, but it may also have beneficial effect contributing for instance to promote angiogenesis and wound healing (13) and also to eliminate inappropriate synaptic connections during development (14).

The role played by the C system in several clinical conditions can now be easily evaluated by functional analysis of the three pathways of C activation and the measurement of activation products recognized by antibodies directed against neoepitopes expressed on cleaved proteins.

THE GROWING IMPORTANCE OF COMPLEMENT IN HEALTHY PREGNANCY

Embryo implantation is a real challenge for the maternal immune system which is confronted with paternal antigens

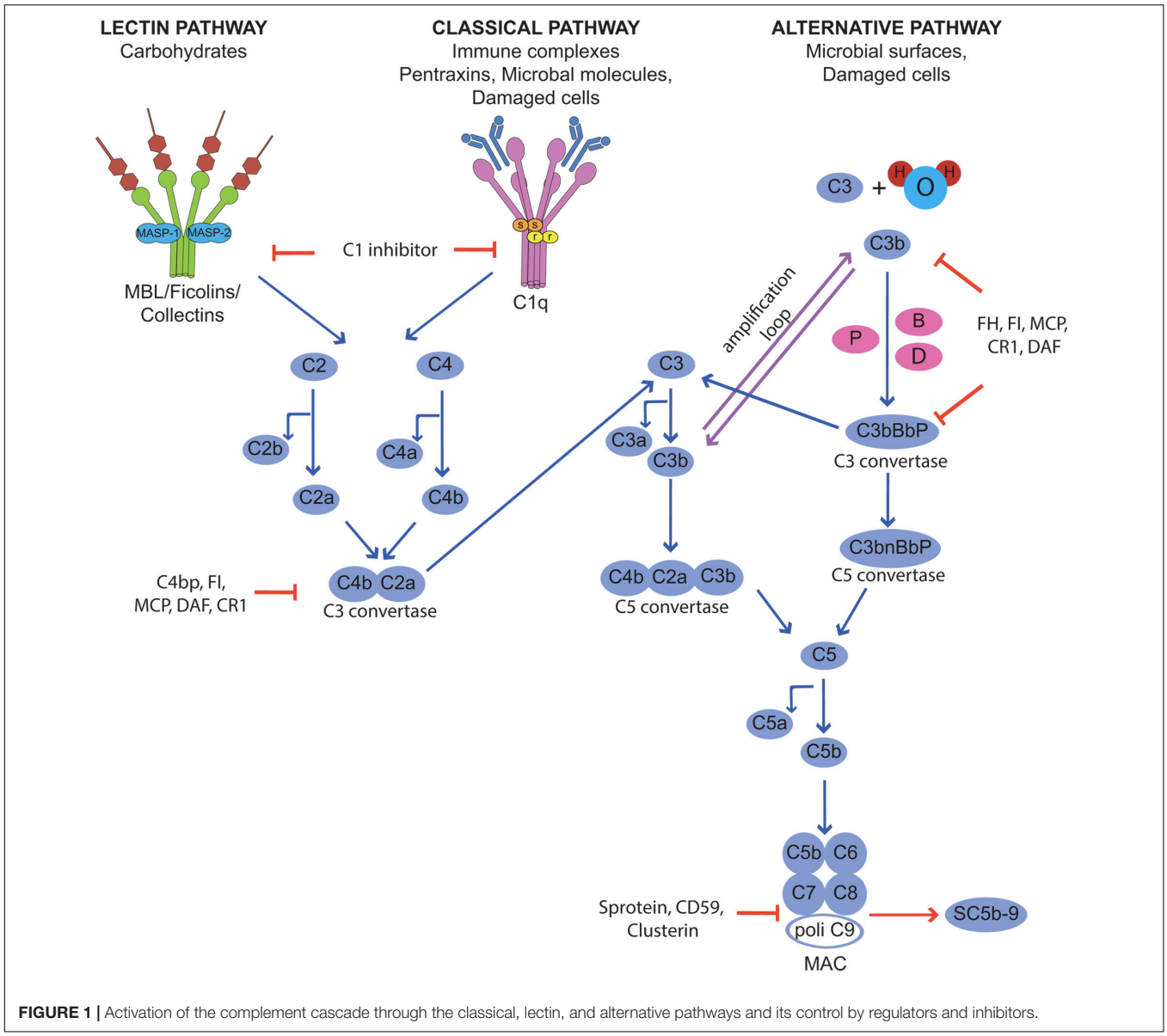

FIGURE 1 | Activation of the complement cascade through the classical, lectin, and alternative pathways and its control by regulators and inhibitors.

expressed on the embryo and the fetus and yet does not mount an immune response leading to its rejection, as it would happen with incompatible organ transplants. Both the trophoblasts that cover the villi bathed into maternal blood and the extravillous trophoblasts invading the maternal decidua represent the main source of these antigens. Villous trophoblasts form a physical double-layer barrier between the fetus and the mother and serve the important function to protect the fetus from maternal immune attack allowing only selective passage of nutrients and defense factors from the mother. Conversely, the extravillous trophoblasts depart from the anchoring villi attached to maternal decidua and contribute to tissue remodeling required for successful implantation. Besides the important role in local defense against infectious agents that may damage the fetus, C has attracted particular attention in recent years for the involvement in the physiologic changes that occur in placenta. The system is present in the maternal blood that circulates in the intervillous space and may be activated by cell-debris of trophoblasts and possibly immune complexes that have been detected in healthy pregnancy (15). Higher levels of MBL, C4, and C3 and of the activation products C4d, C3a, and SC5b-9 have been reported in pregnant women compared to non-pregnant controls (16), while the circulating levels of C1q do not fluctuate and remain relatively stable throughout normal pregnancy (17, 18). C activation in maternal blood represents a continuous risk for villous trophoblasts and may cause cell damage and impairment of the barrier integrity. This dangerous situation is kept under control by the expression of C regulatory proteins on trophoblast surface including CD55, CD46, and CD59, that act at different steps of the C sequence promoting the decay of the C3 convertases, favoring the inactivation of C3b and C4b and preventing the assembly of C5b-9 (19, 20). C components are also

synthesized by different types of cells present in decidua including macrophages, trophoblasts and endothelial cells (21) and form a local system that may operate as a local defense system. Embryo implantation in maternal uterus is associated with an inflammatory-like process induced by proteolytic enzymes that are released by extravillous trophoblast invading the decidua (21). The extensive tissue remodeling caused by trophoblast invasion leads to local recruitment of natural killer cells (NK) and other cells of the innate immune system and activation of the C system, which has limited damaging effect due to the widespread distribution of C regulators. Data collected in recent years have revealed an important role of C1q in the physiological remodeling of decidual spiral artery characterized by partial replacement of endothelial cells by endovascular trophoblasts that migrate upward from the anchoring villi. C1q is synthesized and expressed on the cell surface of both decidual endothelial cells lining the inner side of the spiral arteries and endovascular trophoblasts and serve the important function to establish a molecular bridge between the two cell types (22) (**Figure 2**). The C1q-mediated cellular crosstalk leads to the formation of mosaic vessels with an inner layer formed by the mixture of endothelial cells and trophoblasts. C1q is also synthesized and secreted by the extravillous trophoblasts as soon as they start moving away from the anchoring villi and is required for trophoblast invasion of the decidua (**Figure 2**). By binding to the extracellular matrix, C1q promotes the adhesion and the migration of extravillous trophoblasts that reach the spiral arteries forming cuffs and contribute to the vascular remodeling (23).

COMPLEMENT AND ADVERSE PREGNANCY OUTCOME

Evidence collected over the years has revealed that C plays a dual role during pregnancy. On one hand, the system promotes the physiologic changes at fetal-maternal interface required for a successful pregnancy, and on the other hand it may cause placental damage leading to impairment of the regular progression of gestation. C abnormalities have been reported in several obstetric complications including early pregnancy loss, pre-term birth, and pre-eclampsia. The relevance of C in recurrent miscarriages is supported by data obtained from animal models suggesting a key role for C5a. C activation and interaction of this anaphylatoxin with C5a receptor have been shown to induce the release of soluble vascular endothelial growth factor receptor ultimately resulting in impaired angiogenesis and adverse pregnancy outcomes (24, 25). A role in mediating abortion has also been advocated for anti-C1q antibodies as suggested by the findings that these antibodies administered to pregnant animals induce fetal loss, and that both the prevalence and the titre of anti-C1q antibodies are significantly higher in women with unexplained recurrent pregnancy loss than in healthy parous women (26). Conflicting data have been reported on the circulating levels of C components and C activity in women with early pregnancy loss. While increased levels of C3 and C4 were found in one study and proposed as predictor of fetal loss (27), hypocomplementemia was documented in another study in women with recurrent miscarriages (28). Interestingly, a significant decrease in the placental expression of C regulators

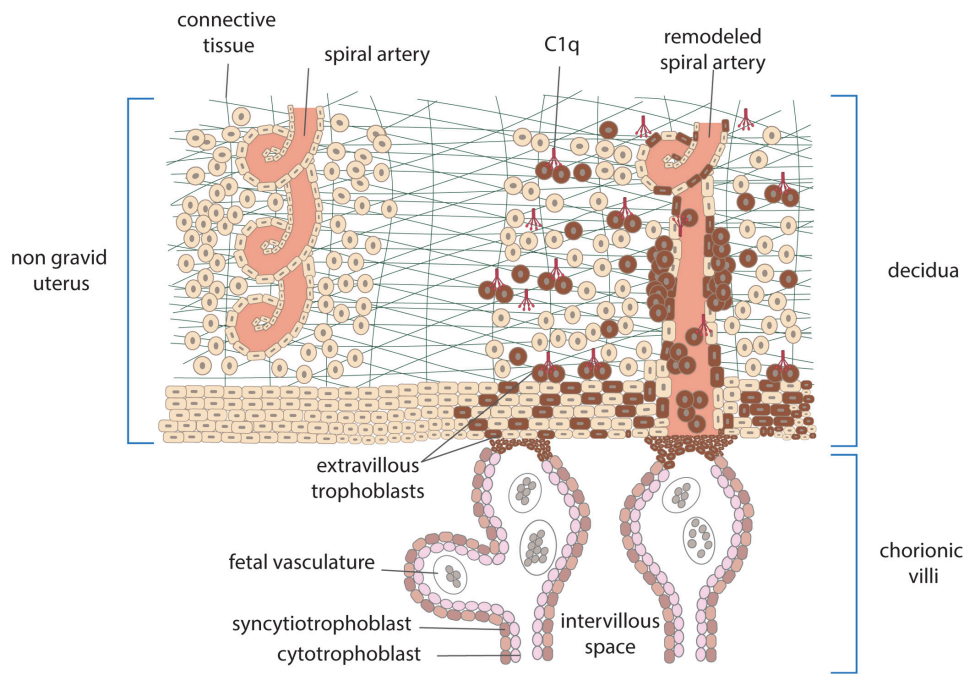

FIGURE 2 | Schematic representation of human placenta showing anchoring villi attached to maternal decidua. Extravillous trophoblasts depart from the villi and invade the decidua surrounding the spiral arteries. Other cells enter the lumen of the arteries as endovasculat trophoblasts and partially replace lining endothelial cells. C1q produced by trophoblasts is used to promote their migration and interaction with endothelial cells.

CD46 and CD55 associated with excessive C activation has been observed after spontaneous abortion, reaffirming the importance of inhibiting C activation to ensure a successful pregnancy (29). Local C activation is supported by the finding of C4d deposits documented by immunohistochemical analysis of the placentae of women with recurrent miscarriages (25). It has been estimated that approximately 20% of otherwise unexplained early pregnancy loss are due to hypocomplementemia (25).

A wealth of data has been collected over several years on the involvement of C in pre-eclampsia, a disorder of pregnancy that affects 3 to 5% of women in the late phase of pregnancy and is characterized by hypertension and proteinuria. Dysregulated angiogenesis is believed to be implicated in the pathogenesis of the disease, as documented by elevated circulating levels of soluble vascular endothelial growth factor receptor 1 (sFlt-1) (30). Animal models have shown that C1q-deficient pregnant mice manifest the key features of human disease such as hypertension, albuminuria, endothelial dysfunction, decreased placental vascular endothelial growth factor, and elevated levels of sFlt-1 providing convincing evidence that C1q protects against pre-eclampsia (31). Consistent with this *in vivo* observation, Agostinis, and colleagues (17) published data indicating that the serum levels of C1q was markedly decreased in both early and late onset forms of pre-eclampsia. Likewise, women with early onset pre-eclampsia are twice as likely to carry deficiency in C4A or C4B suggesting that C4 may also contribute to prevent the onset of pre-eclampsia (32). The reduced concentration of C1q observed in patients with overt pre-eclampsia cannot be used as predictive marker of the disease because the analysis of serum samples collected at an early phase of pregnancy from women who later developed preeclampsia failed to show a decrease in C1q level (17). Mannose binding lectin (MBL) seems to have an opposite effect to that of C1q since the level is elevated in patients with severe pre-eclampsia (33, 34). Furthermore, MBL strongly inhibits the interaction of extravillous trophoblast with C1q and interferes with the process of cell migration (34), suggesting the contribution of MBL to the pathogenesis of the disease.

It has been postulated that C activation following placental ischemia may induce hypertension and impair fetal growth via the endothelin pathway (35). Analysis of C activation products in patients with pre-eclampsia has revealed increased serum levels of C3a, C5a, and SC5b-9 (16, 36, 37) and C activation products have also been detected in the urine of patients with a severe form of the disease as a result of C-mediated renal injury (38). High levels of the activation product of the alternative pathway Bb have been observed in the early phase of pregnancy in women who later developed pre-eclampsia and proposed as an early biomarker of this disease. The finding of high mRNA expression of the membrane C regulators CD55 and CD59 in placenta specimens from pre-eclamptic women has been interpreted as a compensatory attempt to limit local C activation (32). C4d is the C split product most frequently seen in pre-eclamptic placentae, particularly on syncytiotrophoblast, with focal or diffuse staining patterns (39), and the degree of C4d and MAC deposition in the placental tissue is strongly correlated with sFlt1 levels in pre-eclamptic patients (40). Available evidence suggests that activation of the C system is involved in spontaneous preterm birth. Lynch and coworkers (41) measured the circulating levels of Bb, a marker of alternative pathway activation, in pregnant women in the early phase of gestation and found that those with elevated levels were more likely to experience preterm delivery. They propose Bb as a predictor of this adverse pregnancy outcome that develops in late gestation before 34 weeks. An essentially similar conclusion was reached measuring the levels of C3a under the same experimental conditions and again higher levels were associated with preterm birth (42). The increased levels of C5a observed in women with preterm delivery suggest that C5a, by reacting with C5aR, plays a role in the pathogenesis of preterm labor (43).

COMPLEMENT AND OBSTETRIC ANTI-PHOSPHOLIPID SYNDROME

Anti-phospholipid syndrome is an acquired prothrombotic condition characterized by vascular occlusive events occurring in vessels of different size and/or obstetric complications. Adverse pregnancy outcomes include three or more spontaneous abortions before 10 weeks of gestation, one or more unexplained fetal death at or beyond week 10 of gestation, one or more preterm delivery before 34 weeks due to severe pre-eclampsia, HELLP syndrome (hemolytic anemia, elevated liver enzymes, low platelet count) or placental insufficiency. aPL are the serum biomarkers of APS, routinely detected by a functional assay, named lupus anticoagulant (LA), and two solid phase assays identifying IgG and IgM antibodies against cardiolipin (aCL) and beta2-glycoprotein I (anti-β2GPI). β2GPI, the main antigenic target of aPL, is a five domain (D) glycoprotein comprising four C control protein (CCP)-like domains (DI-DIV) and one domain (44) with a large lysine loop which allows β2GPI to interact with anionic phospholipids and other molecules on cell surfaces, coagulation factors, platelets, and complement (45). Antibodies against β2GPI co-localize with their target antigen on trophoblasts and decidual endothelial cells in immunized animals that had received fluorescein-labeled β2GPI (46) and interfere with pregnancy progression by impairing the function of the developing placenta. The antibodies exert their effect on the maternal side, promoting a negative imbalance of angiogenic factors that inhibits endometrial angiogenesis. Furthermore, they act on trophoblasts inducing apoptosis and inhibiting the secretion of β human chorionic gonadotropin and matrix metalloproteinases (MMP) required for invasion of decidua, and in complex with β2GPI activate the classical pathway of the C cascade (47). Several clinical studies have examined the activation of the C system in pregnant patients with APS and its contribution to pregnancy complications. Decreased serum levels of C4 and C3 have been reported in approximately one third of patients with APS (48) and the follow-up of these patients throughout pregnancy revealed that the C4 and C3 levels remained persistently low compared to the values of control pregnant women when normalized for the trimester of gestation (49). As shown in **Table 1**, lower levels of C3 and C4 were found to correlate with adverse obstetric outcomes in some studies (50, 51), but not in others (49, 52). Data obtained from a prospective

TABLE 1 | Studies assessing the correlation of C3 and C4 serum levels with obstetric outcome in pregnant women with anti-phospholipid syndrome.

Author, year	Number of APS patients/ pregnancies	Study design	Timing of C testing	Control group	Main findings
Ruffatti, 2011 (50)	114/114 All PAPS	Retrospective	Baseline and at the end of pregnancies	None	- Hypocomplementemia was associated with adverse pregnancy outcome at univariate analysis.
De Carolis, 2012 (51)	47/47 PAPS/SAPS (No SLE)	Prospective	Within 20 gestational weeks	None	- Hypocomplementemia was associated with fetal loss and preterm delivery at univariate analysis. - Women with hypocomplementemia had lower neonatal birth weight. - Hypocomplementemia was not associated with PE and IUGR at univariate analysis.
Reggia, 2012 (49)	45/57 PAPS	Retrospective	I-II-III trimester	49 women with UCTD/SjS 175 healthy pregnant women	- Hypocomplementemia was not associated with adverse pregnancy outcome in PAPS women. - Women with PAPS had lower C3 and C4 than healthy women, but similar to UCTD and SjS.
Deguchi, 2017 (52)	69/81 PAPS/SAPS (mainly SLE)	Retrospective	NS	None	- Hypocomplementemia was not associated with pregnancy loss, premature delivery and IUGR. - Hypocomplementemia was associated with hypertension at multivariate analysis.

PAPS, primary anti-phospholipid syndrome; SAPS, secondary anti-phospholipid syndrome; C, complement; NS, not specified; SLE, systemic lupus erythematosus; UCTD, undifferentiated connective tissue disease; SjS, Sjögren syndrome; PE, pre-eclampsia; and IUGR, intra-uterine growth restriction.

study of APS pregnant women led De Carolis et al. (51) to suggest that reduced C3 and C4 levels should be regarded as predictors of lower neonatal birth weight and preterm delivery. A multicenter study performed in Japan showed that low levels of C3 and C4 represent a risk factor for hypertensive disorders of pregnancy (52). Evaluation of biologically active circulating products of the C system in APS pregnant patients offers more direct insights on C activation in this clinical condition. Blood samples from 161 aPL positive women including 60 with SLE were analyzed for the presence of Bb and SC5b-9 and increased levels of both activation products were found in all patients with adverse obstetric outcome (53). This finding has been confirmed by a more recent study, reporting higher C5a and C5b-9 levels in APS pregnant patients with pregnancy complications compared to healthy pregnant women (54). More convincing evidence supporting the role of C in inducing aPL-dependent placental damage and pregnancy failure has been obtained from the immunohistochemical analysis of placental tissue for C deposits. The presence of C4d in placentae from APS women has been documented at the fetal-maternal interface, in particular on syncytiotrophoblast basement membrane, and to some extent also on extravillous trophoblasts of the basal plate by three groups (55–57). C4 deposits were found to be associated with intrauterine fetal death (55) and placental abnormalities including decidual vasculopathy, increased syncytial knots, and villous infarcts (57). Two groups have documented deposits of C5b-9 mainly localized on extravillous trophoblasts of placentae from aPL-positive women and observed no difference in the staining intensity between APS and control groups (56, 57). These findings are in contrast with the data obtained by Scambi et al. who reported higher levels of C5b-9 solubilized from APS placentae compared to controls, in particular in APS patients who experienced a pregnancy complication (54). Our group has conducted a prospective study on 13 APS patients with medium to high titers of anti-β2GPI antibodies and positive LA who had pregnancies that resulted in one abortion, four fetal losses, and eight preterm deliveries (58). Histological and immunohistochemical analysis revealed placental abnormalities characterized by decidual vasculopathy and intervillous thrombi and deposition of IgG, IgM, C1q, C4, and C3 suggesting C activation through the classical pathway. Interestingly, C5b-9 was detected in all placentae and was localized on the surface of syncytiotrophoblasts, intervillous fibrin and decidual vessels supporting its contribution to tissue damage. Taken together, these findings suggest that complement activation is involved in placental pathology acting both on villous trophoblast and on endothelial cells of decidual vessels (**Figure 3**). Animal models of APS developed by infusing patient's IgG have provided key information on the role played by the C system in eliciting placental abnormalities and adverse pregnancy outcomes. Mice deficient in C3, C4, C5, and factor B were found to be resistant to aPL-induced fetal loss (59–61) and similar results were obtained using a C3 convertase inhibitor, a C5a receptor antagonist or anti-C5 antibodies (60). C5a has been identified as the main mediator of fetal injury by interacting with C5aR expressed on polymorphonuclear leukocytes and by stimulating the release of tissue factor and tumour necrosis factor (TNF)-α, which in turn promotes inflammation (62, 63). Given the growing evidence implicating C activation as key contributor to the pathogenesis of the clinical manifestations of APS, C inhibitors have been considered good candidates for the therapy of APS. Although the neutralizing anti-C5 antibody eculizumab has been used successfully in treating patients with catastrophic APS and in preventing re-thrombosis in patients undergoing surgical intervention (58, 64), very few information is available on its use in APS pregnant patients except for an anecdotal report of a patient who received eculizumab to prevent severe pregnancy complications (65). A non-C fixing anti-β2GPI monoclonal antibody that was shown to prevent fetal loss in aPL-treated pregnant mice offers an alternative therapeutic approach (66). The advantage of this antibody is to target the β2GPI protein constitutively expressed on villous and extravillous trophoblasts as well as on the endothelium of decidual vessels with relatively high affinity and to compete with antibodies from APS patients.

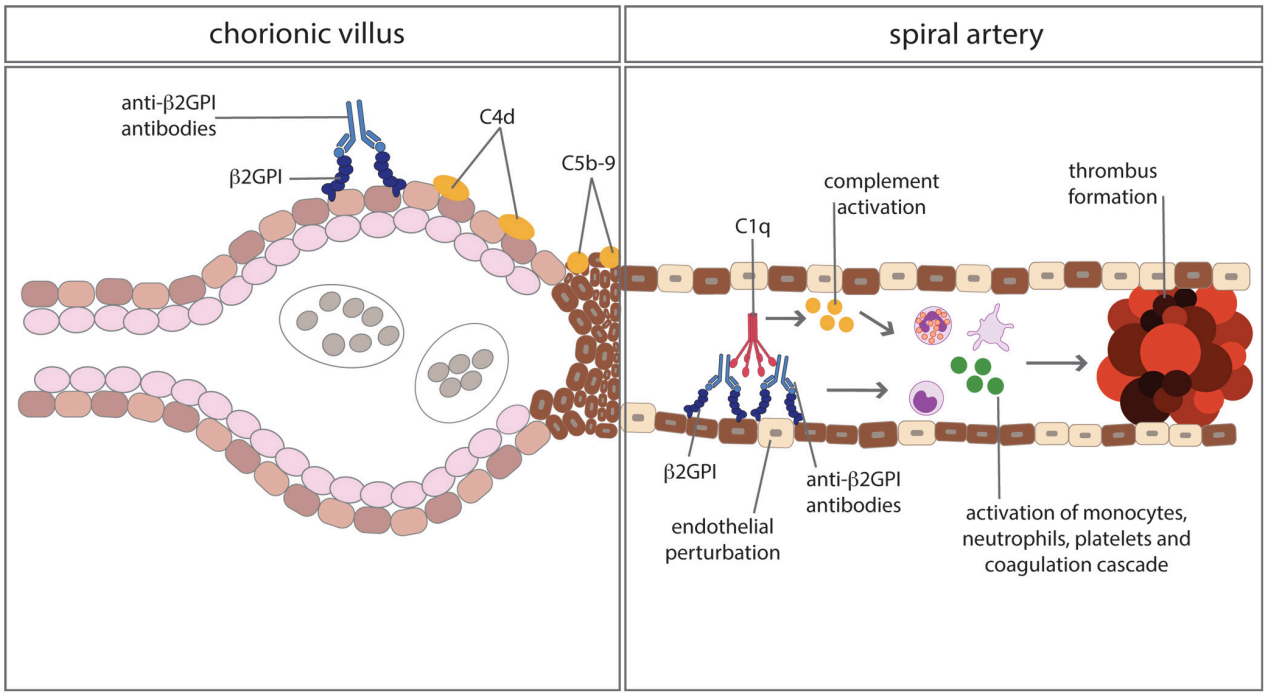

FIGURE 3 | Complement-mediated biological effects at placental level in anti-phospholipid syndrome. Complement activated by antibodies interacting with β2GPI bound to trophoblasts and vascular endothelium of decidual vessels leads to cell damage and promotion of thrombus formation.

COMPLEMENT AND SYSTEMIC LUPUS ERYTHEMATOSUS IN PREGNANCY

Systemic lupus erythematosus is a complex multisystem autoimmune disease with a highly heterogeneous presentation ranging from laboratory abnormalities to multi-organ inflammation and failure and characterized by the production of autoantibodies directed against double stranded DNA and several other autoantigens. The clinical manifestations of SLE are underpinned by several etiopathogenic mechanisms, such as deregulated production of autoantibodies against cellular constituents, abnormal cytokine release, innate, and adaptive immune alterations. Impaired clearance of apoptotic debris is believed to mediate sustained antigen presentation to B cells, ultimately resulting in exaggerated autoantibody production (67). C activated by immune complexes formed in the circulation and at tissue level following interaction of autoantibodies with their target antigen is involved in the pathogenesis of the clinical manifestations of SLE. Some of these autoantibodies are directed against C components, as is the case of anti-C1q antibodies detected in nearly one third of patients with lupus nephritis and thought to have important pathogenic effects in the development of the disease (68). Immune complexes containing anti-C1q antibodies were found to be more potent C activators than classical immune complexes (69). Deposits of C components and C activation products including the terminal complex have been documented in the kidney of SLE patients co-localized with immune complexes suggesting the involvement of C in tissue damage (70). Data accumulated over several years have shown that C plays a paradoxical role in SLE. On the one hand, C activated by immune complexes stimulates inflammation, and causes tissue lesions. The important role played by C is suggested by the beneficial effect of eculizumab in patients with severe lupus nephritis resistant to conventional therapy (71, 72). Further evidence supporting the contribution of C activation to SLE pathogenesis is provided by lupus-prone mouse models, such as NZB/W and MRL/lpr mice, that share with the human disease similar features including autoantibodies production, hypocomplementemia, circulating and glomerular-bound immune complexes, and severe nephritis (73). Treatment of these mice with anti-C5 monoclonal antibodies resulted in improvement of nephritis, reduced proteinuria and prolonged survival (74). On the other hand, C deficiency is now recognized to be a risk factor for SLE development, based on the finding that genetic deficiencies of the early components of the classical pathway from C1q to C4 are associated with the onset of SLE (75). C1q-deficient individuals have the highest susceptibility to SLE due to the role of C1q in the removal of apoptotic cells. The disease occurs in up to 55–75% of individuals with this genetic defect and presents with characteristic clinical features including early age of onset, no gender predilection, low frequency of anti-dsDNA antibodies, prominent photosensitivity, and fewer renal symptoms (76). Given this background, it is not surprising that the C system has attracted particular attention as an important marker of disease activity in SLE patients. It's long been known that C activation in SLE is accompanied by a secondary reduction in circulating C levels and increase in C split products. Importantly, the decrease in C1q, C3, and C4 levels

correlates with disease activity and precedes clinically evident flare (77), even though the decrease in C levels are not invariably associated with disease flares (78). Despite the technical and biological limitations, measurements of C3 and C4 have been included not only in the recent classification criteria for SLE, but also in the disease activity indexes such as SLEDAI (79). Recently, C deposition on immune cells was proposed as a more robust method to diagnose and monitor SLE and a panel of parameters including C4d-deposition on B cells and erythrocytes was suggested (80, 81).

Pregnancies in patients with SLE have always been regarded to be at risk, even though the rates of fetal loss and maternal mortality have steadily decreased over the years (82). However, pregnant women with SLE still display an increased hazard of premature delivery and IUGR irrespectively of disease activity, while the odds for pre-eclampsia is elevated in women with active disease only (83). Hypocomplementemia during gestation has been identified as one of the multiple predictors of poor pregnancy outcome in SLE pregnancies including high disease activity in the 6 months before conception, use of anti-hypertensive medications, non-white ethnicity, aPL positivity, and a history of nephritis or active nephritis at conception. Data on C levels in SLE pregnant women vary considerably in different studies, depending on the composition of the study cohort. Thus, hypocomplementemia is prevalent in certain disease manifestations, such as lupus nephritis. C4 level is a more reliable marker of renal involvement in SLE since low C4 at baseline and a history of previous kidney disease have been found to be independently associated with a higher risk of developing active nephritis in pregnancy (84). Pregnant women with lupus nephritis display significantly lower levels of C3 and C4 more often than other SLE subjects (85). Data on the association of C levels with poor pregnancy outcome can be obtained from studies that assess several clinical and laboratory variables of SLE as predictors of adverse obstetric outcomes, but there is no universal agreement on the clinical significance of complement levels as biomarkers in lupus pregnancies. Indeed, few authors deny a predictive role for C3 and C4 whereas in other studies low C3 and/or C4 levels have been associated with adverse pregnancy outcomes such as spontaneous abortion, premature birth and stillbirth (86–88). Unfortunately, a control group of healthy pregnant women was not included in these studies, and the C levels were not normalized for the gestational age, thus limiting the conclusions on the relationship between C3 and C4 levels and pregnancy outcomes. Changes in C levels were evaluated in 386 SLE patients throughout gestation in the PROMISSE study, and a smaller increase in C3 levels in the second and third trimesters was observed in women with adverse obstetric outcome compared to women with uneventful pregnancy, though the difference was not significant (87). Because of the well-known fluctuation of C levels throughout pregnancy, it is not surprising that low *C* values may not correlate with disease activity in pregnant patients with SLE except when they are lower than those expected in normal pregnant women at the same gestational age (89, 90). The C activation products Ba, Bb and SC5b-9 represent more sensitive indicators of C activation and may be useful to predict and diagnose flares in pregnant SLE patients (89). In the PROMISSE cohort, these markers of C activation (Bb and SC5b-9) were detected in the circulation in early gestation among those SLE/aPL + women who later developed pregnancy complications (53). Analysis of placentae from aPL-negative SLE patients by Matrai and colleagues revealed signs of tissue malperfusion, infarction and intervillous thrombi and increased deposits of C4d and C5b-9 on syncytiotrophoblasts and extravillous trophoblasts compared to controls (56). The extent of C4d deposition was found to be inversely correlated with low placental and birth weight (91).

CONCLUSION

Complement is a versatile system that shows exquisite adaptation to environmental changes and is able to recognize dangerous exogenous and endogenous agents and structures. Besides exerting protective functions, C is now recognized to promote functions unrelated to host defense including tissue repair and remodeling. Data collected in recent years have shown that C plays an important role in the structural organization of placenta at fetal-maternal interface contributing to vascular remodeling of spiral arteries in maternal decidua, a critical process required for the regular progression of pregnancy. However, placenta is not exempt from potential damage that may derive from activation products released as a result of general or local C activation leading to adverse pregnancy outcomes. C exerts a direct damaging effect in clinical situation such as APS as suggested by the failure of antibodies to induce fetal loss in C-deficient animals or treated with neutralizing antibodies to C components. The finding of C components at placental level both in APS patients and in animal models further supports the involvement of C in the onset of placental alterations and has both diagnostic and therapeutic implications. Measurement of C levels is routinely performed in many obstetrics/rheumatology joint clinics to monitor APS pregnancies, but hypocomplementemia does not seem to be a reliable marker to predict pregnancy loss in these patients. More sophisticated and sensitive methods have been proposed to monitor C activation, as is the case of cell-bound C split products. The recent report of a higher percentage of C4-positive B lymphocytes, erythrocytes, and platelets in patients with obstetric and thrombotic manifestations compared to controls (92) suggests that this assay may be an interesting tool to explore C activation in pregnant women with APS or SLE. Preventive treatment with neutralizing antibodies or other reagents aimed at controlling C activation is a promising therapeutic approach in APS. Indeed, heparin currently used as treatment of choice for pregnant APS women together with low dose aspirin was shown to inhibit C activation and to prevent cell binding of β2GPI as a result of interaction with the heparin-binding site located on DV (93). C is most likely involved in the adverse pregnancy outcome observed in patients with SLE, a prototypical C-mediated disease. Currently, measurement of C levels is requested by the obstetricians to differentiate between nephritis and pre-eclampsia in SLE patient with proteinuria. C3 and C4 levels normally rise in patients with pre-eclampsia, while drops

in C3 and C4 levels, coupled with a rising anti-dsDNA titre, are more likely associated with disease flares (94). However, despite many studies conducted to identify predictors of adverse outcome in lupus pregnancies, there are no clear data supporting the association between fluctuation of C levels and disease flare during gestation. Conclusive data on C-mediated tissue damage associated with adverse pregnancy outcomes can be provided by the histological analysis of placenta samples from patients. To be informative, the results should be compared with those of healthy controls of the same gestational age as the phenotype changes with the progression of gestation. To date, C4d has emerged as the most interesting biomarker of C activation in placenta specimens. This is not surprising since C4d, like C3d, binds covalently to the target cell surface and, being highly stable, acts as a fingerprint of C-mediated activation leading to tissue injury. In conclusion, there are indications to suggest that C is involved in complicated pregnancies, although the precise mechanism by which C is activated is not always clear and remains to be determined.

AUTHOR CONTRIBUTIONS

PM and FT designed the study. CC, PL, and LT retrieved the relevant literature. CC and FT drafted the manuscript. PL prepared the figures. PM, LT, and FT revised the literature critically for important intellectual content. All authors approved the final version of the article.

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REVIEW
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Innate Immune Mechanisms to Protect Against Infection at the Human Decidual-Placental Interface

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During pregnancy, the placenta forms the anatomical barrier between the mother and developing fetus. Infectious agents can potentially breach the placental barrier resulting in pathogenic transmission from mother to fetus. Innate immune responses, orchestrated by maternal and fetal cells at the decidual-placental interface, are the first line of defense to avoid vertical transmission. Here, we outline the anatomy of the human placenta and uterine lining, the decidua, and discuss the potential capacity of pathogen pattern recognition and other host defense strategies present in the innate immune cells at the placental-decidual interface. We consider major congenital infections that access the placenta from hematogenous or decidual route. Finally, we highlight the challenges in studying human placental responses to pathogens and vertical transmission using current experimental models and identify gaps in knowledge that need to be addressed. We further propose novel experimental strategies to address such limitations.

Keywords: innate immunity, uterine-placental interface, trophoblast, decidua, vertical transmission

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INTRODUCTION

The human placenta is the temporary extra-embryonic organ that is present only during pregnancy and is the anatomical boundary between the mother and fetus. It has a range of functions including transport of nutrients and gases, and hormonal production (1). The placenta forms a physical, selective barrier between the maternal and fetal circulations, preventing transfer of pathogens. The uterine mucosal lining, the endometrium, is transformed into the decidua during early pregnancy (2). A range of innate immune mechanisms can respond to pathogens in both the decidua and the placenta (3, 4). The maternal-fetal interface is a protective barrier against pathogens, but some pathogens can transfer from the mother to fetus by different routes and cause fetal infection (3, 4).

Vertical transmission during pregnancy can occur on distinct boundaries between the mother and the fetus: (i) the intervillous space (IVS), where placental villi is in direct contact with the maternal blood, (ii) the implantation site or decidua basalis, where maternal cells are in direct contact with the invading fetal trophoblast, and (iii) the fetal membranes, which are in direct contact with the uterine cavity (5). Defense mechanisms in the cervix, such as the production of mucus and antimicrobial peptides (AMP), limit ascending infection from pathogens present in the lower genital tract, that otherwise may access the uterine cavity (6). However, some pathogens can escape antimicrobial strategies at the cervix and ascend to the uterus, where they can bypass the fetal membranes and lead to the inflammation of the membranes- also known as chorioamnionitis- and infection of the amniotic fluid (7, 8). Pathologic and immune features of chorioamnionitis and

intra-amniotic infection are generally associated with bacterial invasion and inflammation [refer to (8, 9) for a comprehensive review on these mechanisms]. Here, we focus on infections and innate immune mechanisms at the uterine-placental interface—cases (i) and (ii) (**Figure 1**).

Infections at the uterine-placental interface are commonly associated with viruses, parasites and few bacteria (**Table 1**). Viral pathogens such as human cytomegalovirus (HCMV), Zika (ZIKV), and rubella virus are the most common vertically transmitted pathogens through the decidual-placental interface (**Table 1**) (26, 27). Non-viral pathogens, such as *Toxoplasma gondii* and *Listeria monocytogenes*, can cross the placental barrier via cell-to-cell transmission (**Table 1**) (28, 29). Fetal infection can result in various forms of congenital anomalies in humans (**Table 1**). Understanding the pathogenic mechanisms used by infectious agents is central to preventing vertical transmission and controlling infection during pregnancy.

How the innate immune cells and mechanisms in the placenta and the uterus recognize and respond to protect both the fetus and mother remains controversial due to technical and ethical constraints. However, there are several different models currently used to interrogate the uterine-placental interface in pregnancy. Firstly, mice are frequently used as a pregnancy model for infection. Although the murine models have provided important insights into the pathogenesis of various infection agents in the context of pregnancy, there are still limitations with this approach. The anatomy of placentation, length of gestation, and use of inbred strains, make extrapolation to humans problematic (30, 31). Secondly, a range of human trophoblast and choriocarcinoma cell lines are used as *in vitro* models for infection with pathogens. In contrast to the first trimester trophoblast *in vivo*, these cell lines do not recapitulate normal human trophoblast characteristics such as expression of the human leukocyte antigen (HLA) class I and methylation of *ELF5* (32, 33). Thirdly, human primary placental explants are frequently used. The syncytium dies rapidly in these cultures and it is virtually impossible to standardize the types of villi sampled (30). Therefore, these *in vitro* experimental factors should be taken into careful consideration when interpreting studies of infection of trophoblast.

In this review, we cover the innate immune features of the decidual-placental interface throughout gestation. We identify the gaps in knowledge and highlight the limitations of current studies and experimental models. Finally, we discuss novel experimental strategies for understanding how infection affects pregnancy in humans.

Physiology of the Placenta Throughout Gestation

The trophoblasts of the placenta are the barrier between fetal and maternal tissues. They are derived from the trophectoderm, the outer layer of the blastocyst that forms an inner mononuclear layer with an outer primary syncytium following implantation (34). The trophoblast in contact with the maternal cells can be: (i) syncytiotrophoblast (SCT), a single layer multinucleated, syncytial layer formed by fusion of the underlying villous cytotrophoblast (VCT), and (ii) extravillous trophoblast (EVT), that invade from the cytotrophoblast shell and anchoring villi into the transformed maternal endometrium, the decidua (2).

The function of EVT is to transform the uterine spiral arteries so that maternal blood is delivered to the intervillous space at low pressure. The arteries are surrounded by interstitial EVT that destroys the smooth muscle cells of the arterial media, known as "fibrinoid" change (35, 36). Subsequently, endovascular EVT (eEVT) moves down the spiral arteries from the placenta-decidua boundary (35). These eEVT form a plug of cells, limiting surges of arterial blood from damaging the delicate villi. EVT invasion transforms the arteries to support optimal regulation of blood flow into the placenta during fetal development (36). The plugs dissipate between 8 and 10 weeks of gestation when the full hemochorial circulation is established (37). Maternal blood then flows into the IVS, and establishes direct contact with the SCT allowing for proper nutrient and gas exchange between the mother and the fetus.

HOFBAUER CELLS: THE TISSUE RESIDENT IMMUNE CELLS OF THE PLACENTA

Hofbauer (HB) cells are fetal macrophages of the human placenta (38). HB cells can be detected in the placental villous stroma as early as 3 weeks post-conception and are present throughout pregnancy (1, 39). They are likely to have a variety of functions including control of villous remodeling and differentiation, hormonal secretion, and trophoblast turnover (1, 40). Several lines of evidence have led to the postulation that HB cells may have a role in infection during pregnancy. HB cells with ZIKV viral particles detected intracellularly have been shown (41, 42). Human immunodeficiency virus 1 (HIV-1) has also been detected in HB cells from first trimester infected placenta (43). Whether the HB cells can serve as a reservoir or limit virus replication is still unknown. Isolated HB cells from healthy term placenta show elevation of pro-inflammatory cytokines such as IL-6, MCP-1, IP-10, and IFN-α upon *in vitro* infection with ZIKV (44). HB cells from the first trimester placenta are also permissive for ZIKV infection and replication (23). However, this must be interpreted with caution because *in vitro* culture of HB cells do not entirely recapitulate the complexity of villous stromal microenvironment, such as presence of hormone and growth factors, all of which will influence the function and activity of HB cells (45).

MATERNAL BLOOD AND SCT INTERFACE

The SCT is the barrier between maternal blood and the placental core as it separates the IVS from the underlying fetal villous stroma. Blood-borne pathogens such as viruses and parasites can potentially be transmitted through the SCT barrier (**Figure 1**).

How can pathogens cross the SCT barrier and the VCT to infect the villous stroma? Although the SCT is an efficient barrier due to its stiff, highly dense actin cytoskeleton network and continuous membrane (46), the syncytium undergoes

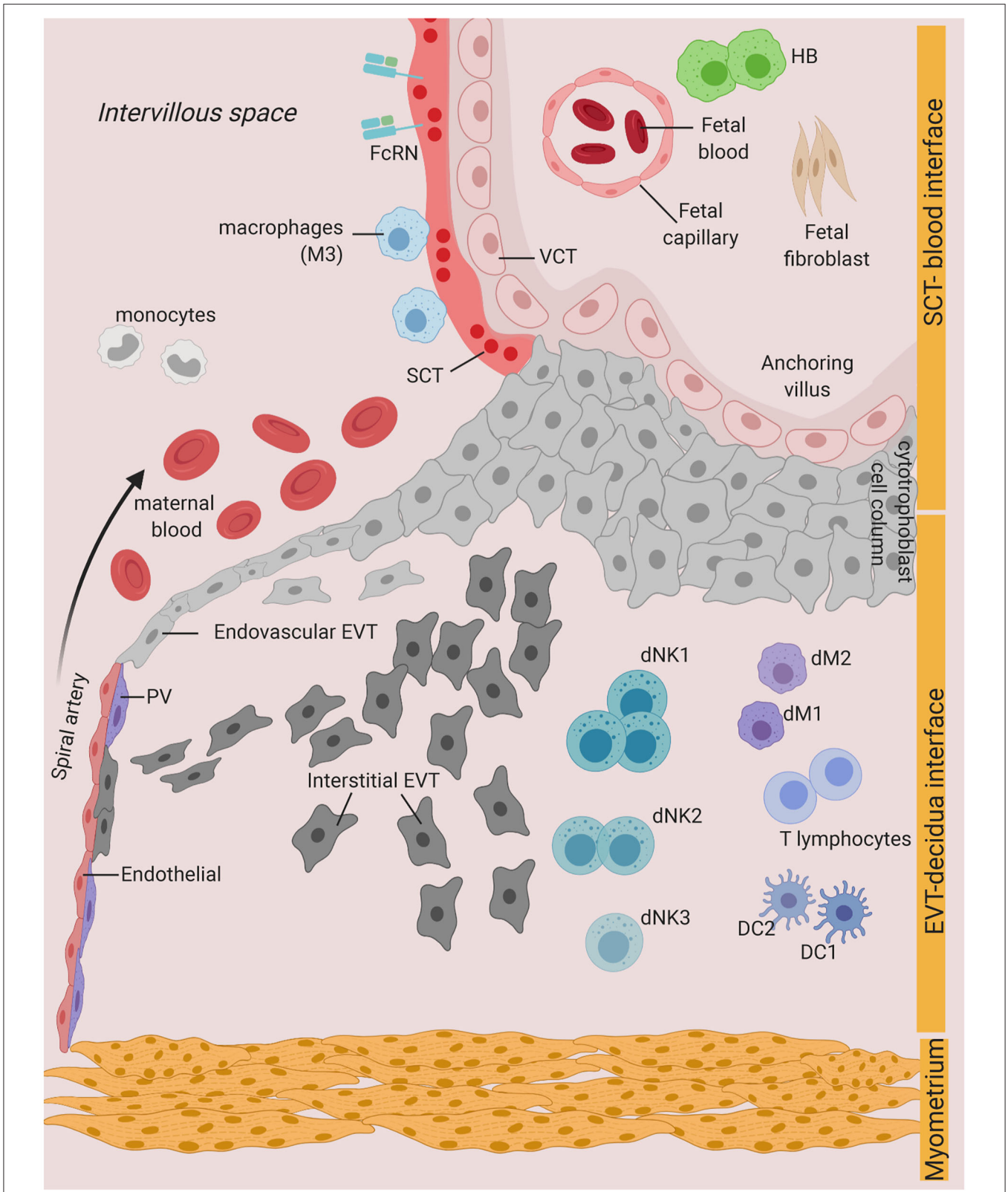

FIGURE 1 | Possible infection and vertical transmission route at the maternal-fetal interface. Illustration representing the anchoring placenta villi of early pregnancy, with onset of maternal blood circulation bathing the intervillous space. SCT-blood interface represents the SCT barrier exposed to maternal blood and immune cells. EVT-decidua interface represents the interface between EVT and maternal decidua cells. Major cell types of placenta trophoblast and decidua from Vento-Tormo et al. (10) are represented. SCT, syncytiotrophoblast; VCT, villous cytotrophoblast; EVT, extravillous trophoblast; DC, dendritic cell; dNK, decidua Natural killer cells; dM, decidua macrophages; HB, Hofbauer cell; PV, perivascular cells; FcRN, neonatal Fc receptor. Figure is created by BioRender.com.

TABLE 1 | Vertically transmitted pathogens with clinical evidence from natural human infection.

Species	Lifestyle	Life cycle and pathogenesis	Clinical manifestations	Evidence of cellular tropism in the human placenta or decidua by histology or PCR	References
Chlamydia trachomatis	Intracellular bacteria	Formation of reticulate body inside host cell allows for rapid replication Conversion of reticulate body to elementary body inside host cell promotes the release of infectious bacteria to neighboring cell	Ectopic pregnancy, stillbirth, preterm labor, blinding corneal injury in neonates, neonatal pneumonia	Whole placenta, glandular epithelial cells, unidentified decidual cells	(11)
Group B Streptococcus (*Streptococcus agalactiae*)	Non-motile, extracellular bacteria	Beta-hemolytic Strong adherence to epithelial layer Able to form biofilm	Neonatal GBS (sepsis and meningitis), preterm birth	Amniotic epithelium, amniotic fluid, chorion, decidua	(12)
Listeria monocytogenes (Listeriosis)	Motile intracellular bacteria	Utilize two bacterial surface proteins (internalin A and B) to invade various non-phagocytic cell types Able to escape phagosome-mediated lysis and multiply rapidly in host cytoplasm Able to spread to adjacent cell through host cell actin polymerization	Spontaneous abortion, stillbirth, preterm labor	Placenta trophoblast	(13)
Coxiella burnetii (Q fever)	Intracellular bacteria	Able to escape phagosome-mediated lysis in macrophage	Spontaneous abortion, preterm delivery, fetal death	Placenta (unknown cell type)	(14)
Treponema pallidum	Motile spirochaete, extracellular bacteria	Able to transverse tight-junction between endothelial cells Highly motile	Congenital syphilis	Placenta (unknown cell type)	(15, 16)
Toxoplasma gondii (Toxoplasmosis)	Intracellular parasite	Able to infect and replicate within various host cell types Able to switch between non-motile (for replication) and motile state (for egress and invasion into new host cell)	Congenital toxoplasmosis, stillbirth	Placenta trophoblast	(17)
Trypanosoma cruzi (Chagas)	Intracellular and extracellular parasite	Able to propagate in various host cells and escape Progeny released by host cells are motile, and able to infect distal tissue or organs	Stillbirth, preterm labor	SCT, villous stroma, placenta basal plate	(18)
Herpes simplex virus 1, 2 (HSV-1/2)	dsDNA virus	Able to cross through skin lesions and epithelial mucosal cells Poor antibody neutralization to viral glycoprotein D (gD) Vertical transmission rate is very low	Spontaneous abortion, intrauterine growth restriction, preterm labor, neonatal herpes	Decidua	(11)
Human cytomegalovirus(HCMV)	dsDNA virus	Easily transmitted through bodily fluid Poor antibody neutralization to viral glycoprotein B (gB) Can establish lifelong latency in myeloid cells	Variable; neonatal neurodevelopmental damage and hearing loss	VCT, decidua, amniotic membrane	(19)
Rubella	ssRNA virus	Able to enter the lymphatic system from the respiratory tract Can lead to a systemic infection Viral capsid can evade host immune recognition	Significant birth defects, neonatal deafness, miscarriage	Placenta basal plate and endothelial cells	(20)
Parvovirus B19	ssDNA virus	Spread through respiratory droplets Preferential tropism for human erythroid progenitor	Fetus is usually unaffected, may result in severe fetal anemia	Whole placenta, placenta villi	(21)
Varicella zoster virus (Chicken pox)	ssDNA virus	Vertical transmission is very rare and only happens in primary infection	Congenital varicella syndrome, intrauterine growth restriction, low birth weight	No evidence, but chronic villitis has been described	(22)
ZIKA virus (ZIKV)	ssRNA virus	Mosquito borne infection transmitted from blood meal Preferentially to invade blood monocytes	Congenital fetal anomalies (microcephaly), miscarriage, stillbirth	Whole placenta, amniotic epithelium, VCT, Hofbauer cells, decidual macrophages, decidual fibroblast	(23–25)

continuous breaks or gaps and dynamic repair processes (47). Breaks in the syncytium could potentially lead to transmission of pathogens into the underlying VCT. Our recent work showed that a novel population of maternal macrophages (M3) is associated with the SCT in early pregnancy and might be involved in repairing the breaks in the syncytium (10). It is intriguing that M3 macrophages infected with intracellular pathogens could possibly gain access to the underlying VCT via the syncytial breaks (**Figure 2**).

Only a few viral entry receptors on the SCT are described. Notably, the SCT lacks expression of ZIKV entry receptors, Axl, and Tyro3 (48) and the HCMV entry co-receptor integrin α/β (49). This is further supported by the transcriptomic expression of viral receptors in placental cells (10, 50, 51). Expression of surface receptors commonly used by ZIKV such as *AXL* and *HCMV* such as *NRP2* and *PDGFRA* are lowly expressed by the SCT (50). In addition, there is minimal co-expression of ACE2, the receptor gene for human severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), and TMPRSS2, the viral spike protein serine protease gene (50, 52). In line with this, there is no conclusive and direct evidence of vertical transmission of SARS-CoV-2 in a placenta from a healthy individual. There are some reports showing SARS-CoV-2 is predominantly localized at the SCT of the second trimester placenta (53, 54) and can lead to severe inflammatory infiltrate in the IVS (55). However, these findings are presented in a very small number of patients with severe disease or pre-existing pregnancy complications (54, 55).

Alternative transplacental mechanisms have been postulated at the syncytial barrier. Neonatal Fc receptor (FcRn) is expressed on the apical surface of the SCT and functions to selectively

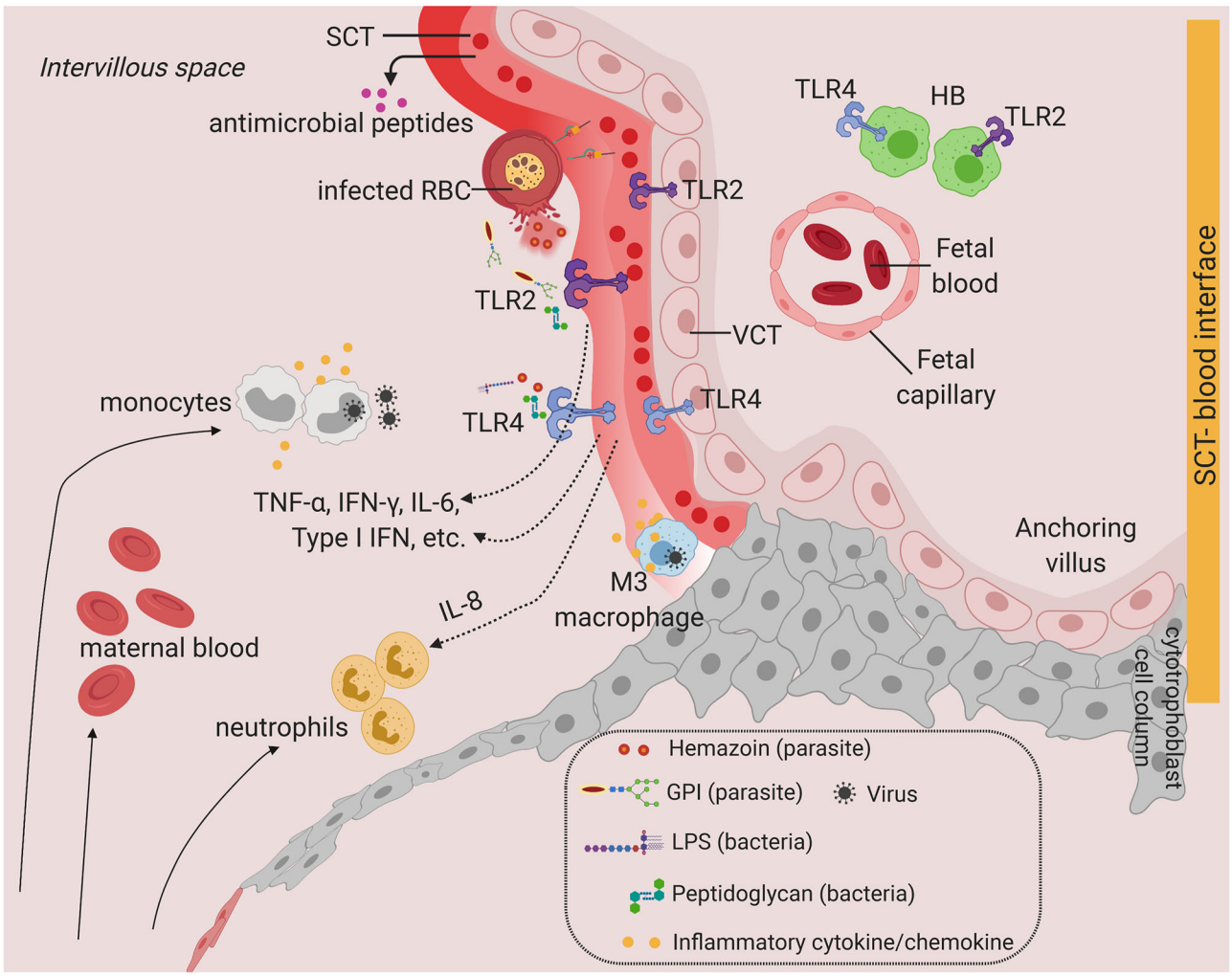

FIGURE 2 | Toll-like receptors and potential inflammatory response at the SCT-blood interface. Predominant TLRs found in the human placenta from early and term pregnancies. TLR2 and TLR4 are expressed in human placenta SCT, VCT, and in HB cells. Infiltration of infected maternal blood, infected immune cells, or release of pathogenic determinant such lipopolysaccharide (LPS), peptidoglycan, or parasite materials such as hemozoin or GPI (glycosylphosphatidylinositol) into the IVS will activate TLR-mediated signaling, leading to the production of a wide range of cytokines and chemokines. Severe infection is characterized by massive immune cell infiltration including monocytes and neutrophils from systemic circulation and overproduction of inflammatory cytokines upon TLR activation. This may lead to SCT inflammation and damage. SCT also secretes antimicrobial peptides as innate immune mechanisms. Figure is created by BioRender.com.

transport maternal IgG (56). FcRn could be exploited by certain viruses to enter the placenta including ZIKV, HIV-1, and HCMV (19, 57, 58). Transferrin receptor 1 (TfR1) is expressed on the apical end of the SCT, and functions as the primary iron transporter into the basal side of the SCT to provide sufficient iron stores into fetal circulation (59). TfR1 has been associated with viral entry into a broad host cell range, including Hepatitis C virus (60, 61) suggesting a possible mechanism of viral transport across the SCT barrier. Some pathogens, although unable to cross the SCT barrier, can still adhere to the syncytium and cause further pathology. For instance, *Plasmodium falciparum* infected red blood cells can bind with high affinity to chondroitin sulfate A expressed on the SCT, resulting in local inflammation, syncytial breaks, and damage (62–64).

Although the SCT is an effective barrier to most pathogens, local inflammation, tissue damage, and FcRN or TfR1-mediated viral entry at the SCT can potentially allow pathogen to breach the syncytial barrier, giving opportunity for transmission from maternal blood into placental villi (**Figure 2**).

MATERNAL DECIDUA AND EVT INTERFACE

During the first trimester of pregnancy, fetal EVT invades deeply into the uterus. The decidua basalis, the region located at the implantation site, is populated at this time by a distinctive subset of innate lymphocytes, decidual Natural Killer cells (dNK), which constitute up to 70% of leukocytes. We have identified three major populations of dNK by single-cell RNA-sequencing with unique phenotypes and functions in early pregnancy (10). In addition, there are populations of decidual macrophages (dMs) (∼20%), conventional dendritic cells (DCs) and small proportions of T cells (∼10–15%), whereas B cells, plasma cells, mast cells, and granulocytes are virtually absent (10) (**Figure 1**). The proportion of immune cells will vary throughout pregnancy, with an increase in the proportion of T cells at term (51).

Systemic infections will reach all organs including the decidua. Whether pathogens can also access the decidua via the cervix is still unclear. *Chlamydia trachomatis*, a common sexually-transmitted intracellular bacteria, was detected in glandular epithelial cells and unidentified decidual cells in decidual biopsies (11). This suggests the possibility of infections ascending and spreading from cell-to-cell from the lower genital tract into endometrial glands and vascular endothelium. The decidua basalis is in close contact with fetal cells and the maternal vasculature (**Figure 1**). First trimester dMs and decidual stromal cells are susceptible to ZIKV infection and replication *ex vivo* (23). Hence, infection could possibly spread from infected maternal immune and non-immune cells at the decidua, into uninfected VCT in the columns of the anchoring villi, and finally into the fetal compartment. However, this is likely to be limited to certain microorganisms which are capable of cell-to-cell spread, have an intracellular host niche, and are able to escape host innate defense mechanisms (**Table 1**).

HCMV, the most common cause of congenital infection, is mostly reported to infect from the decidua (11, 65). Women with primary HCMV infection and first pregnancy are more likely to transmit the virus to their fetus, compared to multiparous women with previous infection and demonstrable antibodies (66–68). Low affinity maternal antibodies against HCMV correlate with higher viral loads detected in the decidua, whereas patients with intermediate to high neutralizing antibodies have minimal viral replication (65), suggesting that maternal immunity against HCMV reduces risk of vertical transmission. HCMV protein was also detected in a range of cells within the decidua including endothelial, decidual stromal cells, DCs and macrophages (11, 65), suggesting that that infected maternal leukocytes could initiate transmission through contact and infection of endothelial cells that line decidual blood vessels.

Despite the evidence of decidual infection, the mechanism of vertical transmission for HCMV is still in debate. dNKs have been proposed to play a protective role against HCMV infection through several mechanisms including modulation of their cytotoxic effector function (69) and the interactions between the killer-cell immunoglobulin receptors (KIRs) expressed by dNK and HLA molecules expressed in the infected cells (70, 71). Activating KIR2DS1 by dNKs has been demonstrated to be more cytolytic against HLA-C2 HCMV-infected maternal decidual stromal cells (70). Similar cytotoxic response was also observed when peripheral blood NK cells expressing KIR2DS1 were exposed to HCMV-infected fibroblasts (71). Hence, this implies that in the decidua, dNKs are capable of eliminating harmful infection depending on the combination of KIR/HLA interactions between dNK and infected cells. dNKs are also able to control HIV-1 infection *in vitro* through production of IFN-γ (72). The role of dNK in controlling viral infection may protect against potential risk of vertical transmission from the decidua.

TRANSMEMBRANE PATTERN RECOGNITION RECEPTORS: TOLL-LIKE RECEPTORS

Pattern recognition receptors (PRR) are encoded in the germ-line and recognize specific, conserved pathogen-associated molecular patterns (PAMPs). These include Gram-negative bacteria lipopolysaccharide (LPS), Gram-positive bacteria lipoteichoic acids, lipoprotein, DNA, RNA, glucans, and peptidoglycans (73, 74). Pathogen recognition is not only an essential component of the innate immune response against infection, but also plays an important role in bridging the innate and adaptive systems by Toll-like receptors (TLR) activation of antigen presenting cells by up-regulation of major histocompatibility complex (MHC) and co-stimulatory molecules (75).

TLRs, the most studied family of PRR, are type I transmembrane proteins with large extracellular domains containing leucine-rich repeats that are expressed at the cell surface or intracellularly (76). Each TLR recognizes distinct PAMPs, leading to the activation of the transcription factor NF-κB and/or the interferon-regulatory factor (IRF) family, and the production of a wide range of cytokines and chemokines,

including type I IFNs (76, 77). TLRs are expressed by immune cells (macrophages, DCs, and B cells) as well as non-immune cells (fibroblasts and epithelial cells) (74).

TLRs at the Human Uterine-Placental Interface

Expression of TLRs is dynamic and changes in response to different pathogens and cytokines (74). TLR2 (which recognizes bacterial proteoglycan) and TLR4 (which recognizes bacterial LPS) are the most well-studied, with immunohistochemical evidence of expression in healthy primary SCT at term (78–80). In contrast, in the first trimester, TLR2 and TLR4 proteins are expressed in VCT and EVT, but minimally in SCT (81, 82) (**Figure 3**). There is therefore variation in TLR2 and TLR4 expression in the different trophoblast lineages across pregnancy. Why and how such dynamic regulation of TLR expression occurs during gestation requires further investigation in a broader range of human placental samples (different donors, gestation stages, genetic background, sampling regions). It is likely that alteration in cytokines profiles in the microenvironment as pregnancy progresses (83) may result in the variation in the expression of TLRs in the placenta. Current evidence is only limited to *in vitro* TLR2/4 stimulation studies using placental explants and primary first trimester trophoblast cells, which drives the expression of

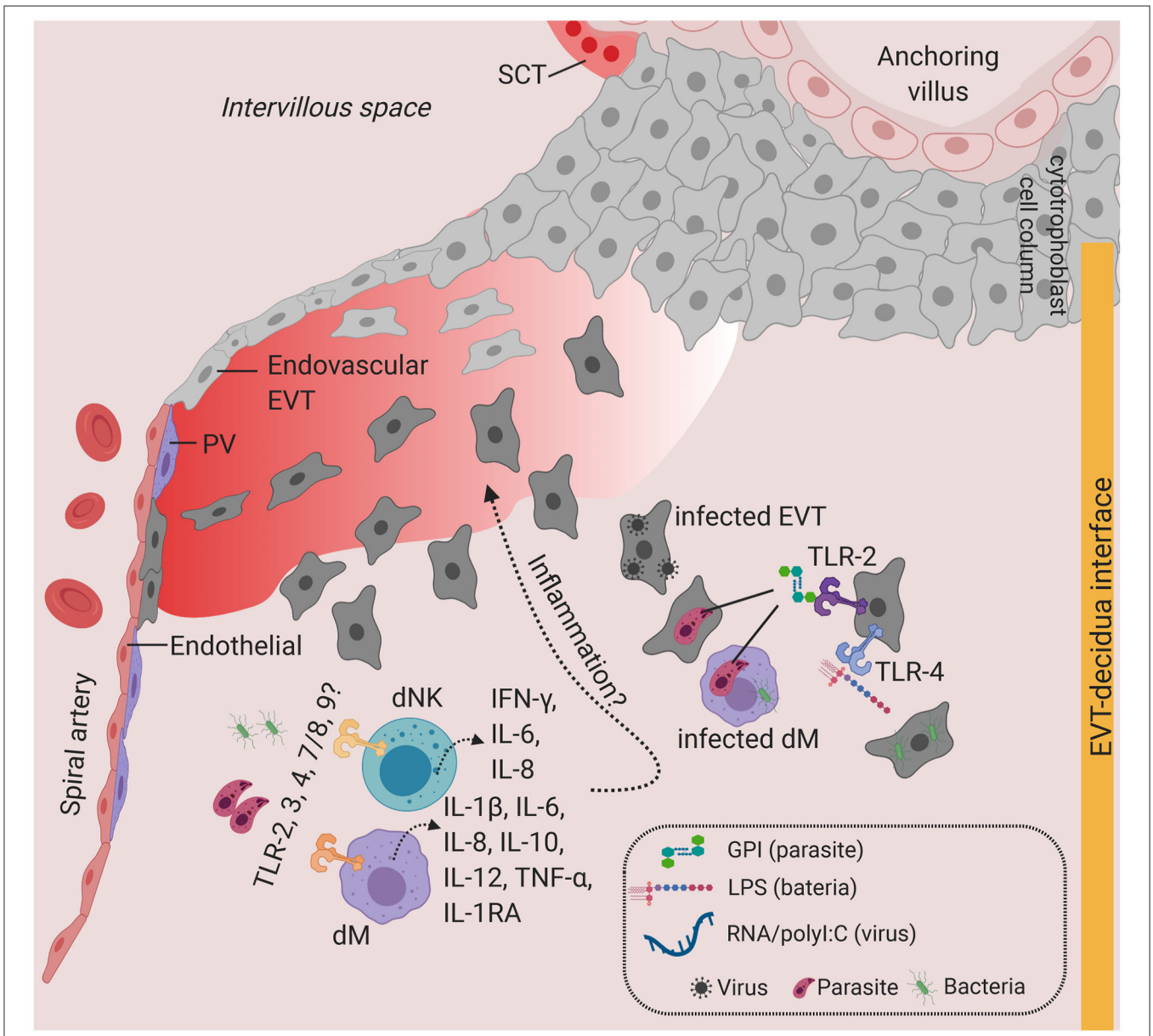

FIGURE 3 | Toll-like receptors and potential inflammatory response at the decidua. Predominant TLRs found in the human placenta from early and term pregnancies. TLR2 and TLR4 are expressed in EVT. dM and dNK also express a wide range of TLR families, where stimulation of TLR agonists lead to the production of a variety of cytokines and chemokines. Infiltration of infected cells and release of PAMPs in the decidua, which will activate TLR-mediated signaling. Overproduction of inflammatory cytokines at the decidua may lead to local inflammation. Figure is created by BioRender.com.

pro-inflammatory cytokines IL-6, IL-8, TNF-α, and IFN-γ (78, 80, 81).

TLR2 and TLR4 proteins are expressed in HB cells, confirmed by co-expression of CD68 in healthy term placentas (78). In early pregnancy, our findings indicate that only *TLR4* but not *TLR2* transcripts are expressed in steady-state HB cells (10) (**Figure 4**). Enhancement of IL-6 and IL-8 secretion upon stimulation of isolated first trimester HB cells with TLR4 agonist, LPS (84), does suggest a role for TLRs on HB cells in bacterial recognition and placental inflammation during early pregnancy. HB cells are postulated to have a role in viral replication (41, 42), however evidence on the expression and function of viral nucleic acid sensing receptors TLR3, TLR7, TLR8, and TLR9 in HB cells is lacking. Our findings show that *TLR7*, which recognizes viral single-strand RNA (ssRNA) (85) is expressed in steady-state HB cells (**Figure 4**) (10).

Other TLRs have also been shown to be expressed in decidua cells. dMs and dNKs isolated from first trimester pregnancies show steady state level expression of *TLR1-9* transcripts and respond to a broad range of PAMPs, including heat-killed bacteria, microbial membranes, and nucleic acids (86). Stimulating primary dMs with these PAMPs produces high levels of TNF-α, IL-1β, IL-6, IL-8, IL-12, IL-10, and IL-1RA, whereas dNKs secrete IL-6, IL-8, and IFN-γ (86). This study suggests that, in addition to the physiological roles of dMs and dNKs in accommodating the uterus for placentation, dMs and dNKs may play a role in pathogen recognition and antimicrobial response via activation of TLR signaling (**Figure 3**). The extent to which subsets of dMs or dNKs population (10) are critical for TLR-mediated response at the decidua is currently unknown.

In malaria endemic populations, single nucleotide polymorphisms (SNPs) within the TLR4 coding and TLR9 promoter regions are associated with variation in disease severity and parasitemia control (87, 88). In the case of pregnancy malaria, primiparous infected mothers with common TLR4 and TLR9 polymorphic variants are correlated with severe complications such as low birth weight and maternal anemia (89). This highlights the importance of studies involving large cohorts of individuals which include genotyping from pregnant mothers living in malaria endemic regions (see section on "Challenges and future perspective").

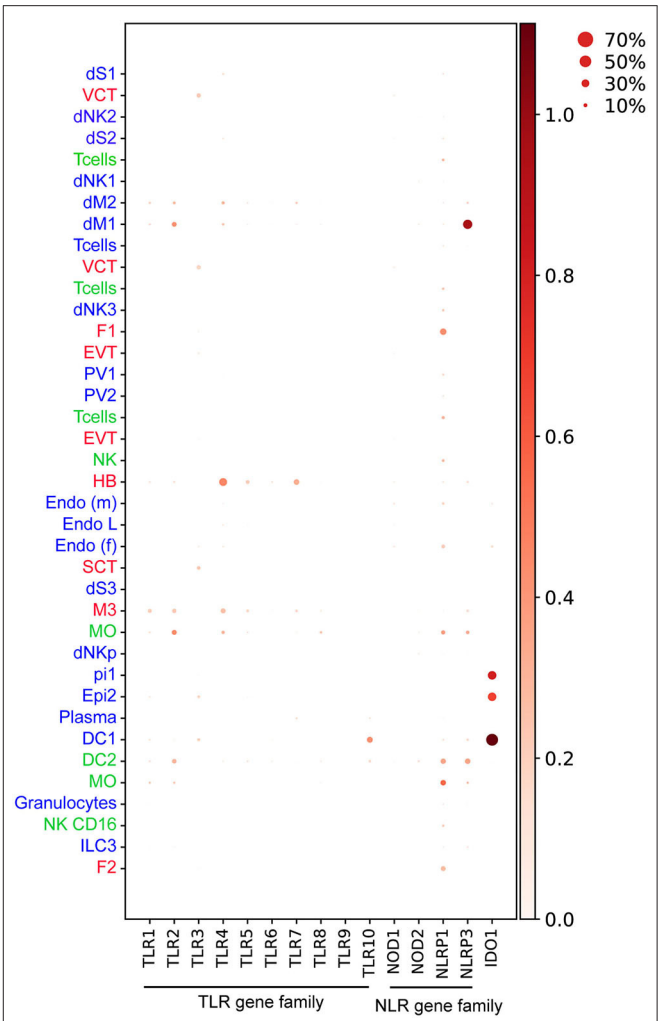

FIGURE 4 | Dotplot representing normalized and log transformed values expression of TLR (*TLR1-10*), NLR genes (*NOD1, NOD2, NLRP1, NLRP3*) and *IDO1* at steady state in early pregnancy from Vento-Tormo et al. (10). Origin of cell types from placenta (red), decidua (blue), and maternal blood (green) are labeled as differences in font color. Dot size represents the fraction of cells from a certain cluster expressing a gene and color scale represents normalized log transformed expression of the gene in that cluster. dS, decidua stroma; F, fibroblast; MO, monocyte; Endo, endothelial; Epi, epithelial; SCT, syncytiotrophoblast; VCT, villous cytotrophoblast; EVT, extravillous trophoblast; DC, dendritic cell; dNK, decidua Natural killer cells; dM, decidua macrophages; HB, Hofbauer cell; PV, perivascular cells. Figure is created by BioRender.com.

TLRs in Animal Models of Placental Parasite Infection

Animal models have also been used to study the functional role of TLR signaling, particularly for pathogens that are intracellular at some stage of their life cycle (**Table 1**). TLR4 and TLR9 are strongly activated by malaria parasite PAMPs such as glycosylphosphatidylinositol (GPI), DNA, and hemozoin (90, 91) (**Figure 2**). In a mouse model of placental malaria, TLR4, and Myd88 signaling activation resulted in placental expression of pro-inflammatory markers, such as IL-6 and TNF-α (92, 93). These studies also demonstrated that malaria parasite infection and inflammation in the mouse placenta lead to reduced fetus growth rate and disorganization of the vascular space in the placenta (92, 93). However, TLR-mediated inflammation and pathology in the human placenta upon malaria infection is unknown and remains to be further investigated.

Studies of congenital toxoplasmosis are also currently limited to animal models. TLR2 and TLR4 are associated with recognition of *T. gondii*'s infection in mice (94). Engagement of the *T. gondii* ligand by TLR2 and TLR4 at the SCT-blood or in the EVT-decidua compartments is plausible, although there is still no direct evidence for such host-parasite interaction in humans. TLR11 has a role in controlling *T. gondii* infection in mice (95, 96), however in humans TLR11 is a pseudogene and is not expressed (97).

CYTOSOLIC PATTERN RECOGNITION RECEPTORS: RIG-I, MDA5, AND NOD-LIKE RECEPTORS AT THE UTERINE-PLACENTAL INTERFACE

Cytosolic PRRs play an important role in fighting against viral infection by eliciting host type I interferons (IFN) antiviral response through recognition of single and double stranded RNA (ssRNA and dsRNA) (98, 99). Examples of PRRs are the cytosolic retinoic acid-inducible gene-I-like (RIG-I) and the melanoma differentiation-associated protein 5 (MDA5) receptors, both expressed in the SCT and VCT of term placenta (100). In the human placenta, there is limited information on the function of RIG-1 and MDA5, but they may play a crucial role in recognizing a variety of RNA viruses, including ZIKV and dengue virus (101).

The Nucleotide binding Oligomerization Domain-like receptors (NOD-like receptors; NLR) recognizes intracellular pathogen products which have entered into the host cytoplasmic compartment (74). Both NOD-1 and NOD-2 receptors, which are known to detect intracellular bacterial peptidoglycans (102), are expressed in the SCT in the first trimester and term placentas (103, 104). The NLR pyrin-containing 1 and 3 proteins (NLRP1 and NLRP3) form the major inflammasome complexes, which contribute to activation of inflammatory caspases and pathogen clearance (105, 106).

Activation of NLRP3 and AIM2 inflammasomes, together with high expression of IL-1R, IL-1β, and caspase-1 was recently shown in the placental tissue of mothers infected with *P. falciparum* with significant pathology (107). In a murine model of intra-amniotic inflammation induced by bacterial LPS, tissue sections from the decidua basalis region expressed high levels of NLRP3, but negligible caspase-1 activation suggesting a possible non-canonical activation of the NLRP3 inflammasome (108). Our analysis shows that decidual dM1 expresses high levels of *NLRP3* transcript at steady state compared to other cell types (10) (**Figure 4**), thus dM1 may play a role in NLRP3-mediated pathogen recognition during early pregnancy.

SECRETED HOST DEFENSES AT THE UTERINE-PLACENTAL INTERFACE

Antimicrobial Peptides

AMP secreted by epithelial and immune cells are small peptides that bind and destroy most groups of pathogens—bacteria, yeasts, fungi, and viruses (109). In addition to direct killing of pathogens, AMPs can rapidly modulate innate host immune responses by recruiting myeloid cells and lymphocytes to the site of infection and mediating activation of TLR (110, 111). The human placenta expresses high levels of β-defensins, a family of broad spectrum antimicrobial peptides which participate in direct bactericidal and anti-viral activity (112). Specific subtypes of β-defensins (HBD-1, 2, and 3) are expressed in SCTs (112), suggesting these AMPs can target potentially bacterial or viral infection from the maternal blood.

Antiviral Interferons

Recognition of PAMPs by PRRs during infection leads to production of pro-inflammatory cytokines that can aid in clearing the pathogen (74). Studies on the direct role of pro-inflammatory cytokines on the placenta in the case of infection is limited. Inflammatory mediators can directly influence infection outcome and fetal development, but they can also cause damage to the placenta if produced in excess (113). Amongst the proinflammatory cytokines associated with uterine-placental infection during pregnancy, the antiviral IFN are the most well-characterized.

IFNs are secreted by a variety of cell types as the first line of defense against viral infection (114). Type I IFNs, including IFN-α and IFN-β, are potent antiviral cytokines. IFN-α and IFN-β bind to the IFNAR1/2 receptor and lead to expression of IFN stimulated genes (ISGs), which control virus infection through a variety of mechanisms (114). Loss of IFNAR in the placenta leads to vertical transmission and fetal mortality in murine herpesvirus-68 (MHV68) infected mice (115). In the mouse model of ZIKV infection, type I IFN-mediated signaling is essential for the control of viral replication in the placenta, but can also lead to significant placental pathology and fetal mortality (116, 117). The mechanism of type I IFN-mediated placental pathology has been recently elucidated. IFN-induced transmembrane (IFITM) protein, which normally blocks viral entry into host cells, impairs syncytin-mediated fusion of VCT to form SCT, leading to aberrant placental development (118).

Type II IFN, IFNγ, predominantly produced by NK and CD4+ T cells is crucial in controlling parasitic infection, such as *T. gondii* in mice (94, 119). However, elevated levels of IFNγ in response to *T. gondii* infection can lead to pathological effects during pregnancy including fetal demise (119, 120). Severe placental pathology and fetal death have also been associated with elevation of IFNγ during pregnancy in a murine model of malaria (121). Hence, proper regulation of type I and II IFN-mediated signaling at the uterine-placental interface is crucial in limiting pathogen replication, whilst preserving a balanced environment for normal placental development (122). Type III IFN, IFNλ, are constitutively secreted by the human SCT, which presumably confers antiviral effects against ZIKV infection (123–125).

INTRACELLULAR HOST DEFENSES AT THE UTERINE-PLACENTAL INTERFACE

Tryptophan Metabolism by IDO

Indoleamine 2,3-dioxygenase (IDO) is a host intracellular enzyme which metabolizes the amino acid tryptophan (126). IDO has been associated with maternal immunoregulation during pregnancy (127). It also plays a key role in the control of bacterial and viral replication, through limiting the bioavailability of tryptophan (128). IDO also inhibits the replication of several parasitic pathogens including *T. gondii* in human fibroblasts (129) and *Leishmania spp* in human macrophages (130). Mouse infection with *L. monocytogenes* showed that IDO is elevated in an IFN-γ-dependent manner in stromal cells of the metrial gland and decidua basalis; a crucial process to resolve bacterial

infection in the mouse placenta (131). Our findings also show *IDO1* expression is enriched in epithelial glandular and DC1 cell type in the first trimester decidua (10) (**Figure 4**). The presence of IDO in decidua suggests that the enzyme might have a central role in limiting parasitic, viral, and bacterial replication, thus preventing their spread to the fetus.

CHALLENGES AND FUTURE PERSPECTIVES

Research on how the human placenta safeguards itself against infections is challenging due to obvious logistical and ethical issues in obtaining tissue from early in gestation (**Box 1**). Although animal experimental models have provided important insights relating to the immune responses to pathogenic infection, major differences between human and animal placentas must be considered (30, 31). Likewise, differences between strains of pathogens adapted for mice compared with human clinical isolates should be taken into account as this may lead to variation in pathogenesis and cellular response. One such example is the use of mouse CMV, which is unable to cross the mouse placental barrier, unlike the HMVC counterpart which can be transmitted transplacentally in humans (132). Therefore, all data obtained from studies of infection in pregnant animals needs careful interpretation and consideration prior to translation to clinical infection in humans.

Inherent properties of trophoblast cell lines, primary cultures or explants vary between donors, and are likely to be confounded by the area of the placenta that is sampled and as well as stage of gestation (133). For instance, villous placental explants will vary depending on the types of villi sampled and the presence of attached decidual tissue (133). Caution is therefore needed when interpreting data using these experimental models.

To overcome such limitations, population-based cohort studies of women with infection during pregnancy with extensive tissue sampling should be performed. These need to include and focus on LMIC where infection is still a major cause of maternal and fetal mortality and morbidity. Cohort studies and epidemiological surveillance on maternal infections can offer significant insights into disease pathogenesis and accelerate clinical interventions (134). Collaborations between clinicians and researchers for population-based cohort collection and sample processing will be instrumental to achieving this goal. Biological samples such as blood or placenta collected from controls and infected pregnant individuals could be stored and cryopreserved retrospectively. To capture the overall heterogeneity of infected and non-infected placenta samples, sampling, and biobanking criteria of different regions of placenta should be considered (135). Protocols are now available to use frozen tissue processed for single-cell/nuclei and spatial genomics (136, 137). Hence, application of single-cell "omics" on infected vs. healthy human placental and decidual samples will enable us to evaluate cellular heterogeneity in response to infection.

The capacity to detect transcripts specific to host or pathogen mRNA from the same tissue using *in situ* nucleic acid hybridization methods will provide direct quantification of infection burden and identification of potential target host cells within the same tissue (138). Recent advances in spatial transcriptomics methods have also allowed gene expression signatures to be quantified and resolved from individual tissue sections (139). Combination of these emerging technologies with new methods to integrate single-cell and spatial data computationally (140) will provide an unbiased approach to characterize and profile the transcriptome of individual cells *in situ* from the placenta and decidua in response to infections. We anticipate that high-throughput datasets generated from cohort sampling studies will unravel novel cell states and tissue spatial localization associated with placental infections and inflammation. This will also allow us to better characterize not only the innate immune response or makers of infection, but also other adaptive immune states in the human placenta (**Box 1**).

The use of *in vitro* models will also further define host responses to infection. The recent generation of human trophoblast stem cells (hTSCs) (141) and three-dimensional (3D) trophoblast organoids (142, 143) offer a great opportunity to study infections in early pregnancy where the access to first trimester placental samples is a concern. More importantly, the hTSCs and trophoblast organoids fulfill the criteria characteristic of human first trimester trophoblast *in vivo* (32). Both hTSCs and trophoblast organoids can differentiate *in vitro* into SCT and EVT with appropriate media (142, 143) allowing infection experiments on both the major trophoblast subpopulations present at the two major sites of contact between maternal and fetal cells. Sequencing of both host and pathogen transcriptomes from infected trophoblast at single-cell resolution will also advance our understanding on host-pathogen interactions in placentas (144, 145).

BOX 1 | Perspective of vertical transmission and innate immune function during pregnancy and infection.

A variety of maternal infections can lead to vertical transmission (**Table 1**). The exact mechanisms these pathogens use to escape host defense and cross the placental barrier into the fetal compartment are not entirely known. Experimental models that recapitulate infection of the human placenta and thus vertical transmission are challenging to set up. More data and representative experimental models are needed to answer these questions: (i) how do different pathogens escape or modulate the maternal-fetal host innate immune barrier (ii) why do some pathogens lead to congenital infection but not others? Studying infected human placentas will be essential in understanding this but access to these samples is difficult especially in low and middle-income countries (LMIC) where maternal infection is particularly prevalent (WHO, Maternal mortality index 2019). Despite evidence of expression in primary placental tissue, functional studies on important innate immune features such as TLRs, AMPs, RIG-I, MDA5, NLRs, and IDO during infection and pregnancy are lacking. Understanding how different cell types at the uterine-placental interface (HB cells, dNKs, and dMs) respond to pathogen challenge is essential, but remains under-researched. A critical obstacle is to also extrapolate the protective and pathological mechanisms of cytokines from mouse to human infection. Therefore, systematic comparison of the innate immune effector mechanisms across gestation, in the placenta and decidua from natural human infection vs. healthy pregnancy, will provide a more accurate representation in clinical settings.

Further refinement of the trophoblast organoid and hTSCs culture system is needed to address key biological questions unanswered by current models. These include studying the effect of infection on cellular crosstalk between trophoblast and other primary placental cells such as HB cells, or decidual cells in culture, such as dNK or decidual stromal cells. Adaptation of CRISPR/Cas9 genome editing technology for the trophoblast organoids or hTSCs will offer novel insights into essential host genes required for vertical transmission and placental defense mechanisms in humans.

CONCLUSION

Major maternal and fetal complications as a result of infection are still a concern, especially in LMIC with highest prevalence reported in countries of sub-Saharan Africa (WHO, Maternal mortality index 2019). Profound limitations on current study models and ethical regulations on studying human placenta have significantly delayed the development of therapies and vaccines for maternal-fetal infection. How vertical transmission occurs and how the uterine-placental innate immune system reacts to infection remain as major unresolved questions. Revolutionary advances in single-cell genomics, imaging, computational, and stem cell biology methods are currently underway to study the molecular and cellular mechanisms of human diseases. Therefore, it is now an exciting time to apply these transformative technologies to comprehensively address fundamental questions on host-pathogen interaction at the human uterine-placental interface.

AUTHOR CONTRIBUTIONS

RH, AN, and RV-T wrote and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Dynamic Changes in the Phenotype of Dendritic Cells in the Uterus and Uterine Draining Lymph Nodes After Coitus

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Dendritic cells (DCs) are essential for successful embryo implantation. However, the properties of uterine DCs (uDCs) during the implantation period are not well characterized. In this study, we investigated the dynamic changes in the uDC phenotypes during the period between coitus and implantation. In virgin mice, we evaluated the expressions of CD103 and XCR1, this is the first report to demonstrate uDCs expressing CD103 in XCR1^{+}cDC1s and XCR1^{+}cDC2s. On day 0.5 post coitus (pc), the number of uterine CD11c^{+}CD103^{-}MHC classII^{high}CD86^{high}–mature DCs rapidly increased and then decreased to non-pregnancy levels on days 1.5 and 2.5 pc. On day 3.5 pc just before implantation, the number of CD11c^{+}CD103^{+}MHC class II^{dim}CD86^{dim}–immature DCs increased in the uterus. The increase in mature uDCs on day 1.5 pc was observed in both allogeneic- and syngeneic mating, suggesting that sexual intercourse, or semen, play a role in this process. Meanwhile, the increase in immature uDCs on day 3.5 pc was only observed in allogeneic mating, suggesting that allo-antigens in the semen contribute to this process. Next, to understand the turnover and migration of uDCs, we monitored DC movement in the uterus and uterine draining lymph nodes (dLNs) using photoconvertible protein Kikume Green Red (KikGR) mice. On day 0.5 pc, uDCs were composed of equal numbers of remaining DCs and migratory DCs. However, on day 3.5 pc, uDCs were primarily composed of migratory DCs, suggesting that most of the uDCs migrate from the periphery just before implantation. Finally, we studied the expression of PD-L2—which induces immunoregulation—on DCs. On day 3.5 pc, PD-L2 was expressed on CD103^{+}-mature and CD103^{-}-mature DCs in the uterus. However, PD-L2 expression on CD103^{-}-immature DCs and CD103^{+}-immature DCs was very low. Furthermore, both remaining and migratory DCs in the uterus and uterus-derived-DCs in the dLNs on day 3.5 pc highly expressed PD-L2 on their surface. Therefore, our study findings provide a better understanding of the dynamic changes occurring in uterine DCs and dLNs in preparation for implantation following allogeneic- and syngeneic mating.

Keywords: feto-maternal tolerance, Kikume Green Red (KikGR), PD-L2, photoconvertible protein, tolerogenic dendritic cells, uterus

INTRODUCTION

Dendritic cells (DCs) play an essential role in successful implantation and placentation in allogeneic- and syngeneic pregnancy (1–4). Moreover, uterus-resident DCs have been proposed to contribute to feto-maternal tolerance by regulating T cell activation (1, 5–8). Uterine DCs (uDCs) take up paternal antigens and present them to T cells in the draining lymph node (dLN), thereby inducing the regulatory T cells (Tregs) at the feto-maternal interface (5, 7, 9–13). The essential role of uDCs in the maintenance of feto-maternal tolerance during pregnancy has been examined. For instance, IDO-expressing DCs and plasmacytoid DCs (pDCs) act as tolerogenic DCs (tDCs) (4). These tDCs have been shown to possess the capability of immunoregulation by inducing Tregs, as well as T cell, anergy and deletion (6, 14, 15). However, few reports have examined the characteristics of uDC subsets in murine pregnancy (2, 4, 16–22).

Based on their morphological features and functions, DCs can be classified into conventional DCs (cDCs) and pDCs (4, 23–26). While each DC subset can present antigens to $CD4^+$ T cells, $CD103^+$ DCs can also present antigens to $CD8^+$ T cells (23). Each DC subset is reported as tDC in food tolerance and tumor immunity (4, 15, 16, 27–31), however, little is known on the type of DC phenotype that increases in the uterus before implantation.

Transient inflammation in the uterine cervix and endometrium is observed after coitus-induced dynamic changes in immune cells (32–34). After insemination, neutrophils migrate rapidly into the uterus and are immediately decreased to non-pregnant levels by day 1.5 post coitus (pc) (35). Subsequently, DCs and macrophages migrate into the uterine endometrium to clear semen debris and make the uterus sterile (33). However, little is known about the phenotype, subsets, and spatiotemporal features of uDCs, as well as the migration of DCs between the uterus and the draining para-aortic lymph nodes. Hence, in the current study we aimed to investigate the dynamic changes in uDCs during allogenic- and syngeneic mating, from coitus to before implantation. To this end, we examined the surface markers of uDCs via flow cytometry, and migration of DCs using mouse line expressing photoconvertible fluorescent protein Kikume Green Red (KikGR) (36–38). We found that approximately 75% of uDCs were transformed to migratory DCs from day 2.5 to 3.5 pc. These migratory DCs may, therefore, play important roles in successful implantation.

RESULTS

Uterine DCs Are Increased Following Coitus and Just Before Implantation

To clarify the dynamic changes in uDC phenotype from coitus to implantation, we analyzed the time course for the classification of uDC subsets in allogeneic pregnancy (**Figure 1A**). Uterine DCs were identified as propidium iodide $(PI)^-$ $CD45^+$ $Gr\text{-}1^-$ $F4/80^-$ $CD11c^+$ MHC class $II^{low-high}$ $B220^-$ cells ($CD11c^+$ DCs) (**Figure 1B** and **Supplementary Figure 1A**). We then subdivided them into $CD103^-$ $CD11b^{-/+}$ ($CD103^-$ DCs) and $CD103^+$ $CD11b^{-/+}$ ($CD103^+$ DCs) cells (**Figure 1B** and **Supplementary Figure 1B**). Moreover, uterine pDCs were identified as PI^- $CD45^+$ $Gr\text{-}1^-$ $F4/80^-$ $CD11c^+$ $PDCA\text{-}1^+$ $CD11b^-$ $Ly6C^+$ $B220^+$ cells.

Compared to the non-mated control virgin mice, the total number of uDCs were increased on days 0.5 and 1.5 pc (9.7 and 4.9-folds, respectively), and returned to the non-pregnancy level on day 2.5 pc (1.7-fold), followed by an additional increase on day 3.5 pc (6.6-fold) (**Figure 1C**). The proportion of each DC subset in virgin mice showed that the majority of uDCs were $CD103^-$ DCs (79.0%), followed by $CD103^+$ DCs (17.8%), while pDCs (3.3%) were in minority (**Supplementary Figure 1C**). Hence the number of $CD103^-$ DCs was similar to that of $CD11c^+$ DCs (**Figure 1C**). Meanwhile, although the proportion of $CD103^+$ DCs was smaller than $CD103^-$ DCs, similar changes were observed in the time course (**Figure 1C**). Additionally, pDCs increased in number beginning on day 1.5 pc, however, continued to only represent a minor population (**Figure 1C**).

Presence of CD103-Expressing uDCs in $XCR1^+$-cDC1s and $XCR1^-$-cDC2s

Recently, the characteristics of different DC subsets have been classified (26, 39). Therefore, here we sought to examine the expression of CD64, CD26, XCR1, and SIRPα to compare the presence of different DC subsets. First, we confirmed the exclusion of macrophages by staining for F4/80 and CD64 expression (**Supplementary Figure 2A**). From the total $F4/80^+$ cell population, $F4/80^+$ $CD64^+$ cells accounted for 68%, while $F4/80^+$ $CD64^+$ cells accounted for 12.5% of the total $CD64^+$ cell population, indicating that $F4/80^+$ gating effectively excluded most of the macrophages (**Supplementary Figure 2B**). Next, we confirmed the proportion of DCs by examining CD26 expression (**Supplementary Figures 2C,D**). The expression of CD26 in $CD11c^+$ DCs, $CD103^-$ DCs, and $CD103^+$ DCs was 69, 54.5, and 95.3%, respectively (**Supplementary Figure 2D**). These results indicate that PI^- $CD45^+$ $Gr\text{-}1^-$ $F4/80^-$ $CD11c^+$ MHC class II^+ $B220^-$ cells may be considered as DCs in the uterus. Moreover, although both CD103 and XCR1 have commonly served as markers of cDC1s, CD103 expression was also recently detected in cDC2s (26). Therefore, we further confirmed the DC subset be detecting XCR1 and SIRPα expression (**Supplementary Figure 2E**). In the $CD11c^+$ DC population, the proportion of $XCR1^+$ DCs and $CD103^+$ DCs was 13.5 and 43%, respectively (**Supplementary Figures 2E,F**). Conversly, CD103 expression was detected in 13.1% of $CD103^+$ $XCR1^+$ DCs, and in 34.7% of $CD103^+$ $XCR1^-$ DCs (**Supplementary Figures 2E,G**), indicating the presence of CD103 in both cDC1s and cDC2s in the uterus.

Mature DCs Increase After Coitus and Decrease to Non-pregnancy Levels Just Before Implantation

We then subdivided each DC subset into $CD86^{low}$ MHC class II^{low}-immature DCs and $CD86^{high}$ MHC class II^{high}-mature DCs

Abbreviations: cDCs, conventional DCs; DCs, dendritic cells; dLN, draining lymph node; KikGR, Kikume Green Red; LNDCs, lymph node DCs; MPA, medroxyprogesterone; pc, post coitus; pDCs, plasmacytoid DCs; PI, propidium iodide; tDCs, tolerogenic DCs; Tregs, regulatory T cells; tSNE, t-distributed stochastic neighbor embedding; uDCs, uterine dendritic cells.

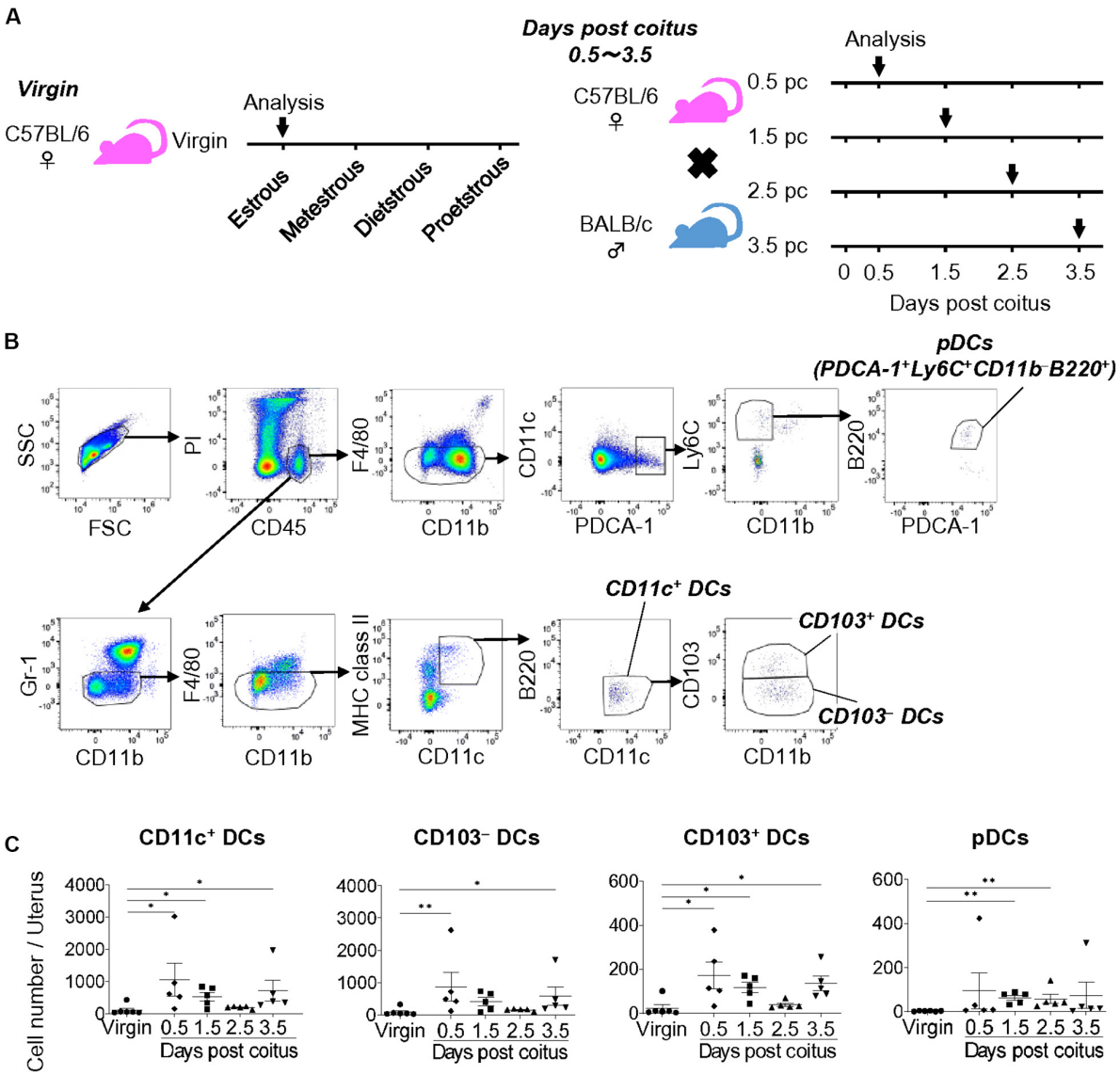

FIGURE 1 | Cell numbers of uDCs after coitus in allogeneic mating. **(A)** Experimental time course. The DCs in the uterus in non-mated control virgin mice and mice on days 0.5, 1.5, 2.5, and 3.5 pc were analyzed by flow cytometry. **(B)** Gating strategy was used to identify CD103$^-$ DCs, CD103$^+$ DCs, and pDCs in the uterus. **(C)** Graphs show number of CD11c$^+$ DCs and each DC subset in the uterus at each time point. A minimum of five samples from each time point were analyzed. Data represent mean ± SEM and are representative of three independent experiments. Statistical comparisons were performed using Kruskal-Wallis test with Dunn's multiple comparisons test (**$P < 0.01$, *$P < 0.05$).

(**Figure 2A**) (15). The frequency of mature DCs among CD11c$^+$-total DCs increased at days 0.5 and 1.5 pc compared to that in virgin mice, and then returned to the level observed in virgin mice on days 2.5 and 3.5 pc (**Figures 2B,D,E**). Meanwhile, the frequency of CD11c$^+$-immature DCs increased on day 3.5 pc compared to that in virgin mice (**Figures 2B,C,E**). CD103$^-$-mature DCs also increased at days 0.5 and 1.5 pc, however, the frequency of CD103$^+$-mature DCs and pDCs did not change on days 0.5 and 1.5 pc. Hence, the increased number of mature uDCs on days 0.5 and 1.5 pc was likely due to increased CD103$^-$ DCs. Moreover, the frequency of CD103$^+$-immature uDCs on day 3.5 pc was significantly elevated compared to that in virgin mice, suggesting that an increase in immature uDCs on day 3.5 was due to increased CD103$^+$ DCs.

Characterization of uDCs Using t-Distributed Stochastic Neighbor Embedding (tSNE)

To define the specific DC subset present during the implantation period, we next analyzed the time course of uDC subsets via dimensionality reduction analysis, using tSNE. The pooled data for individual CD11c$^+$ DCs and pDCs within the uterus across each time point (n = 26) was concatenated and

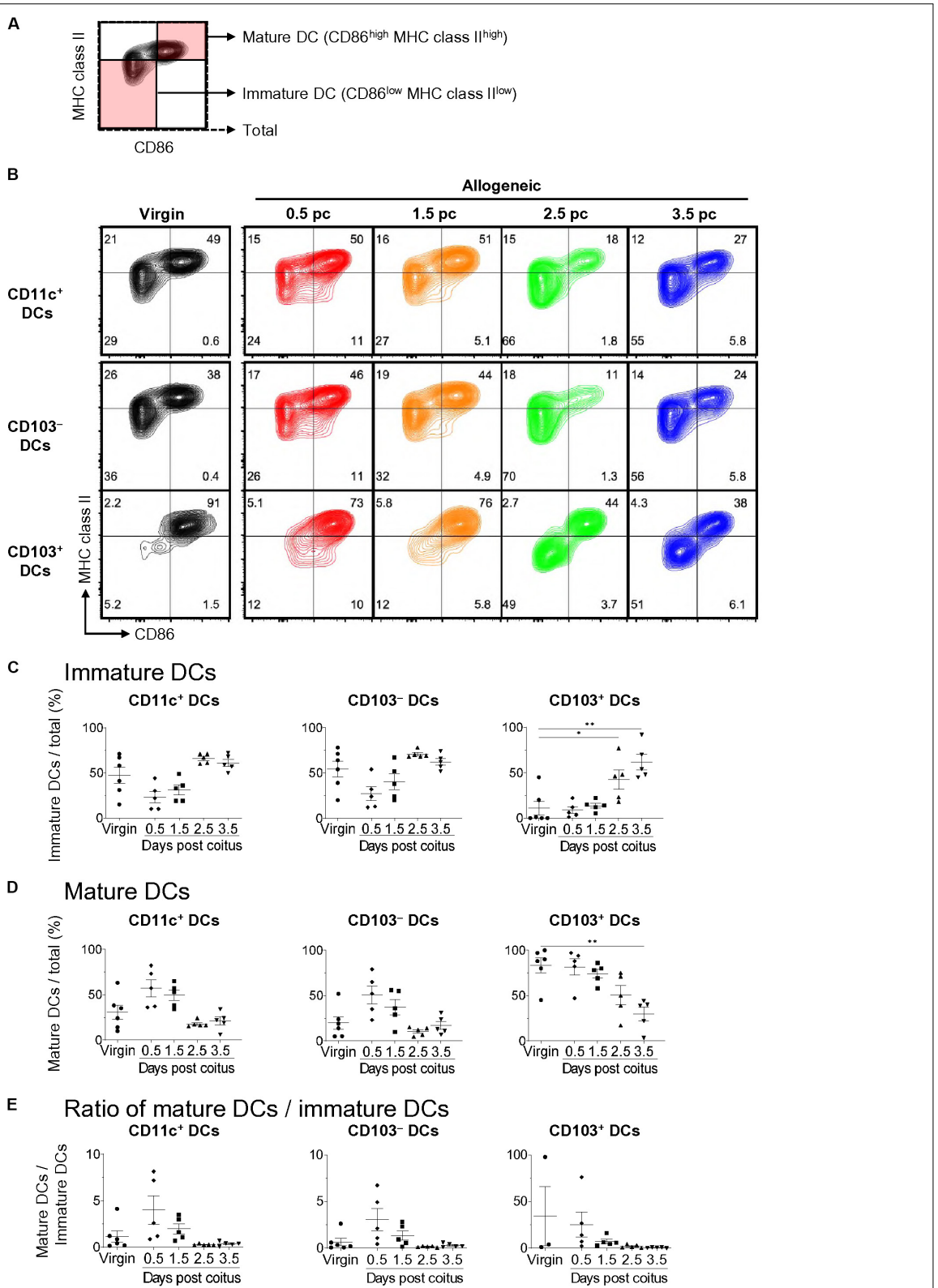

FIGURE 2 | Time course for uterine immature and mature DCs after coitus in allogeneic mating. **(A,B)** Flow cytometry contour plots show immature DCs and mature DCs based on the expression of CD86 and MHC class II **(A)**, and representative plots in proportion to those within each DC subset at each time point **(B)**. **(C–E)** Graphs show the proportion of immature DCs **(C)** and mature DCs **(D)** out of the total DCs, and ratio of mature DCs/immature DCs **(E)** in each DC subset at each time point. A minimum of five samples from each time point were analyzed. Data represent mean ± SEM **(C–E)** and are representative of three independent experiments. Statistical comparisons were performed using the Kruskal-Wallis test with Dunn's multiple comparisons test (**$P < 0.01$, *$P < 0.05$).

visualized as a two-dimensional map by tSNE (**Supplementary Figure 3**). Results show that the clusters of each DC subset—particularly those of CD103$^+$ DCs—were clearly divided into two clusters, MHC class II^{high} CD86^{high} and MHC class II^{dim} CD86^{dim} (**Supplementary Figure 3A**). The changes in distribution demonstrate (**Supplementary Figures 3B,D,E**) that the primary clusters in virgin mice consisted of cluster 6 made up of CD103$^+$ mature DCs (**Supplementary Figures 3A,C**), clusters 8, 9, and 10 comprised of DCs without any specific surface markers, and cluster 12 made up of CD11b$^+$ DCs. After coitus, the increased clusters on days 0.5 and 1.5 pc appeared as cluster 11 (**Supplementary Figures 3D–F**), which contained CD11c$^+$ CD86^{high} MHC class II^{high} Ly6C$^-$ PDCA-1^{dim} CD11b$^+$ CD103$^-$-mature DCs (**Supplementary Figures 3A,C**). Interestingly, new clusters on days 2.5 and 3.5 pc appeared as cluster 7 (**Supplementary Figures 3D–F**), containing CD11c$^+$ CD86^{dim} MHC class II^{dim} Ly6C$^-$ PDCA-1^{dim} CD11b$^-$ CD103$^+$-immature DCs (**Supplementary Figures 3A,C**). These results indicate that these newly appearing DCs before implantation are primarily CD103$^+$ immature DCs.

Differences in uDC Populations Between Allogeneic- and Syngeneic Mating

To examine the differences in the uDC populations in response to paternal antigens, we analyzed the characteristics of uDCs between virgin, allogeneic-, and syngeneic mating on days 1.5 and 3.5 pc (**Figure 3A**). The increase in uDCs on day 1.5 pc was observed in both allogeneic- and syngeneic mating (**Figure 3B**). There were no significant changes in the number or frequencies of uDCs between allogeneic- and syngeneic mating on day 1.5 pc (**Figures 3B–E**). However, an increase in CD103$^-$- and CD103$^+$-immature uDCs was observed in allogeneic mating, but not in syngeneic mating on day 3.5 pc (**Figures 3C–E**). These results suggest that the induction of immature DCs in the uterus before implantation is dependent on paternal antigens.

Immature uDCs Are Increased Among the Infiltrating and Pre-existing DCs Just Before Implantation

Although the migration and pre-existence of uDCs using CFSE labeling in non-pregnant mice has been reported, little is known regarding how resident and migratory uDCs contribute to successful implantation (1). Thus, we elucidated the turnover of uDCs in KikGR mice (**Figure 4A** left). All DCs in the uterus were converted to KikGR-red immediately after photoconversion (**Supplementary Figures 4A–D**). Consequently, under such photoconversion conditions, we analyzed the KikGR-red remaining DCs and non-photoconverted KikGR-green migratory DCs at 24 h after photoconversion of the uterus with KikGR mice from day 0.5 to 1.5 pc, from 1.5 to 2.5 pc, and from 2.5 to 3.5 pc (**Figure 4A** right). This protocol allowed us to monitor the uDC turnover for 24 h on each gestational day. Results show that migratory DCs consisted of equal numbers of immature and mature DCs, with no change the proportion of immature/mature DCs in the migratory DCs over time (**Supplementary Figures 5B, 6A,B** and **Figure 4B**). In general, infiltrating DCs—which are of the immature phenotype—migrate to peripheral organs and are subsequently matured. Thus, equal proportions of mature and immature phenotypes in infiltrating DCs at 24 h from coitus to before implantation implies that the maturation rate of infiltrating DCs is not altered drastically. Alternatively, the remaining DCs were predominantly of the mature phenotype throughout the period after coitus, and just before implantation, with the proportion of mature DCs in the remaining DCs observed to gradually decrease (**Supplementary Figures 5B, 6C** and **Figure 4C**). During the analysis of changes due to cell turnover, the time course study revealed that the proportion of remaining DCs in the uterus gradually decreased by day 3.5 pc (**Figure 4D**). These results indicate that increased immature uDCs before implantation primarily make up the infiltrating DCs.

DC Subsets of Uterine dLNs After Coitus to Just Before Implantation

It has been reported that paternal antigen-specific Tregs increase in the dLNs before implantation (13, 40). Uterus-derived DCs would then stimulate the paternal antigen-specific Tregs by presenting paternal antigens. Thus, it is important to understand dynamic changes in the migratory patterns of uDCs to dLN from coitus to before implantation. To this end, we analyzed the time course of MHC class II^{high} DCs in dLNs (**Supplementary Figure 7A**). Compared with virgin mice, the total number of migratory DCs in dLNs from day 0.5, 1.5, 2.5, and 3.5 pc were increased by 2.5, 14.4, 8.3, and 18.6-fold, respectively (**Supplementary Figure 7B**). In the changes of each DC subset, CD103$^-$ DCs, CD103$^+$ DCs, and pDCs levels were significantly increased on day 3.5 pc compared to those in virgin mice (**Supplementary Figure 7B**). Meanwhile, CD103$^+$ DCs accounted for a minor population in dLNs throughout this period. To clarify the dynamic changes of migratory uDCs in the dLNs, we next analyzed the migration of uDCs using KikGR mice (**Figure 4A**). No KikGR-red DCs were detected in the dLNs immediately after photoconversion, indicating that photoconversion was restricted to the uterus (**Supplementary Figures 4C,D**). However, 24 h after photoconversion, we detected KikGR-red DCs in migratory DCs (CD11c$^+$ MHC class II^{high}) (**Figure 4E**), but not in lymph node DCs (LNDCs) (CD11c$^+$ MHC class II^{int}) (**Supplementary Figure 7C**), suggesting that uterine migratory DCs were exclusively CD11c$^+$ MHC class II^{high}. Furthermore, the time course study revealed that the migration of KikGR-red uterus-derived total DCs, CD103$^-$ DCs, and CD103$^+$ DCs in the dLNs showed an increasing trend by day 3.5 pc (**Figure 4E**).

PD-L2$^+$ Expression on DCs

PD-L2 has been implicated to play a critical role in immune tolerance by negatively regulating the T cell immune response (27, 41). To clarify its contribution to tolerogenic conditioning, we analyzed PD-L2 expression on DCs (**Figure 5A**) and found that it was not expressed on mature and immature uDCs in virgin mice (**Figures 5B,C**). However, more than 30% of CD103$^+$- and CD103$^-$-mature DCs, but not immature DCs,

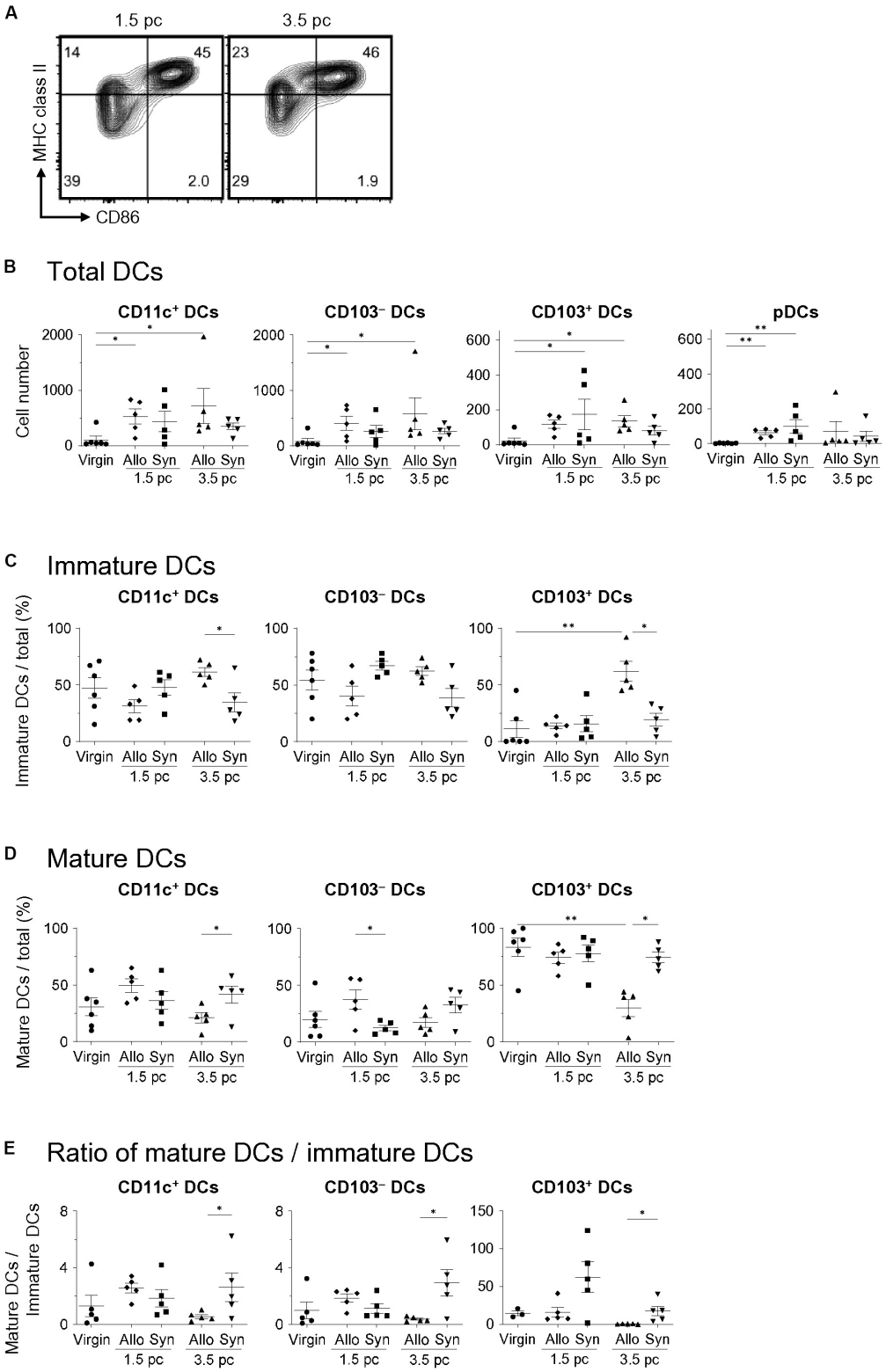

FIGURE 3 | Comparison of uDC phenotype between allogeneic- and syngeneic mating. **(A)** Flow cytometry contour plots show immature DCs and mature DCs in syngeneic mating on days 1.5 and 3.5 pc. **(B,C)** Graphs show total DC number **(B)**, proportion of immature DCs **(C)** and mature DCs **(D)** out of the total DCs, and the ratio of mature DCs/immature DCs within each DC subset **(E)** in virgin, allogeneic-, and syngeneic mating mice at each time point. A minimum of five samples from each time point were analyzed. Data represent mean ± SEM **(B,C)** and are representative of three independent experiments. Statistical comparisons were performed using Mann-Whitney *U*-test (**$P < 0.01$, *$P < 0.05$).

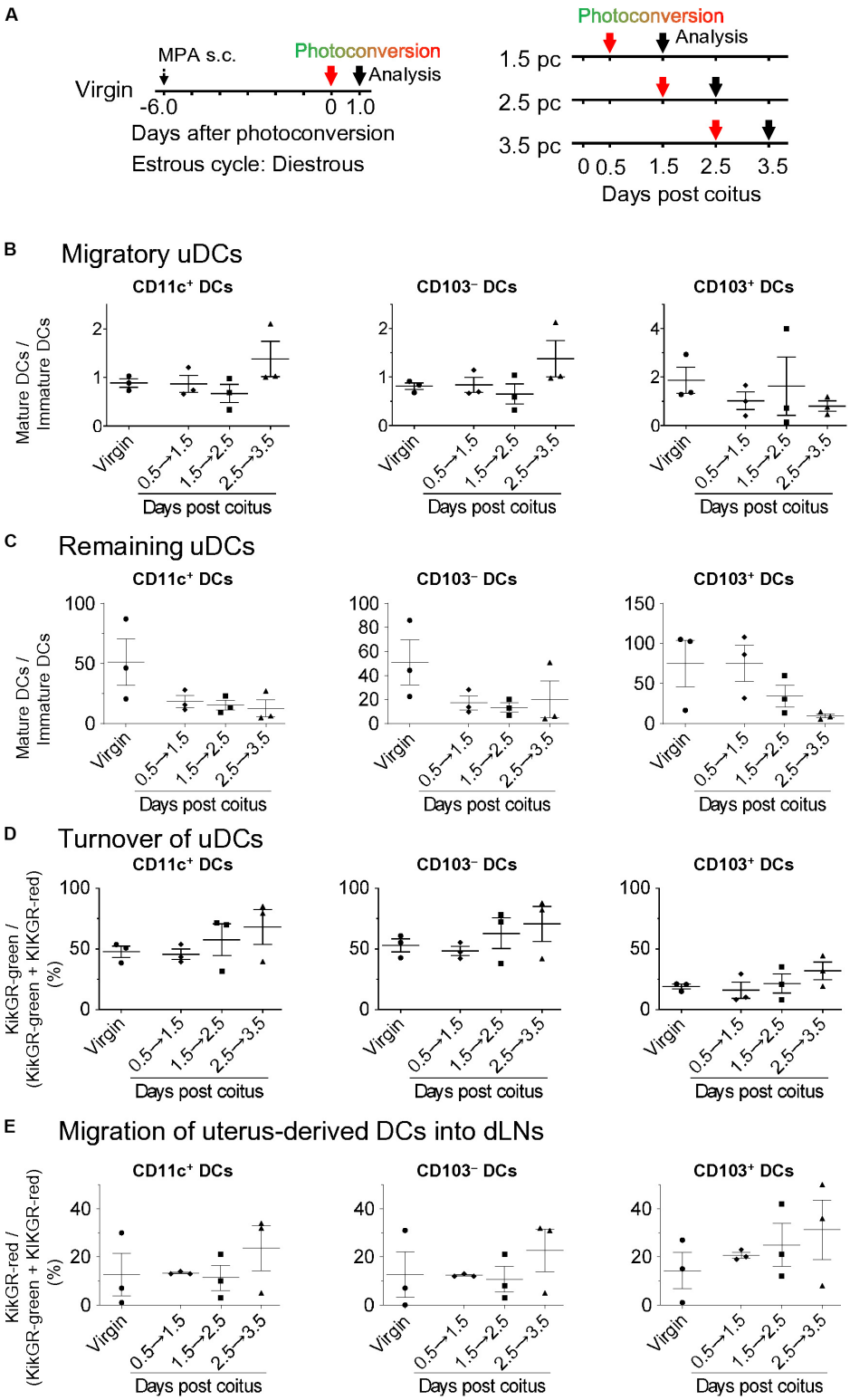

FIGURE 4 | Time course for migratory DCs and remaining DCs in the uterus and dLNs after coitus in allogeneic mating. **(A)** Experimental time course. Virgin mice were synchronized at the diestrous stage, and the uteri of mice after coitus in allogeneic mating were photoconverted on days 0.5, 1.5, and 2.5 pc. The DCs in the uteri and dLNs were analyzed 24 h after photoconversion. **(B–E)** Graphs show the ratio of mature DCs/immature DCs **(B,C)** in each DC subset labeled with KikGR-green **(B)** and KikGR-red **(C)**, and proportion of each DC subset labeled with KikGR-green in the uterus, **(D)** and KikGR-red within MHC class II high DC subset in the dLNs **(E)** out of the total DCs 24 h after photoconversion. Three samples from each time point were analyzed. Data represent mean ± SEM **(B–E)** and are representative of three independent experiments. Statistical comparisons were performed using the Kruskal-Wallis test with Dunn's multiple comparisons test.

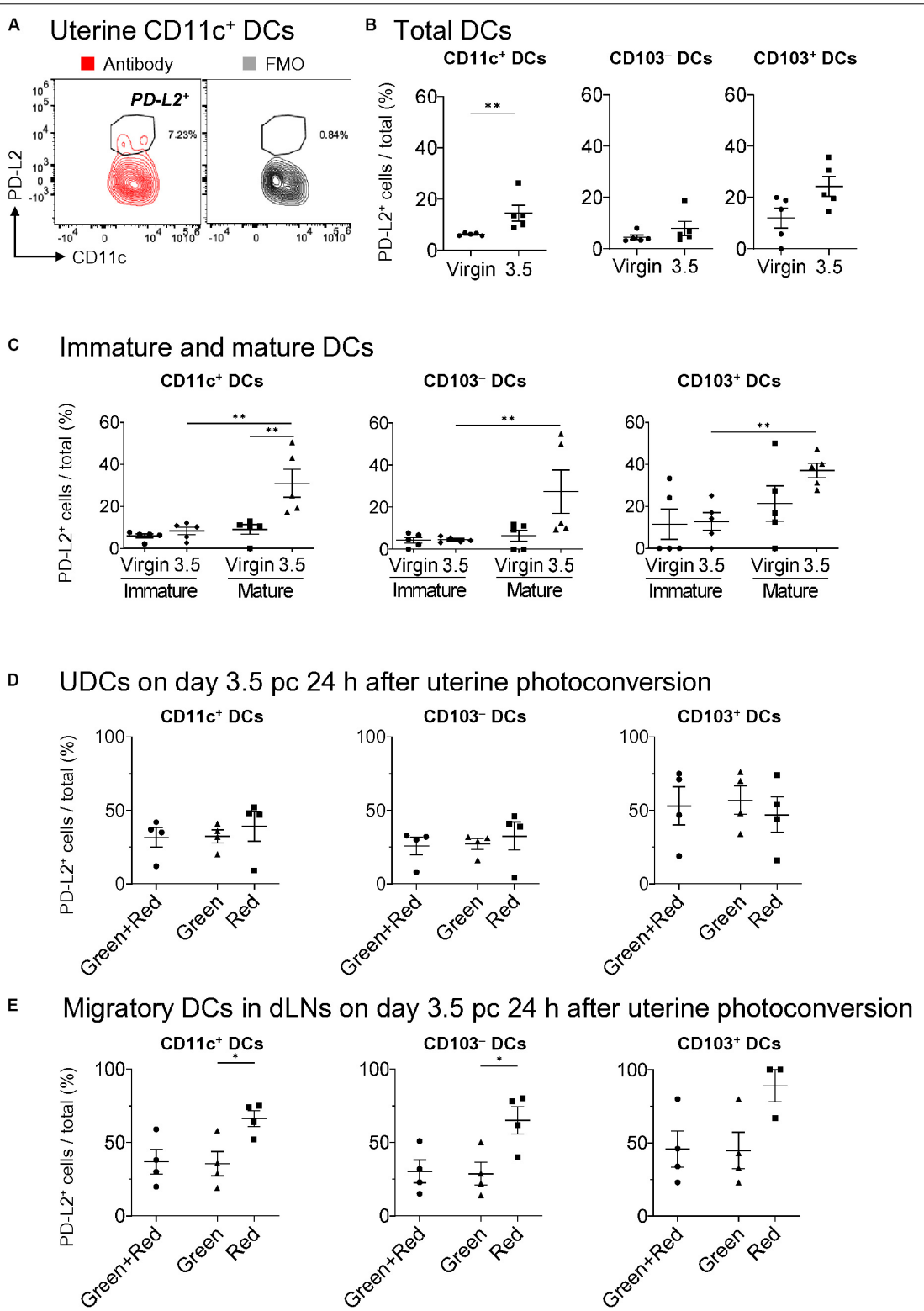

FIGURE 5 | Expression of PD-L2 in uDCs increased immediately before implantation. **(A)** Flow cytometry contour plots show expressions of PD-L2 in uterine CD11c$^+$ DCs with fluorescence minus one (FMO). **(B,C)** Proportion of PD-L2$^+$ DCs of total DCs **(B)**, immature DCs, and mature DCs **(C)** in allogeneic mating at each time point. **(D,E)** Proportion of PD-L2$^+$ DCs within the uterus **(D)** and dLNs **(E)** in allogeneic mating 24 h after uterine photoconversion on day 3.5 pc. A minimum of five **(B,C)** and four **(D,E)** samples from each time point were analyzed. Data represent mean ± SEM **(B,C)** and are representative of three independent experiments. Statistical comparisons were performed using Mann-Whitney *U*-test **(B,C)** and Kruskal-Wallis test with Dunn's multiple comparisons test **(D,E)** (**$P < 0.01$, *$P < 0.05$).

expressed PD-L2 on day 3.5 pc in allogeneic mating (**Figure 5C**). Moreover, we examined results for day 3.5 pc—24 h after uterine photoconversion—to clarify the type of DCs expressing PD-L2. However, no clear differences were observed in PD-L2 expression between the migratory DCs and remaining DCs in the uterus on day 3.5 pc (**Figure 5D**). Meanwhile, a significant increase was observed in the expression of PD-L2 on uterus-derived $CD11c^+$ DCs and $CD103^-$ DCs in the uterine dLNs (**Figure 5E**), suggesting that the DCs expressing PD-L2 in uterine dLNs were actually migratory DCs from the uterus.

DISCUSSION

In this study, we showed that there is a transient increase in $CD11c^+$ $CD86^{high}$ MHC class II^{high} $Ly6C^-$ $PDCA\text{-}1^{dim}$ $CD11b^+$ $CD103^-$-mature uDCs on day 0.5 pc, and $CD11c^+$ $CD86^{dim}$ MHC class II^{dim} $Ly6C^-$ $PDCA\text{-}1^{dim}$ $CD11b^-$ $CD103^+$-immature uDCs on day 3.5 pc. The mature DCs were observed following allogeneic- and syngeneic mating on day 1.5 pc, suggesting that sexual intercourse itself, or semen, induced accumulation of mature DCs in the uterus after coitus. After seminal priming, semen stimulates uterine inflammation through the γδ T cells/IL-17A axis (35). Moderate inflammation is proposed to play an important role in successful implantation (32). Additionally, even mechanical injury of the endometrium can contribute to the receptivity of uterus for implantation with the accumulation of DCs (42, 43). It is proposed that moderate inflammation also contributes to the infiltration of DCs to the uterus, which induces feto-maternal tolerance by recognition of paternal antigens (32, 33, 44). Our study showed that paternal antigens did not affect the increase in uDCs on day 0.5 pc as the increased uDCs on day 1.5 pc were observed in both allogeneic- and syngeneic mating. Hence, the increase in mature uDCs might play a role in successful implantation and decidualization in allogeneic- and syngeneic mating. However, the role of inflammation in successful implantation remains unknown. Moreover, further investigations are required to confirm the role of uDCs on day 1.5 pc in the process of implantation.

The migration and pre-existence of uDCs using CFSE labeling in non-pregnant mice has been reported, however, little is known about the process by which resident and migratory uDCs contribute to successful implantation (1). Our study was the first to show turnover in the uterus and migration of uDCs to the dLNs using KikGR mice. We found that half of uDCs interchanged from day 0.5 to 1.5 pc. Interestingly, uterine remaining DCs were almost mature DCs, and an equal number of immature and mature made up the migratory DC population on day 1.5 pc. In general, infiltrating DCs—which are immature phenotype—migrate to peripheral organs and are subsequently matured. After coitus uDCs are almost matured for 24 h. Importantly, we revealed that uDCs before implantation consist of two types, namely immature DCs and $CD103^-$- and $CD103^+$-mature DCs expressing PD-L2. Immature DCs, which have been previously reported (1, 45), have low antigen presenting capacity. These immature uDCs regulate maternal T cell-activation against fetal antigens and induce paternal antigen-specific Tregs *in vitro* via seminal plasma (13). Our data showed that the frequency of immature uDCs was significantly higher in allogeneic mating as compared to that in syngeneic mating on day 3.5 pc, suggesting that immature uDCs may prevent rejection of the semi-allogenetic fetus by regulating the maternal immune system. Interestingly, our data showed that approximately 68% of uDCs were transformed to migratory DCs from day 2.5 to 3.5 pc. These migratory DCs may play important roles in successful implantation by regulating decidualization and angiogenesis, as these DCs regulate the activation of T cells (1, 2, 5–8). Additionally, we observed the presence of immature uDCs immediately before implantation originating from migratory DCs, however, it was unclear the origins of these immature DCs, which must be investigated in future. Similarly, the mechanism by which DCs migrate from the periphery to the uterus should also be characteraized.

Immature DCs are typically reported to be tDCs (1, 45), however, another type of uDCs found on day 3.5 pc were the $CD103^-$- and $CD103^+$-mature DCs expressing PD-L2 (**Figures 5B,C**). We showed that a large proportion of the uterus-remaining mature DCs express PD-L2 immediately before implantation. These PD-L2-expressing cells may stimulate effector T cells with an inhibitory signal via the PD-L2/PD1 pathway, thereby inhibiting their effector T cell functions allowing for successful implantation during allogeneic pregnancy. However, it has been reported that the number of paternal antigen-specific Tregs increase in the dLNs before implantation during allogeneic, but not syngeneic mating (13, 40). The DCs in the dLNs that migrated from the uterus were of the $CD103^-$- and $CD103^+$-mature phenotype, suggesting that they could take up the paternal allo-antigen from the semen and migrate to the dLNs, wherein they could effectively stimulate both the paternal allo-antigen-specific-$CD4^+$, and -$CD8^+$ effector T cells, as well as the Tregs in dLNs before implantation (23).

Although few reports have detailed DC subtypes in the uterus, we evaluated not only $CD103^-$ DCs, $CD103^+$ DCs, and pDCs in allogeneic- and syngeneic pregnancy, but also $XCR1^+$ DCs and $XCR1^-$ DCs in virgin mice. To our knowledge, this is the first study reporting CD103-expressing uDCs in cDC2s. Thus far, many reports on cDCs have shown cDC1s and cDC2s as $CD103^+$ DCs and $CD11b^+$ DCs, respectively (46–50) and, like the DC classification of intestines (26, 39, 51), it would be necessary to investigate the expressions of XCR1 and SIRPα before detecting the expression of CD103, although, the distribution of cDC1s and cDC2s in the uterus was not determined in our present study. Additionally, as a limitation of this study, the functional analysis of migratory DCs was not examined, thus, further examination will be required to determine the relative abundance of proliferation, activation markers, and functional analysis of the uterine cDC1s and cDC2s. During DC turnover analysis we did not examine uterine photoconversion from day 0 to 1.0 pc, as there was a limitation of natural sexual intercourse after photoconversion. In addition, as a topic for further study, we plan to examine the role of sperm and seminal plasma in the induction of DC differentiation. Further research is also required to investigate the induction of Tregs and anti-inflammatory cytokines, or tolerogenic functions of each DC

subset, as well as the properties of human uDC subsets associated with implantation failure.

In conclusion, we comprehensively demonstrated the coitus-induced uDC dynamics associated with preparing tolerogenic conditions in the uterus and dLNs at the time of implantation. Importantly, we revealed that uDCs before implantation consist of two types of DCs: the immature DCs, as it has been previously proposed, as well as the $CD103^-$- and $CD103^+$-mature DCs expressing PD-L2, which may present paternal allo-antigens to $CD4^+$ and $CD8^+$ T cells with an inhibitory signal, thereby inhibiting their effector T cell functions. Our findings deepen the current understanding regarding the reproductive immune response before implantation, and may serve to provide new targets for the prevention of implantation failure.

MATERIALS AND METHODS

Mice

C57BL/6 and BALB/c mice were purchased from CLEA Japan. Knock-in mice carrying KikGR cDNA under the CAG promoter (KikGR mice) were generated as previously described (36, 37). Mice were bred and maintained in a specific pathogen-free facility at Osaka Ohtani University. All animal procedures were performed in accordance with the institutional guidelines of the Animal Research Committee of Osaka Ohtani University. C57BL/6 female mice aged 8–10 weeks were mated with BALB/c or C57BL/6 male mice. KikGR knock-in C57BL/6 female mice aged 8–10 weeks were mated with BALB/c male mice. The presence of vaginal plugs was determined the next morning and females were then separated from the males. The presence of vaginal plugs marked day 0.5 of pregnancy. Pregnant mice were euthanized on days 0.5, 1.5, 2.5, and 3.5 pc. Six days before photoconversion, non-pregnant virgin mice were synchronized in the diestrous stage via subcutaneous injection with 2 mg medroxyprogesterone (MPA) (Tokyo Chemical Industry, Osaka, Japan).

Photoconversion

During photoconversion of the uterus, non-photoconverted regions were protected from light using aluminum foil, while the region of the uterus targeted for photoconversion was exposed to violet light (405 nm, 100 mW/cm^2) for 2 min from the front and behind following laparotomy. Following photoconversion, the abdominal wall was closed. To keep the exposed tissues moist during exposure to light, warmed PBS was applied to the region of photoconversion. To prevent hypothermia, mice were warmed with a heater during the perioperative stages.

Reagents, Antibodies, and Flow Cytometric Analysis

Mononuclear cells were isolated from dLNs and the uterus on days 0.5, 1.5, 2.5, and 3.5 pc. Resected uteri were minced with scissors, and the tissues were then passed through a 100-μm cell strainer. The antibodies used were as follows: purchased from BD, eBioScience, or BioLegend: FITC-conjugated anti-I-A/I-E (clone M5/114.15.2), plycoerythrin (PE)-conjugated anti-CD103 (clone 2E7), PE-Dazzle594-conjugated anti-CD11c (clone N418), PE-cyanine5-conjugated anti-CD45R/B220 and streptavidin (clone RA3-6B2), PE-cyanine7-conjugated anti-Gr-1 and I-A/I-E (clone RB-6-8C5, and M5/114.15.2, respectively), allophycocyanin (APC)-conjugated anti-PDCA-1, CD26,and SIRPα (clone 927, H194-112, and P84, respectively), Alexa Flour 700-conjugated anti-CD45 (clone 30-F11), APC-cyanine7-conjugated anti-F4/80 and CD45 (clone BM8 and 30-F11, respectively), APC-R700-conjugated anti-CD103 (clone M290), Brilliant Violet (BV) 421-conjugated anti-CD86, CD64, and XCR1 (clone GL-1, X54-5/7.1, and ZET, respectively), Pacific blue-conjugated anti-CD11b (clone M1/70), BV510-conjugated anti-Ly6C, CD45, and CD11c (clone HK1.4, 30-F11, and N418, respectively), and biotin-conjugated anti-CD273 (clone TY25). For flow cytometric analysis, cells were washed with Dulbecco's PBS supplemented with 2% fetal calf serum (FCS), and 0.02% sodium azide. Next, cells were incubated with 2.4G2 hybridoma culture supernatant to block Fc binding. Dead cells were labeled with PI. Stained samples were acquired using SP6800 (SONY, Tokyo, Japan). KikGR-green and red signals were detected using 530/60 and 595/50 bandpass filters, respectively. Flow cytometry data were analyzed using the FlowJo software (Tree Star, Ashland, OR, United States).

Data Analysis

Dimensionality reduction was performed using tSNE analysis, followed by FlowJo. First, we exported PI^- $CD45^+$ $Gr\text{-}1^-$ $F4/80^-$ $CD11c^+$ MHC class II^+ $B220^-$ ($CD11c^+$ DCs) and PI^- $CD45^+$ $Gr\text{-}1^-$ $F4/80^-$ $CD11c^+$ $PDCA\text{-}1^+$ $CD11b^-$ $Ly6C^+$ $B220^+$ (pDCs) compartments from each dataset, and the cell numbers were adjusted to be the same as those in the minimal sample for each gestational age, including a minimum of five uterine samples. Next, the data was concatenated and visualized as a two-dimensional map by tSNE.

Mann-Whitney *U*-test and Kruskal-Wallis test with Dunn's multiple comparisons test were performed using GraphPad Prism version 8.4.3 (GraphPad Software, San Diego, CA, United States). Data in bar graphs represent mean ± standard error of mean (SEM). *P*-values <0.05 were considered to be statistically significant.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by Osaka Ohtani University.

AUTHOR CONTRIBUTIONS

IY designed the study, performed the experiments, analyzed the data, and wrote the manuscript. TM, RI, YK, AU, TS, and AN

performed the experiments and analyzed the data. MT, TS, AN, and SS designed the study and wrote the manuscript. All authors read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

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REVIEW
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Innate Immune Responses to Acute Viral Infection During Pregnancy

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Immunological adaptations in pregnancy allow maternal tolerance of the semi-allogeneic fetus but also increase maternal susceptibility to infection. At implantation, the endometrial stroma, glands, arteries and immune cells undergo anatomical and functional transformation to create the decidua, the specialized secretory endometrium of pregnancy. The maternal decidua and the invading fetal trophoblast constitute a dynamic junction that facilitates a complex immunological dialogue between the two. The decidual and peripheral immune systems together assume a pivotal role in regulating the critical balance between tolerance and defense against infection. Throughout pregnancy, this equilibrium is repeatedly subjected to microbial challenge. Acute viral infection in pregnancy is associated with a wide spectrum of adverse consequences for both mother and fetus. Vertical transmission from mother to fetus can cause developmental anomalies, growth restriction, preterm birth and stillbirth, while the mother is predisposed to heightened morbidity and maternal death. A rapid, effective response to invasive pathogens is therefore essential in order to avoid overwhelming maternal infection and consequent fetal compromise. This sentinel response is mediated by the innate immune system: a heritable, highly evolutionarily conserved system comprising physical barriers, antimicrobial peptides (AMP) and a variety of immune cells—principally neutrophils, macrophages, dendritic cells, and natural killer cells—which express pattern-receptors that detect invariant molecular signatures unique to pathogenic micro-organisms. Recognition of these signatures during acute infection triggers signaling cascades that enhance antimicrobial properties such as phagocytosis, secretion of pro-inflammatory cytokines and activation of the complement system. As well as coordinating the initial immune response, macrophages and dendritic cells present microbial antigens to lymphocytes, initiating and influencing the development of specific, long-lasting adaptive immunity. Despite extensive progress in unraveling the immunological adaptations of pregnancy, pregnant women remain particularly susceptible to certain acute viral infections and continue to experience mortality rates equivalent to those observed in pandemics several decades ago. Here, we focus specifically on the pregnancy-induced vulnerabilities in innate immunity that contribute to the disproportionately high maternal

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mortality observed in the following acute viral infections: Lassa fever, Ebola virus disease (EVD), dengue fever, hepatitis E, influenza, and novel coronavirus infections.

Keywords: pregnancy, innate antiviral immunity, Lassa virus, Ebola virus, dengue virus, hepatitis E, influenza virus, emerging coronavirus

INTRODUCTION

Pregnancy creates a unique immunological paradox: the maternal immune system must undergo complex adaptations to permit tolerance of the semi-allogeneic fetus while simultaneously maintaining robust defenses against invasive pathogens. Initial theories of maternal-fetal tolerance proposed that a temporary state of maternal immunosuppression was vital to allow successful implantation and development of a pregnancy (1–3). With the advent of technologies including microscopy, advanced cytometry and single-cell sequencing, these models have been superseded by new data that suggest a tightly regulated balance between inflammatory and tolerogenic states during the "immune chronology" of normal pregnancy (4–8). Longitudinal studies of peripheral, decidual, and amniotic fluid cytokine profiles and immune cell subsets demonstrate that a pro-inflammatory environment predominates during early trophoblast invasion and at parturition, while the second and third trimesters require an anti-inflammatory bias to facilitate fetal growth (5, 8–11). The balance between innate and adaptive immunity shifts in favor of innate mechanisms, particularly in the first trimester; as pregnancy progresses, silencing of chemokine genes inhibits accumulation of effector T cells in the decidua, peripheral B cells are depleted and pregnancy-specific hormones skew B cell polarization toward a tolerogenic IL-10-producing phenotype (12–14).

These changes are orchestrated by the sentinel innate immune cells of the maternal decidua—neutrophils, macrophages, dendritic cells, and natural killer cells—and their molecular interactions with invading fetal trophoblast at the maternal-fetal interface. Activation of decidual innate immunity is crucial in the establishment of a pregnancy-specific immune environment: it recruits additional populations of leukocytes to the decidua, educates adaptive cells to refine appropriate effector and memory responses, and modulates the phenotype and functions of peripheral immune cells (15–18). Dysregulation of this complex bi-directional relationship has been implicated in several obstetric and perinatal complications, including recurrent miscarriage, pre-eclampsia, fetal growth restriction, chorioamnionitis, and preterm birth (19–26).

The corollary of this capacity for immunological tolerance is an increased susceptibility to infection (27–29). The innate cells that mediate maternal-fetal crosstalk and induction of tolerance also constitute the frontier of defense against infection through a wide repertoire of effector mechanisms (30–32). Chief amongst these are the expression of pattern-recognition receptors that detect pathogen-specific molecular signatures and soluble mediators such as the complement system. The World Health Organization estimates that sepsis accounts for 10.7% of maternal deaths globally (33) and there is evidence of a particular vulnerability to acute viral infection. For example, pregnant women suffered disproportionately high mortality rates in the influenza pandemics of 1918, 1957, and 2009 (34–36), and hepatitis E, typically a mild and self-limiting illness, has a 26% case-fatality rate in pregnant women (28). These disparities are likely to arise from a combination of immunological, hormonal, and physiological adaptations that are specific to pregnancy (37–39).

Viral infection in pregnancy carries four distinct risks:

1. Adverse pregnancy outcomes: acute viral infection is consistently associated with a broad spectrum of obstetric complications [reviewed in (40–42)]
2. Acute severe maternal disease with consequent morbidity and/or mortality
3. Vertical transmission to the fetus (during pregnancy), resulting in congenital infection that can cause intrauterine death or permanent disability
4. Perinatal transmission to the fetus (during delivery), which can cause severe neonatal disease.

Although congenital infection is a major public health concern, this article will not cover viruses that cause fetal damage through vertical or horizontal transmission as these have been recently and comprehensively reviewed (42, 43). Instead, we focus on the following six viruses listed in **Table 1** that cause acute severe maternal disease, reviewing the innate immune mechanisms and viral evasion strategies that contribute to their effects in pregnancy.

DECIDUAL INNATE IMMUNITY IN PREGNANCY: AT THE FRONTIER OF MATERNAL-FETAL TOLERANCE AND INFECTION

Pregnancy is a unique immunological state. Alterations in systemic maternal immunity and cellular dialogue at the maternal-fetal interface combine to maintain tolerance of the fetal allograft while simultaneously preserving the ability to respond to infection. Shortly after conception, rising progesterone levels trigger decidualization, the transformation of the endometrium into a specialized tissue that promotes implantation of the blastocyst (62). Intensive study of the cellular composition of the decidua over recent years has identified diverse immune cell populations, including natural killer (NK) cells, macrophages, dendritic cells (DC) and T cells (63–65) (**Figure 1**). Interactions between these decidual immune cells and the invading fetal extravillous trophoblast exert a critical influence on subsequent placentation, fetal growth and pregnancy outcome (23, 25, 67–69).

TABLE 1 | RNA viruses that cause severe disease in pregnancy.

Virus	Family	Genome	Global burden of disease	Overall case-fatality rate (CFR)	Case-fatality rate (CFR) in pregnancy
Lassa virus	*Arenaviridae*	ssRNA	>500,000 cases annually, endemic in West Africa (44)	1% (44)	33.7% (29)
Ebola virus	*Filoviridae*	ssRNA	>28,000 cases during the 2013–2016 epidemic in West Africa (44)	45–90% (44)	84.3% (45)
Dengue virus	*Flaviviridae*	ssRNA	390 million cases annually (46)	1.1% (47)	3% (48)
Hepatitis E virus	*Hepeviridae*	ssRNA	20 million cases annually (49)	0.2–4% (42)	26% (28)
Pandemic influenza	*Orthomyxoviridae*	ssRNA	1918: >500 million cases 1957: 2 million cases 2009: 1.6 million cases	1918: 2.5% (50) 1957: 0.1% 2009: 2.5% (27)	1918: 27–50% (34, 51) 1957: 30–50%* 2009: 1.7–11% (27, 36, 52–54)
Novel coronavirus infections: - SARS-CoV - MERS-CoV - SARS-CoV-2	*Coronaviridae*	ssRNA	SARS: 8,437 cases 2002–03 MERS: 2,494 cases 2012–13 COVID-19: >21 million cases 2019–20	SARS: 11% (55) MERS: 34% (55) COVID-19: 1.3–4.2% (56)	SARS: 30–40% (57, 58) MERS: 54% (59) COVID-19: 1.2% (60)

SARS-CoV, severe acute respiratory syndrome coronavirus; MERS-CoV, Middle East respiratory syndrome coronavirus; COVID-19, syndrome resulting from acute infection with novel coronavirus SARS-CoV-2; ssRNA, single-stranded ribonucleic acid.

**Maternal mortality in the 1957 "Asian" influenza pandemic is not well described, but half the women of reproductive age (15–44 years) who died were pregnant and the disease became the leading cause of maternal death in Minnesota (35, 61).*

The unique non-classical human leukocyte antigen (HLA) class I molecule HLA-G is exclusively expressed on extravillous trophoblast (EVT) (70, 71). Invading EVT comes into direct contact with maternal cells when it infiltrates through the decidua into the myometrium, remodeling maternal spiral arteries into dilated low-resistance channels that maximize blood flow to the developing feto-placental unit (42).

The EVT is capable of both immune evasion *and* induction of tolerance due to its unique HLA expression profile, consisting of only the class I molecules HLA-C, HLA-E, and HLA-G. Trophoblast HLA-G undergoes high-affinity binding with leukocyte immunoglobulin-like receptor B1 (LILRB1), an inhibitory receptor widely expressed on decidual antigen-presenting cells. This interaction modulates decidual DC signaling, suppresses production of pro-inflammatory cytokines and inhibits proliferation of maternal T cells. HLA-G therefore provides a critical tolerogenic signal at the maternal-fetal interface (72–74).

The role of decidual innate immune cells in defense against infection is an emerging and rapidly evolving field. Current knowledge is summarized in a recent review by Yockey et al. (75) and relevant aspects of the decidual innate immune response to viral infection will be discussed in detail below. Despite significant advances, our understanding of the recalibration of the innate-adaptive equilibrium in pregnancy remains incomplete. This is due to the practical and ethical difficulties of obtaining decidual tissue samples and the uncertain correlation between peripheral and decidual immune cell activity (6, 25, 76). Renewed focus on the role of the maternal innate immune system in the apparent conflict between fetal tolerance and robust defense against intracellular pathogens will provide further insights into how these two priorities interact in pregnancy. For the purposes of this review, the term "tolerance" refers to the pregnancy-specific state of tolerance of the semi-allogeneic fetus, rather than to the specific T cell phenomenon.

SYSTEMIC INNATE IMMUNITY IN PREGNANCY

Systemically, there is a global upregulation of innate immune cells and effector mechanisms in normal pregnancy (6, 77). Complement activity increases compared to the non-pregnant state (78, 79) and there is a substantial rise in circulating phagocytes and type I interferon (IFN)-producing plasmacytoid DC with advancing gestational age (9, 80). Longitudinal studies of serial blood samples from pregnant women show specific enhancement of innate pathways that mediate antiviral immunity: for example, IFN-α-induced STAT1 signaling, a critical response to viral challenge, increases throughout gestation in NK cells, monocytes and myeloid DC (6, 81). This state must be finely calibrated: excessive activation can be associated with tissue damage during response to acute viral infection (9, 77, 82, 83) and adverse obstetric outcomes [such as complement overactivity in antiphospholipid syndrome (84, 85)], while an attenuated immune response could predispose to overwhelming infection. Whether maternal susceptibility to RNA viral infections is due to over- or under-activity of the innate immune system is not yet clear; it is likely that some effector mechanisms are upregulated while others are suppressed. Data specific to individual viruses will be discussed below.

Of all the viruses discussed in this review, influenza has received the most scientific attention with regards to its propensity for pregnancy, but precise mechanisms underlying this vulnerability remain uncertain. While some animal models (86, 87) and a few *ex vivo* human studies (82, 83) have

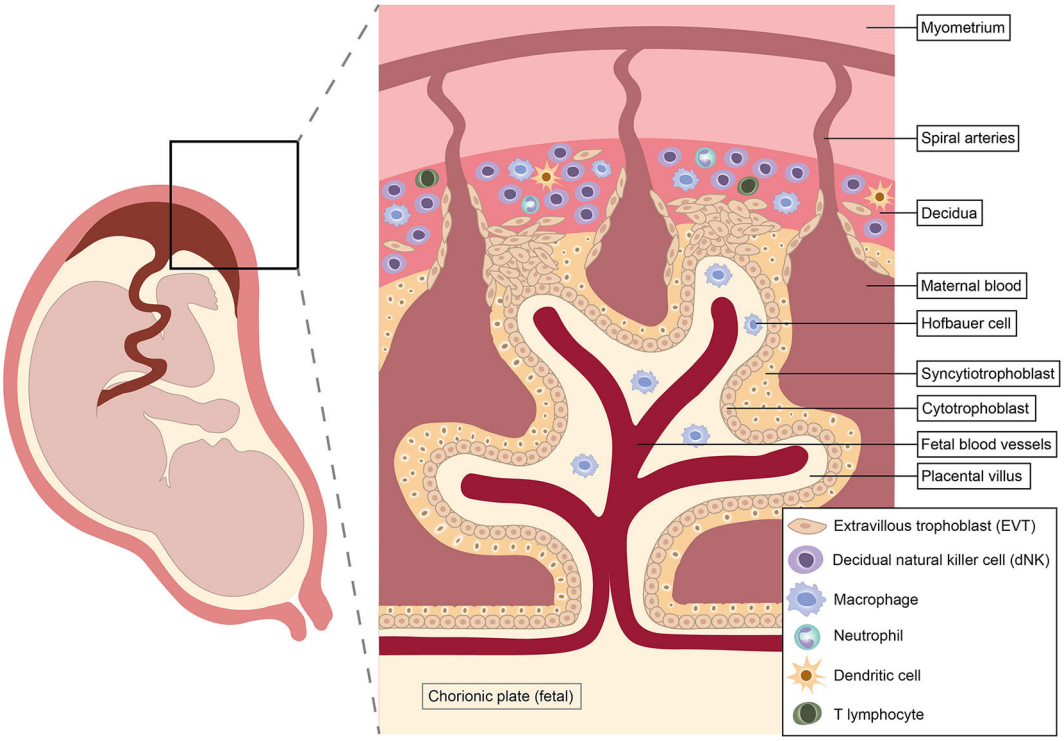

FIGURE 1 | Architecture and immunological repertoire of the maternal-fetal interface. Immune cells shown reflect approximate abundances of different populations in the first and second trimesters of pregnancy. Natural killer cells predominate in early pregnancy, but disappear from mid-gestation onwards (62, 66).

determined specific innate immune correlates of the increased severity of influenza in pregnancy, much less is known about the other viruses included in this article. This review will synthesize available knowledge on the nature, magnitude, and timing of innate immune responses to viral infection in pregnancy and how these interact with decidual, hormonal and physiological influences.

MOLECULAR MECHANISMS OF INNATE IMMUNITY TO VIRUSES

Human antiviral immunity is a two-step process. Non-specific innate mechanisms are activated immediately, predominate during the first 5–7 days of infection, and are then superseded by T and B cell-mediated antigen-specific adaptive responses. Rapid-onset innate responses may be sufficient to eliminate the virus, but if not, they limit replication during the critical temporal gap between onset of viral challenge and development of adaptive virus-specific cytotoxic lymphocytes, reducing the likelihood of disseminated disease (88).

The innate antiviral response is activated when pattern-recognition receptors (PRRs) detect pathogen-associated molecular patterns (PAMPs), particularly viral nucleic acids. Toll-like receptors (TLRs) and RIG-I-like receptors (RLRs), which are expressed on innate cell membranes and throughout the intracellular compartment, are the primary viral sentinels (89). This PRR-PAMP interaction triggers activation of latent transcription factors that upregulate a vast repertoire of antiviral effector proteins: type I interferons, other pro-inflammatory cytokines such as TNF and IL-1β, chemokines and the antimicrobial peptides (e.g., defensins, cathelicidins, surfactant proteins) (88, 90, 91).

The Interferon Response

Interferons, which are classified according to their cell surface receptors, are grouped into three types. Type I (including IFN-α and IFN-β) and type II (IFN-γ) are produced by virtually all cells, while the more recently discovered type III (IFN-λ) is produced by epithelial and dendritic cells (92). The production of type I IFNs is the hallmark of effective antiviral immunity. Once secreted by virally infected cells, they act in a paracrine manner to induce IFN-stimulated gene (ISG) expression in neighboring cells, creating an antiviral state in the surrounding environment (shown in **Figure 2**). IFN binding to the type I IFN receptor, IFNAR, leads to receptor endocytosis and activation of the receptor-associated tyrosine kinases Janus kinase I (JAK-1) and tyrosine kinase 2 (TYK2). These in turn activate transcription factors STAT1 and STAT2, which associate with interferon regulatory factor 9 (IRF9). IRF9 is translocated to the nucleus and triggers IFN-stimulated response elements (IRSEs) to upregulate expression of ISG, the definitive effectors of the antiviral response (91–93). Type III IFN can also induce ISG but their effects appear to be limited to sites of epithelial damage at anatomical barriers, exerting a localized antiviral response that is superseded

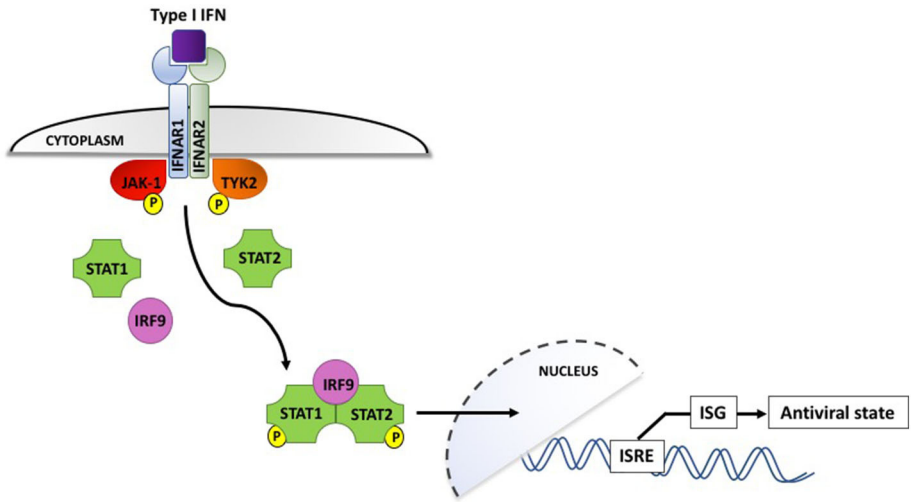

FIGURE 2 | Type I interferon signaling via the IFNAR receptor induces phosphorylation and activation of the JAK-1 and TYK2 tyrosine kinases, which interact with IRF9 to upregulate interferon-stimulated genes and induce an antiviral state in the surrounding cellular microenvironment. IFN, interferon; IFNAR, interferon α/β receptor; IRF9, interferon regulatory factor 9; ISG, interferon-stimulated genes; ISRE, interferon-stimulated response elements; JAK-1, Janus-associated kinase I; STAT1/2, signal transducer and activator of transcription 1/2; TYK2, tyrosine kinase 2. P denotes phosphorylation.

by potent systemic type I IFNs if the infection is not successfully contained (94).

Cellular Interactions With RNA Viruses

RNA viruses, the focus of this review, are detected by a variety of PRRs. Plasmacytoid dendritic cells (pDC) detect viruses via endosomal TLR7-9 and are responsible for the first wave of type I IFN production, releasing large quantities through the MyD88-IRF7 pathway [reviewed in (95)]. In contrast, macrophages and conventional dendritic cells (cDC) sense viral challenge through RLRs, specifically the cytoplasmic helicases RIG-I and MDA-5. These receptors signal via mitochondrial antiviral-signaling protein (MAVS) and IRF3/7, culminating in a second wave of type I IFN production (91, 95) (**Figure 3**). The emerging role of NK cells in antiviral immunity and pregnancy, recently reviewed in this journal (66), has provoked controversy and is discussed in more detail below.

Viral Interactions With Programmed Cell Death Pathways

Since viruses depend on host cells for replication, programmed cell death pathways—including apoptosis, necroptosis, and pyroptosis—are a crucial component of antiviral defense. In apoptosis, infected cells undergo an orderly caspase-mediated degradation and are rapidly cleared by surrounding phagocytes (96). This highly regulated disassembly minimizes the release of damage-associated molecular patterns (DAMPs) that could trigger harmful auto-inflammatory responses, but also fails to induce robust antiviral immunity (97).

Necroptosis and pyroptosis differ from apoptosis in that they are powerfully immunogenic. They induce lytic cell death, triggering release of DAMPs and pro-inflammatory cytokines, as detailed in a recent review (98). Although these mechanisms have been implicated in a wide range of autoimmune disorders [reviewed in (99)], they are also a key component of antiviral immunity.

Necroptosis is a caspase-independent process that can be triggered through several different mechanisms, including TNF signaling and TLR detection of viral molecular signatures. These pathways converge on receptor-interacting protein kinase-3 (RIPK3), which phosphorylates and activates the pseudokinase mixed lineage kinase domain-like protein (MLKL). Activated MLKL undergoes conformational changes that expose its pore-forming 4-helical bundle domain, leading to rapid cell lysis (97, 100).

Pyroptosis, a swift and powerfully pro-inflammatory form of programmed cell death, results from activation of the cytosolic NLRP3 inflammasome in virally infected cells. Its caspase-1 effector domain cleaves and activates gasdermin-D, triggering lethal pore formation in the host cell membrane and efflux of pro-inflammatory cytokines (101, 102).

Complement Antiviral Responses

The complement system is another crucial component of antiviral defense. It bridges the innate-adaptive divide through its diverse roles: opsonization and lytic destruction of pathogens, clearance of apoptotic cells and immune complexes, phagocyte chemotaxis and mast cell activation. A highly conserved cascade of over 50 circulating and membrane-bound protein components, it can be activated through three separate pathways: classical, alternative, and lectin. All converge on the formation of C3 convertases, which cleave C3 into active fragments C3a and C3b. Deposition of C3b on cell or pathogen surfaces triggers formation of the C5 convertase, which splits C5 into C5a and C5b, catalyzing the formation of the membrane attack

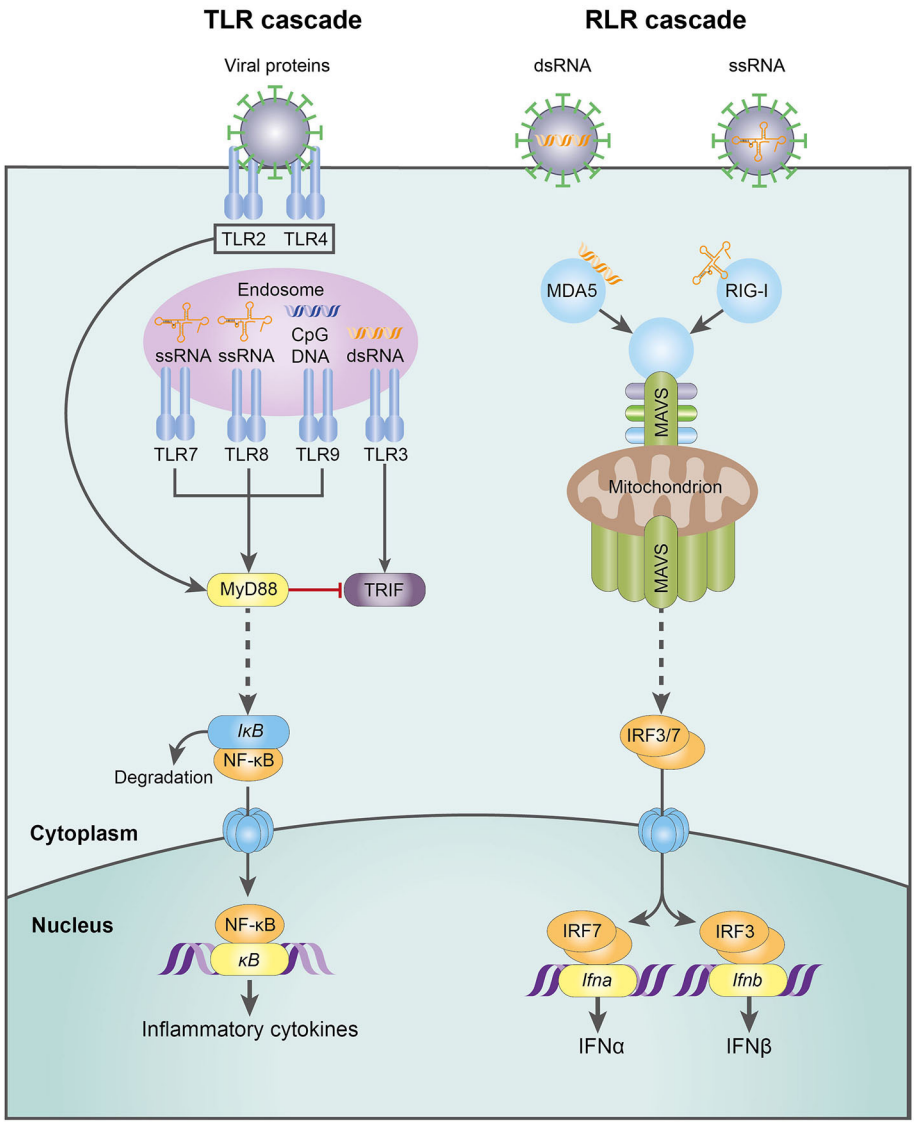

FIGURE 3 | The molecular mechanisms of the TLR- and RLR-mediated innate response to RNA virus infection. CpG DNA, cytosine-guanine oligodeoxynucleotides; dsDNA, double-stranded deoxyribonucleic acid; IFN-α, interferon-α; IFN-β, interferon-β; IkB, inhibitor of NFkB; IRF3, interferon regulatory factor 3; IRF7, interferon regulatory factor 7; MAVS, mitochondrial antiviral-signaling protein; MDA5, melanoma differentiation-associated protein 5; MyD88, myeloid differentiation primary response 88; NFkB, nuclear factor kappa-light-chain-enhancer of activated B cells; RIG-I, retinoic acid-inducible gene I; RLR, RIG-I-like receptor; ssRNA, single-stranded ribonucleic acid; TLR, Toll-like receptor; TRIF, TLR-domain-containing adapter-inducting interferon-β.

complex (MAC) that penetrates viral membranes to induce lytic destruction (26, 103–105).

Regulation of the Antiviral Response

The pro-inflammatory antiviral response is tightly calibrated: inadequate responses lead to overwhelming infection, while excessive activity causes host tissue damage and is associated with autoimmune disease. A variety of inbuilt negative feedback post-translational modification systems orchestrate spatial and temporal regulation of the antiviral response. For example, ubiquitin-specific peptidase 18 (USP18, also known as ISG43) displaces JAK1 from IFNAR to block type I IFN signaling and also deconjugates ISG15 (one of the most potent inhibitors of viral replication) from its target proteins (91, 106–108).

Such mechanisms are not always sufficient to prevent dysregulation of type I IFN activation, which has been implicated in several auto-inflammatory conditions including systemic lupus erythematosus (91, 109), a disease which shows excessive complement deposition. Similarly, excessive complement activation contributes to acute lung injury in mouse and human studies of influenza and novel coronaviruses, through over-production of the anaphylatoxins C3a and C5a (110–112).

The following sections examine the individual cellular components and effector mechanisms of the innate antiviral response that undergo significant adaptations in pregnancy.

PREGNANCY-INDUCED MODIFICATION OF ANTIVIRAL IMMUNITY

Antimicrobial Peptides

Antimicrobial peptides (AMP) are small molecules that create a microbicidal shield at mucosal surfaces with high pathogen exposure, such as the small intestine, the renal epithelium, and the chorionic membranes. They are secreted by leukocytes and disrupt pathogen membrane integrity, leading to lytic destruction. Three classes of AMP that contribute to antiviral immunity exhibit differential expression in pregnancy: defensins, cathelicidins, and surfactant proteins.

Human defensins are categorized into three families (α, β, and θ). Alpha-defensins are found in neutrophil granules and Paneth cells while beta-defensins are expressed in epithelial cells, including those of the female reproductive tract, and are upregulated by cytokine secretion in response to pathogen-mediated epithelial injury (113, 114).

During pregnancy, the AMPs human-beta-defensin-1 (HBD-1) and HBD-3 are elevated in the amniotic fluid of women who develop preterm labor with coexistent infection (115, 116). HBD1-3 are also expressed in the trophoblast and decidua, suggesting a critical physiological role for defensins in innate immune defenses at the maternal-fetal interface; they also participate in innate-adaptive crosstalk through chemotaxis, recruiting T cells and immature DC (117).

Like defensins, cathelicidins are small AMP secreted by innate cells and mucosal barriers. The sole human cathelicidin, LL-37, is vitamin D-inducible and promotes wound healing, angiogenesis and clearance of cell debris; it can also regulate macrophage and DC responses to pro-inflammatory stimuli (118). LL-37 expression in first-trimester cervicovaginal secretions is significantly higher in women with bacterial vaginosis and incubation of endocervical epithelial cells with LL-37 *in vitro* induces a pro-inflammatory milieu with enhanced secretion of IL-8 (119). Circulating serum LL-37 is also elevated during pregnancy: LL-37 levels rose consistently in serial samples from Ugandan pregnant women, peaking in the third trimester (120). These studies suggest a role for LL-37 in decidual and systemic pregnancy-specific innate immune defenses.

AMP have been implicated in the innate response to influenza A virus (IAV). LL-37 neutralizes IAV in mouse models by directly damaging viral membranes, whereas surfactant protein-D (SP-D) works by triggering viral aggregation and inhibiting haemagglutinin activity (121–124). In human monocytes, LL-37 and SP-D readily block replication of seasonal IAV, but both had strikingly impaired inhibitory activity against the pandemic H1N1 strain, whereas antiviral activity of a related AMP, H-ficolin, was unchanged (125, 126). The significance of this strain-specific discrepancy in AMP-mediated anti-influenza immunity in pregnancy requires further clarification.

Toll-Like Receptors

The placenta expresses the full repertoire of human TLRs (TLR1-10) (127). Of these, TLR3, TLR7, TLR8, and TLR9 contribute to antiviral immunity: TLR3 binds to double-stranded viral RNA; TLR7 and 8 detect single-stranded RNA viruses; and TLR9, which recognizes unmethylated cytosine-guanine (CpG) motifs in bacterial genomes, can also respond to herpesvirus infection. Antiviral PRR signaling mostly converges on the canonical MyD88 pathway, stimulating activation of NFκB and production of pro-inflammatory cytokines (128, 129). The exceptions are TLR3 and RIG-I, which signal through an alternative MyD88-independent pathway that uses an adapter protein, TRIF, to generate large amounts of IFN-β (130).

TLR expression at the maternal-fetal interface exhibits both temporal and tissue-specific fluctuations in expression levels and functionality (131–134). This suggests a potential contribution to the observed differences in severity of fetal and maternal viral infections in different trimesters (42, 44, 48, 66, 135). In human trophoblast, TLR3 is highly abundant in the first trimester and forms a defensive barrier along the cytotrophoblast with TLR2 and TLR4 (136). TLR3 has dual roles at the placental interface, inhibiting viral replication to protect the developing fetus from vertical transmission but also promoting tolerance through release of indoleamine 2,3-dioxygenase (IDO) (137–139). In the decidua, Duriez and colleagues have demonstrated differential expression of the four antiviral TLRs in decidual macrophages and NK cells, with each cell type producing a distinct cytokine signature in response to TLR7/8 ligation (140). These TLR-mediated responses to viral challenge are at the frontier of the critical balance between tolerance and immunity, which ultimately dictates whether or not a pregnancy will be successful (141).

Complement

In pregnancy, complement activity is increased systemically but suppressed at the maternal-fetal interface (26, 142). While some complement components contribute to normal placentation [C1q, for example, promotes adequate EVT invasion and remodeling of maternal spiral arteries (143, 144)], the majority must be inhibited to ensure successful pregnancy. Synergistic action of regulatory molecules at the placenta impairs formation of the classical and alternative C3 convertases, preventing downstream activation of the C5 convertase and the MAC (104). Failure to suppress these pathways is associated with a wide range of adverse obstetric outcomes, including recurrent miscarriage, fetal growth restriction, preterm birth and pre-eclampsia (26, 104, 145).

The complement system mediates neutralizing antiviral immunity through multiple effector mechanisms, including:

1. MAC-induced pore formation in viral envelopes, leading to lytic destruction;
2. MAC-independent virolysis through deposition of complement components on non-enveloped viruses or direct binding to MBL;
3. Opsonization of virions: this induces aggregation, decreasing the total infectious burden for the host, and may be followed by phagocytosis (103, 146, 147).

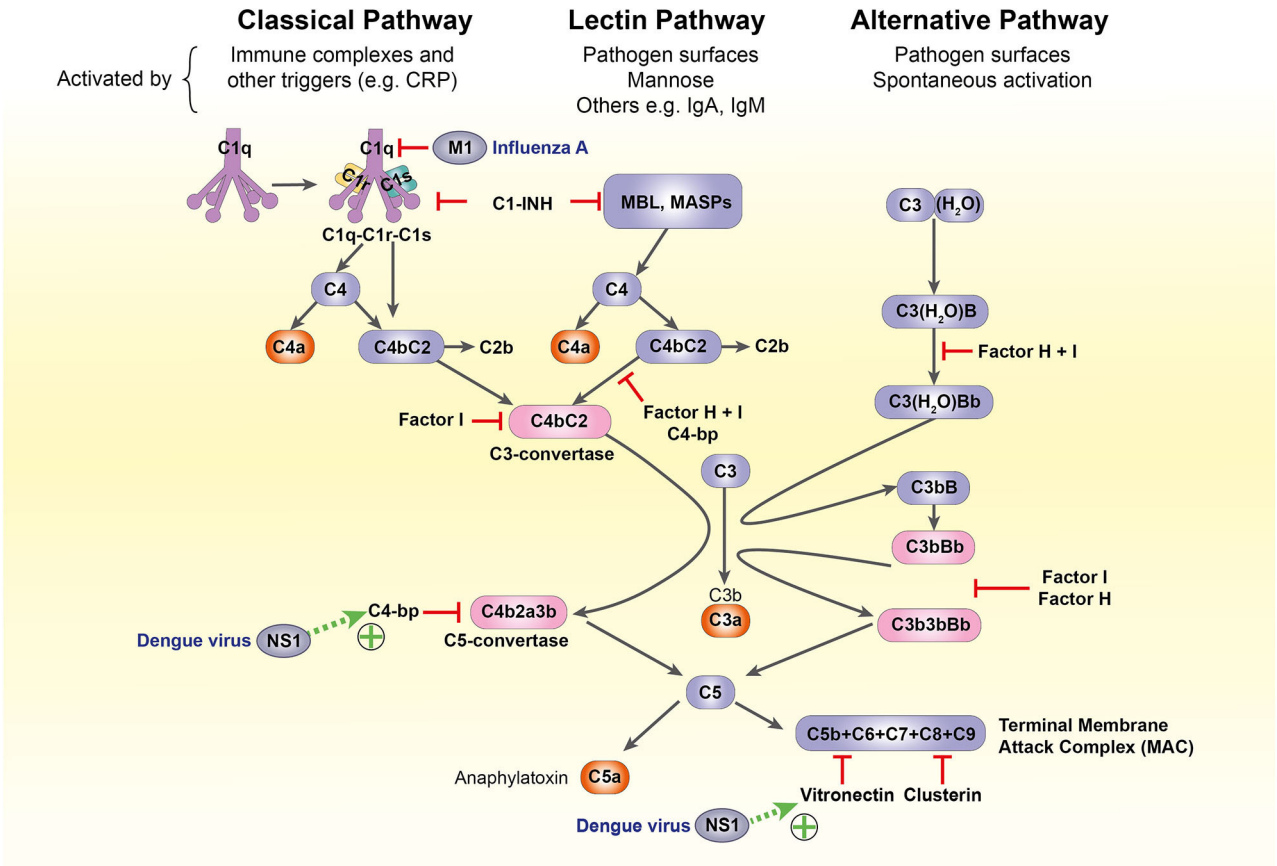

FIGURE 4 | The complement system and its subversion by dengue and influenza A viruses. C1-INH, C1-inhibitor; C4-bp, C4-binding protein; CRP, C-reactive protein; IgA/IgM, immunoglobulin A/M; M1, influenza virus matrix protein 1; MASP, MBL-associated serine protease; MBL, mannose-binding lectin; NS1, non-structural protein 1.

However, the diversity of methods for complement-mediated viral attack is mirrored and in some cases surpassed by viral evasion mechanisms, which vary widely according to species and virion structure. For example, Dengue virus non-structural protein 1 is extruded from infected cells and recruits terminal complement inhibitors C4b-binding protein and vitronectin to the cell surface, inhibiting MAC assembly (148, 149); the influenza virus matrix protein 1 inhibits C1q-mediated activation of the classical pathway (150) (**Figure 4**). These mechanisms have not been characterized in pregnancy and a complete discussion of complement subversion by viruses is beyond the scope of this article, but further detail is available in a recent review (103).

Cellular Innate Immunity

Various cellular mechanisms are exploited by viral infection, a number of which are altered during pregnancy.

Innate Lymphoid Cells

Innate lymphoid cells (ILC), which account for up to 70% of all leukocytes present in first-trimester decidua (62), originate from the common lymphoid progenitor and bridge the innate-adaptive divide. Like other innate cells, they mount rapid responses to infection, lack antigen-specific receptors, and do not exhibit conventional clonal expansion; like T cells, they can modulate adaptive immune responses through production of specific cytokines and regulation of B cell and DC activity. On this basis, ILC have been described as "innate counterparts" of T cells (151). Their abundance at the maternal-fetal interface suggests pivotal roles in both innate immune defense and normal placental development (152). **Table 2** summarizes the classification and functions of decidual ILC:

Given the recent discovery of ILC, data on their antiviral properties are still accumulating. In the liver, rapid ILC1-mediated IFN-γ production is essential for early suppression of cytomegalovirus (CMV) viremia (156); in the lungs, production of amphiregulin by ILC2 is critical for restoration of epithelial integrity and lung function following influenza infection, while excessive remodeling can predispose to allergy (157, 158). In pregnancy, investigations have focused on NK cells, both peripheral and decidual.

Peripheral Blood Natural Killer Cells

The majority (90%) of peripheral blood NK (pbNK) cells exhibit a predominantly cytotoxic $CD56^{dim}$ $CD16^{+}$ phenotype, while

TABLE 2 | The roles of decidual innate lymphoid cell subsets in pregnancy (152–155).

Subset name		Phenotype	Greatest abundance	Key effectors	Roles in the decidua
ILC1	dNK cells	$CD56^{bright}$ $CD16^{-}$ $KIR^{++/-}$ $CD9^{+}$ $Tbet^{+}$ $Eomes^{+}$	Early pregnancy	Perforin Granzyme B Granulysin Cytokines	Tolerance Trophoblast invasion Tissue remodeling Antiviral immunity
	Non-cytotoxic ILC1	$CD56^{-}CD127^{-}$ $CD117^{-}Tbet^{+}$ Eomes-	Early pregnancy	IFN-γ	Defense against intracellular bacteria and parasitic infection Allergy
ILC2		$CD56^{-}CD127^{+}$ $CD161^{+}Tbet^{-}$	Late pregnancy	IL-4, IL-5, IL-13, IL-22	Tolerance Tissue repair Homeostasis
ILC3	NCR^{+}	$CD127^{+}CD117^{+}NCR^{+}$	Early pregnancy	IL-8, IL-22, GM-CSF	Tissue remodeling Neutrophil recruitment and activation
	NCR^{-}	$CD127^{+}CD117^{+}NCR^{-}$	Early pregnancy	IL-17A, TNFα	Renewal of NCR^{+} population through ILC3 plasticity
	LTi-like	Defined by RORγt expression	Early pregnancy	IL-17A, TNFα	Tissue remodeling Recruitment of other immune cells

dNK cells, decidual natural killer cells; Eomes, eomesodermin; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL-, interleukin-; ILC, innate lymphoid cells; KIR, killer-cell immunoglobulin-like receptors; LTi, lymphoid tissue inducer, NCR, natural cytotoxic receptor; RORγt, RAR-related orphan gamma receptor; T-bet, T-box expressed in T cells; TNF, tumor necrosis factor.

the remaining 10% constitute the main cytokine-producing $CD56^{bright}$ $CD16^{-}$ subset (25). Although not antigen-specific, NK cells can utilize the Fc-receptor CD16 to target cells for destruction. Aside from CD16, NK cells integrate signals from an array of germline-encoded activating and inhibitory receptors. Inhibitory signals transmitted through interaction with the ubiquitously expressed HLA class I and the non-classical HLA-E dominate in the healthy human. This balance can be shifted in the case of diseases such as cancer, in which HLA-I and HLA-E may be downregulated, and instead ligands for activating receptors can be induced on transformed cells (e.g., NKG2D ligands) (159).

NK cell receptors can distinguish between "self" and "non-self" through detection of HLA class I and related molecules: their absence can be sensed and triggers degranulation and release of cytotoxic components. Through the same mechanism, viruses, which downregulate HLA-I expression in the cells they infect, become more susceptible to NK-mediated cytotoxicity (76). In a recent study, Le Gars et al. used mass cytometry to compare the responses of isolated pbNK cells from pregnant and non-pregnant women to *ex vivo* challenge with IAV. In the pregnant cohort, production of IFN-γ by both pbNK subsets was significantly upregulated, as was their capacity to kill influenza-infected monocytes (83). Although robust NK cell activity is usually important for viral clearance, this pregnancy-specific NK enhancement may actually be detrimental: IL-15-deplete mice, who cannot mount NK-mediated responses to influenza, show significantly improved survival compared to controls (160).

Decidual Natural Killer Cells

Decidual NK (dNK) cells are a unique population of $CD56^{bright}$ $CD16^{-}$ $KIR^{++/-}$ cells that expand in the peri-implantation window and remain highly abundant until the end of the second trimester, after which their numbers gradually diminish (66). Despite some phenotypic overlap with pbNK, dNK exhibit a unique set of surface markers and are functionally distinct: their cytotoxic capacity toward allogeneic "non-self" cells, namely the trophoblast with which they are in direct contact, is completely abrogated (62). The lack of dNK cytotoxicity was initially attributed to an attenuation of cytotoxic granule components. However, this has proved wrong: paradoxically, dNK in fact possess equivalent or higher levels of granzyme B, perforin, and granulysin than pbNK (30, 62). The issue is one of translocation: dNK fail to polarize cytotoxic granules to the immunological synapse with target non-self-cells (161). Similarly, it was presumed that these non-cytotoxic dNK would lack the ability to kill virally infected cells. This was disproved when Siewiera et al. demonstrated that dNK isolated from first-trimester decidua rapidly developed into cytotoxic effectors on exposure to CMV-infected autologous decidual fibroblasts, efficiently mobilizing cytolytic apparatus to the immunological synapse and infiltrating CMV^{+} trophoblastic tissue in culture (31). Importantly, this cytotoxic effect was lost when experiments were repeated with CMV-positive primary EVT, which dNK were unable to kill (162). Whether a similar pattern occurs with other viruses currently remains unproven, and observations specific to CMV should not be extrapolated to other infections.

These findings have led to renewed interest in NK-mediated antiviral immunity and the capacity of viruses to subvert molecular mechanisms of NK activity. In the case of CMV, viral infection can exert unique selective pressures on the pbNK cell compartment, influencing KIR acquisition and triggering clonal expansion of $CD57^{+}$ populations expressing specific repertoires of the C-type lectin NKG2C (163, 164). However, it is important to emphasize that currently these data refer only to CMV and equivalent effects with other viruses have not been demonstrated. Similarly, expansions of this kind have not been observed among uterine NK cells and it remains uncertain whether the pbNK cell pool (where such proliferation may exist) influences the endometrial or decidual NK cell composition (164). Whether this imprint on NK cells induces long-term immunological

memory against repeated viral challenge has proved controversial in humans, where peripheral and lymphoid tissue-resident NK appear to develop memory, as demonstrated in studies of CMV (165), hantavirus (166), and varicella-zoster virus (167). However, an equivalent effect in the uterus remains to be clarified. Overall, the precise role of dNK in antiviral immunity remains controversial and largely unknown.

Neutrophils

Pregnancy induces a physiological neutrophilia both systemically and at the decidua. In pregnant women, neutrophils account for up to 95% of peripheral blood leukocytes, compared to 50–70% outside pregnancy (80, 168). This reflects a state of enhanced innate immune vigilance against infection: neutrophils, the most abundant of all human phagocytes, are recruited to sites of infection or injury by chemokines such as complement C5a and form the first line of defense against microbial challenge. Engulfed micro-organisms are internalized in phagolysosomes and destroyed using a wide range of effector mechanisms including release of serine proteases (e.g., neutrophil elastase, cathepsin G) and production of reactive oxygen species (141, 169). Pathogens that evade phagocytosis can be controlled by the release of neutrophil extracellular traps (NET), large reticular structures containing cytotoxic granule proteins and AMP that sequester and neutralize microbes (170). While the role of NET in defense against bacterial and fungal infections is well-established, their contribution to antiviral immunity has been more difficult to ascertain (171, 172). NET formation may actually be detrimental in viral infections (173), with recent data showing that high levels of NET production exacerbate acute lung injury in influenza (174, 175) and can worsen prognosis in dengue virus infection (176).

The origins of this pregnancy-specific neutrophilia are incompletely understood but are likely to include progesterone-induced upregulation of granulocyte-macrophage colony-stimulating factor (GM-CSF) (177, 178). Mass cytometry analysis of circulating neutrophils in pregnancy by Aghaeepour et al. revealed not only increased abundance but also a hyper-activated state, with progressively enhanced sensitivity to pro-inflammatory stimuli including IL-6 and TNFa (6). However, the same group have also demonstrated a linear increase in "immature-like neutrophil signatures" (denoted by CD10 and CD15 expression) with advancing gestational age, which they suggest could contribute to the increased mortality observed in acute influenza and hepatitis E infections during late pregnancy (179).

Chemokine synthesis in the decidua leads to an influx of innate immune cells. Contact with invading trophoblast during implantation stimulates production of neutrophil chemo-attractants IL-8, CXCL1, and CXCL2 by decidual stromal cells, a process potentiated by the addition of progesterone *in vitro* (180, 181). However, the existence of a decidual neutrophil population remains controversial. Although one study from 2014 identified neutrophils in second-trimester decidua (182), this has not proved reproducible in subsequent single-cell reconstructions of the maternal-fetal interface (183, 184).

Macrophages

Monocytes are myeloid leukocytes that are released from the bone marrow, mature in the peripheral circulation and infiltrate into tissues, where they can differentiate into specialized populations of macrophages or myeloid DC. As with neutrophils, the monocyte-macrophage lineage expands both systemically and in the uterus during pregnancy (24, 185): macrophages account for up to 25% of the decidual leukocyte population and are outnumbered only by NK cells (80, 141). Macrophages have long been recognized as key phagocytic immune sentinels at mucosal surfaces. At the maternal-fetal interface their roles include antigen presentation to adaptive immune cells, promotion of tolerance through clearance of apoptotic trophoblast debris, secretion of pro-angiogenic factors and direct participation in spiral artery remodeling through the phagocytosis of maternal vascular smooth muscle cells (24).

The diverse functions of macrophages arise from their capacity for polarization into two antagonistic phenotypes, M1 (pro-inflammatory) and M2 (anti-inflammatory) (186). In normal pregnancy, the M1/M2 equilibrium is in constant flux, with M1 predominating during early trophoblast invasion, a shift toward tolerogenic M2 in the second and third trimesters, and a reversion to M1 at the onset of parturition (24). Differential TLR activity in decidual macrophages may contribute to protection of both mother and fetus against infection: stimulation of decidual macrophages with TLR2-4 and 7/8 agonists restricts viral replication and leads to overproduction of IL-10, an anti-inflammatory cytokine that suppresses trophoblast TLR signaling (140, 187).

Pregnancy-specific adaptations in the M1/M2 balance have also been implicated in the response to systemic viral infection. A study comparing the response of peripheral blood monocytes from pregnant and non-pregnant women to *in vitro* challenge with IAV found that the pro-inflammatory response was exaggerated in pregnant women, although trimester-specific effects were not reported (82). In a mouse model of pandemic H1N1 IAV in pregnancy, mice infected in mid-gestation exhibited higher clinical severity scores and a strong phenotypic shift toward the M2 phenotype in alveolar macrophages obtained from broncho-alveolar lavage samples, compared to those from non-pregnant matched females (87).

Dendritic Cells

Like macrophages, DC are critical for innate-adaptive crosstalk. DC can be broadly divided into two subsets:

1. Myeloid DC (mDC, $CD14^{-}CD11c^{+}$), which exist in an immature state in the spleen and lymph nodes and can migrate into various tissues where they are associated with Th1-type pro-inflammatory responses
2. Plasmacytoid DC (pDC, $CD123^{+}CD11c^{-}$), which reside in non-lymphoid tissues and induce Th2-type responses and proliferation of Tregs (25).

Circulating mDC from pregnant women demonstrate a skewed cytokine response to inflammatory stimuli, with increased

production of IL-10, programmed death-ligand 1 and CD200, favoring Th2 and Treg polarization over Th1 (6, 188). DC of myeloid origin are recruited into the decidua from a variety of progenitor populations. Decidual DC (dDC) account for <2% of decidual leukocytes, are a highly proliferative and relatively immature population, and are phenotypically distinct from peripheral DC: they display a universal reduction in expression of T cell costimulatory molecules and thus fail to induce specific T cell responses *in vitro* (189). dDC help to prevent fetal rejection by promoting tolerogenic responses to endocytosed trophoblast antigens, recruit NK cells through production of IL-15 and potentiate decidual angiogenesis via close dialogue with decidual stromal cells (141).

The integral role of pDC in the innate antiviral response to RNA viruses, which centers on their unrivaled capacity for type I IFN production (95), may be further upregulated in pregnancy. In a study comparing pDC isolated from pregnant and non-pregnant women, pDC from pregnant women were more abundant at baseline and demonstrated increased chemokine production (including IFN-inducible protein 10 and CCL4) in response to IAV challenge. The authors note that excessive chemokine release is associated with higher disease severity and mortality from influenza A *in vivo* (190, 191) and suggest that the exaggerated pDC response they observed could contribute to the disproportionately high fatality rates of influenza in pregnancy (82).

Decidual Stromal Cells

Trophoblast invasion induces dramatic remodeling of the uterine mucosa, transforming the glands, arteries and stroma of the late-secretory-phase endometrium in a progesterone-dependent process known as decidualization (62, 164, 192). The specialized decidual stromal cells (DSC) generated as a result are fundamental for the establishment and homeostasis of the decidual immune system. In particular, DSC are in constant dialogue with dNK and exert critical regulatory influence over their various functions. Progesterone-induced IL-15 production by DSC is essential for the early expansion of dNK (193) and potentiates their cytotoxic degranulation in response to virally infected cells (194). Conversely, DSC-derived IL-33 inhibits dNK perforin and granzyme A synthesis, reduces their cytotoxic capacity and shifts their cytokine effector profile toward the immunosuppressive IL-4, IL-10, and IL-13 (195–197). The observation that T cell chemokine genes are epigenetically silenced in DSC highlights their role in maintaining a tolerogenic environment (12); indeed, they have even shown promise as a novel therapy for acute and chronic graft-versus-host disease in allogeneic stem cell transplant patients (198, 199). Their roles in viral infection remain poorly defined, but recent data show that murine DSC undergo necroptosis in response to transfection with a synthetic analog of viral RNA (200).

It is therefore clear that DSC, instead of merely functioning as a cellular scaffold that hosts the decidual immune system, are vital contributors to its key functions of simultaneous fetal tolerance and immune defense.

ACUTE VIRAL INFECTIONS THAT CAUSE SEVERE MATERNAL DISEASE

Lassa Virus

Lassa virus (LASV) is an enveloped ssRNA arenavirus endemic to West Africa that is acquired through contact with infected rodents (*Mastomys natalensis*, the multi-mammate rat). It causes regular seasonal outbreaks of Lassa fever, responsible for over 500,000 cases and 5,000 deaths per year. In the general population:

- 80% of patients will be asymptomatic or experience a mild non-specific illness;
- 15–20% require hospital admission due to severe disease (which may manifest as mucosal hemorrhage, hepatitis or multi-organ failure);
- Up to 29% will develop sensorineural hearing loss during their recovery;
- 1% will die (44, 201, 202).

However, these figures are strikingly altered in pregnancy. Cohort studies have consistently estimated maternal mortality rates as disproportionately high: 7% in the first two trimesters, rising as high as 87% in the third trimester, with fetal loss rate reported between 75 and 100% (44, 201, 203). The first systematic review of Lassa fever outcomes in pregnancy, published this year, identified just seven studies eligible for inclusion in the meta-analysis. This incorporated a total of 236 women in whom the absolute risk of maternal death was 33.7%, the fetal CFR was 61.5% and the neonatal CFR was 30.2%. Pregnancy conferred a 2.86-fold increase in risk of death (95% confidence interval 1.77–4.63) compared to non-pregnant women of reproductive age (29).

This disparity "underscores the need to prioritize pregnancy" in studies of the immune response to LASV (29). Currently, however, our understanding is almost entirely limited to data from animal models or cultured human cells (29, 204). These have provided insights into the key components of the innate immune response to LASV. The importance of the type I IFN response to LASV has been demonstrated in a non-human primate model: animals who survived viral challenge generated a robust IFN-α response shortly after inoculation, whereas those who died failed to upregulate IFN-α until the terminal phase of illness (205). Given that the type I IFN response to other RNA viruses such as influenza is less effective in pregnancy (206), it is possible that a similar attenuation in the case of LASV infection contributes to the higher mortality in pregnant women.

LASV has also been shown to directly antagonize the type I IFN response through its Z matrix protein, which is common to all arenaviruses, and its nucleoprotein (NP). LASV-NP possesses a unique exonuclease domain that degrades viral dsDNA—usually a potent ligand for RLR—to prevent type I IFN production (207).

In cultured human cells, LASV replicates in DC and macrophages but is remarkably adept at concealing its presence: it does not activate the host cells, induce apoptosis, alter their cell surface expression profile or trigger antigen presentation to T cells. This creates immune-privileged reservoirs that facilitate unchecked LASV replication in early infection, with subsequent

systemic spread when the infected cells enter draining lymph nodes (202). It may also be a critical determinant of LASV's pathogenicity—in contrast, infection of macrophages and mDC with Mopeia virus, which shares 75% sequence homology with LASV but is non-pathogenic, leads to rapid activation of both cell types with upregulation of T cell costimulatory molecules and production of pro-inflammatory cytokines including type I IFNs and IL-6 (208–211).

The fact that LASV uses elaborate evasion mechanisms to avoid activation of innate immunity, and that this capacity correlates directly with suppression of adaptive responses, reinforces the role of the innate immune system in defense against Lassa fever. However, no single animal or human study to date has focused specifically on innate anti-LASV responses in pregnancy. The question of why pregnant women experience such high mortality from this disease therefore remains unsolved and should be urgently prioritized in future research.

Ebola Virus

Ebola virus (EBOV) caused over 11,000 deaths in the 2013–2016 outbreak in West Africa and had catastrophic implications for the region's already fragile public health infrastructure. Cases of Ebola virus disease (EVD) were concentrated in Guinea, Sierra Leone and Liberia, which have among the highest maternal mortality ratios in the world, and it is estimated that at least 100 pregnant women died (212–214).

EBOV causes abrupt onset of a febrile illness that can progress to profuse diarrhea, hemorrhage, meningo-encephalitis and hepatic or renal failure (44). Overall CFR is high (45–90%) and if acquired in pregnancy it is almost always lethal for the fetus (213, 215). However, a recent synthesis of available studies demonstrated no significant difference between mortality rates in pregnant vs. non-pregnant women (214). While this may be the case, caution is required on two counts: firstly, as the authors acknowledge, their analysis is limited by the small size, retrospective nature and considerable heterogeneity of included studies; and secondly, while pregnant women may not be intrinsically more vulnerable to EVD, the fact that they are often carers for sick relatives and make frequent visits to health facilities places them at increased risk of acquiring the disease (212).

Like LASV, EBOV targets myeloid DC and macrophages for entry and replication. Both viruses arrest mDC in an immature state that is permissive for viral replication, but their effect on macrophages is different: unlike LASV, EBOV does activate macrophages (44, 216). This allows them to present antigen to T cells, meaning that adaptive responses are at least partially activated in EVD: paradoxically, rather than being protective, this may exacerbate the strong and rapid upregulation of both innate and adaptive immunity that is characteristic of fatal disease (217).

The role of the IFN response in EVD has proved difficult to elucidate. On one hand, EBOV, like LASV, exhibits complex mechanisms that specifically counteract IFN: its VP35 protein contains an inhibitory domain that can scavenge viral dsRNA to prevent it from binding to RIG-I receptors (218) and its VP24 protein blocks nuclear accumulation of phosphorylated STAT1, a critical transcription factor in IFN signaling (219). On the other hand, certain ISGs such as tetherin have been found to actively suppress EBOV replication (220) and the IFN response is massively exaggerated in patients who die of EVD compared to those who survive, suggesting a contribution of host inflammatory response to disease similar to that seen in influenza (217, 221).

The West African epidemic revealed an unexpected tropism for the placenta: women who survived EVD in early pregnancy with complete resolution of viremia and no fetal loss were found to have unusually high rates of miscarriage and stillbirth weeks or months later, with abundant EBOV RNA detected in placental and fetal tissues (222, 223). Immuno-histochemistry analysis of placentas from EBOV-positive mothers shows accumulation of EBOV antigen within the intervillous space, where it co-localizes with a population of atypical maternal macrophages, and in extravillous trophoblast (224). This may reflect shared dependence on specific endocytic mechanisms: EBOV entry into target cells depends on the Niemann-Pick cholesterol transporter protein Niemann-Pick C1 (NPC-1), a protein that is also expressed on the syncytiotrophoblast (225, 226). These findings raise the suspicion of an EBOV predilection for pregnancy, even if the maternal death rate is comparable to that of the non-pregnant population, and support a rationale for close follow-up of female survivors (214).

Dengue Virus

The arboviruses are a group of over 100 arthropod-borne RNA viruses including dengue, Zika, Chikungunya, and yellow fever viruses. They constitute a major threat to global public health: 90% of pregnant women live in areas with either endemic or epidemic transmission of arboviruses (47).

Dengue, a spherical enveloped flavivirus with four ssRNA serotypes (DENV1-4), is the most abundant of the arboviruses and occupies two separate environmental niches: a sylvatic cycle, in which DENV circulates between arthropod vectors and non-human primate reservoirs, and an urban cycle, in which humans and *Aedes* mosquitoes are the only hosts. Following inoculation, the majority of people remain asymptomatic, but a minority will develop an acute febrile illness accompanied by severe headache, retro-orbital pain, arthralgia, and a rash. Approximately 1% develop severe dengue, a potentially lethal manifestation of disease that results from a sudden increase in systemic vascular permeability and can cause shock, profound thrombocytopenia, hemorrhage, and multi-organ failure. Mortality in severe dengue can be reduced from over 20 to $<$1% with good supportive care, but there are no specific antiviral therapies with proven benefit (227).

Quantifying the risks associated with DENV infection in pregnancy has proved particularly challenging due to the high proportion of asymptomatic cases, the lack of large prospective studies with comparison groups, the inevitable reporting bias in small case series and the inherent difficulties in accurate diagnosis of acute dengue fever (dengue IgM cross-reacts with other flaviviruses and coinfections are common) (48). Machado and colleagues were the first to definitively establish the association between pregnancy and severe dengue. They analyzed all 151,604 cases of suspected DENV in Rio de Janeiro from 2007 to 2008,

compared outcomes in 99 pregnant women to 447 matched non-pregnant women of reproductive age, and found an increased risk of severe dengue in the pregnant women (odds ratio 3.38) with a trend toward higher mortality (3 vs. 1.1%) (47). A 2018 study added to this by investigating correlates of severe maternal disease in French Guiana: they showed an 8.6-fold increase in the risk of postpartum hemorrhage in the presence of severe dengue (228). Whether or not dengue increases the risk of adverse fetal and perinatal outcomes (specifically miscarriage, stillbirth, preterm birth, and low birthweight) remains controversial, with two meta-analyses from 2016 and 2017 reporting conflicting results (229, 230).

The origins of this increased susceptibility to severe dengue in pregnancy remain unknown but are likely to include placental tropism, innate immune adaptations and the physiological increase in vascular permeability that occurs in normal pregnancy, which may both delay diagnosis and exacerbate severe dengue (40). An immunohistochemical analysis of placentas from women with dengue in pregnancy showed accumulation of DENV antigen in the trophoblast and decidua in 92% (22/24) of cases. Microscopy revealed hypoxic changes (including villous edema and infarction) and an unusual observation of sickled erythrocytes in the intervillous space—this occurred despite no history of maternal sickle cell disease and was correlated with maternal death, suggesting that the virus may be able to influence erythrocyte biology in pregnancy (231, 232).

DENV can infect and deplete human megakaryocytes, suppressing their capacity to produce platelets and causing dengue-induced thrombocytopenia. Campbell et al. found that DENV leads to marked upregulation of interferon-induced transmembrane protein 3 (IFITM3) on platelets with corresponding release of type I IFNs, and that the highest levels of IFITM3 expression correlated with mildest disease (233, 234). These mechanisms are yet to be studied in pregnancy, but it is conceivable that enhanced DENV-mediated megakaryocyte depletion or a failure to upregulate platelet IFITM3 could contribute to the higher rates of hemorrhagic complications during pregnancy.

Type I IFN production is central to the innate anti-DENV response, although no specific studies have been conducted in pregnancy. DENV triggers type I IFNs through binding with various PRRs (RIG-I, endosomal TLR3, and endosomal TLR7) and is detected by the mannose-binding lectin complex. This leads to deposition of Cb4 and C2a on the virion surface, formation of the C3 convertase and activation of complement-mediated virolysis, although the virus can subvert this through its non-structural protein NS1 as described above. RNA interference and activation of apoptosis in infected cells are also important contributors to DENV defense, as reviewed by Uno and Ross (96). NS1 also appears to be a critical regulator of the DC and NK-mediated response to dengue infection. Sentinel cutaneous DC detect invading DENV and rapidly recruit NK cells through contact-dependent IFN-mediated upregulation of adhesion molecules, and the NK are key for viral suppression: in humanized mice, depletion of NK cells exacerbates DENV viremia and thrombocytopenia (235).

Although mechanistic insights into DENV pathogenicity with plausible relevance to pregnancy continue to emerge—for example, the observation that vitamin D supplementation reduces cultured human DC susceptibility to DENV2 through downregulation of TLR3, TLR7, and TLR9 signaling (236)—these findings cannot be extrapolated to the pregnant population and dedicated studies are urgently needed.

Hepatitis E

The hepatitis E virus (HEV) is a small, non-enveloped ssRNA hepevirus that causes over 20 million infections annually. Of its four genotypes, HEV-1 and 2 cause human disease and are spread by feco-oral transmission through contaminated water supply, entering the body via enterocytes and replicating in the liver. This route accounts for the wide geographical variation in disease burden of hepatitis E, which is concentrated in areas of poor sanitation (42, 49). In the general population, hepatitis E is asymptomatic in the vast majority of cases, but still causes 3.3 million symptomatic infections (usually mild, self-limiting and clinically indistinguishable from hepatitis A) and 56,000–70,000 deaths per year, a mortality rate of $<0.5\%$ (42).

However, outcomes are much worse for pregnant women: HEV mortality can exceed 50%, particularly if acquired in the third trimester. Several large cohort studies from India have demonstrated that pregnant women with HEV are both more likely to develop fulminant hepatic failure (FHF) and more likely to die from it (237–240). A 2019 systematic review including a total of 1,338 pregnant women with hepatitis E showed a 45% risk of fulminant hepatic failure, a median maternal CFR of 26% and a median fetal CFR of 33%. Other obstetric complications were not consistently reported in the included articles but data from four studies suggest an increased risk of postpartum hemorrhage, ranging from 13.6 to 30% (28). There are no proven drug therapies for hepatitis E (ribavirin, which has shown equivocal benefits in small case series, is contraindicated in pregnancy) and the only available vaccine for prevention of hepatitis E is not manufactured or licensed outside China (28).

Like the other viruses discussed in this review, there is therefore a global imperative to identify the factors that confer increased susceptibility to fatal HEV infection in pregnancy. Common themes emerge: the mechanisms are poorly characterized, difficult to recapitulate through *in vitro* models, influenced by hormonal factors and likely to arise from dysregulation of immune homeostasis at the maternal-fetal interface (241). Studies examining peripheral blood cells and circulating inflammatory mediators from women with HEV-induced FHF have implicated excessive Th2 switching, impaired NFkB-mediated liver regeneration, oxidative stress, and coagulopathy in its pathogenesis; these are reviewed by Perez-Gracia et al. (242), although no convincing individual candidate has emerged. The innate immune response to HEV is also unclear, although it appears to be crucial for prevention of severe disease. In 2015, a comparison of macrophages and DC from pregnant patients with HEV-induced FHF, non-fulminant acute HEV, and healthy pregnant controls found that macrophages from the women who developed fulminant liver disease had significantly impaired phagocytic capacity, with

reduced TLR3 and TLR9 expression impeding MyD88-mediated IFN production (239).

Importantly, although the life cycle of HEV remains enigmatic, extrahepatic replication has been demonstrated in both the placenta (241, 243) and, more recently, in cultured human endometrial stromal cells (244). Gouilly et al. (241) showed that HEV-1 causes severe necrotic tissue injury in both decidua and trophoblast, significantly reduces both tissues' ability to produce type III IFNs and distorts the cytokine secretome of cultured DSC, upregulating potent pro-inflammatory mediators including IL-6 and the chemokines CCL-3 and CCL-4. As the authors suggest, these widespread disruptions to the architecture and homeostasis of the maternal-fetal interface are likely to contribute to viral dissemination, adverse obstetric outcomes and increased disease severity in pregnancy.

Influenza—Seasonal and Pandemic

The *Orthomyxoviridae* family includes three species capable of causing human disease: influenza viruses A, B, and C. Types A and B account for the majority of seasonal influenza, which causes approximately 389,000 deaths per year (245). They have an unusual segmented genome with eight negative-sense RNA strands and, unlike the other viruses discussed in this review, replicate in the nucleus. The pleiomorphic IAV virion consists of a host-derived lipid envelope displaying embedded surface glycoproteins haemagglutinin (HA) and neuraminidase (NA), which account for its antigenicity and are used to classify the different IAV serotypes (e.g., H1N1).

Major influenza pandemics over the last century have consistently shown disproportionately high mortality rates in pregnant women, mostly recently the H1N1 outbreak in 2009 (34–36). A 2011 meta-analysis including a total of 3,110 pregnant women who developed H1N1 showed that rates of hospital admission (52.3%), requirement for intensive care (23.3%) and death (4.1%, of which two thirds occurred in the third trimester) were all significantly increased in pregnancy. Despite only 1% of the susceptible population being pregnant, these women accounted for 5.7% of all deaths in the pooled analysis (52). Although maternal mortality outside a pandemic setting is low, these data prompted the WHO to recommend seasonal influenza vaccination for all pregnant women (52, 246). A subsequent larger systematic review and individual participant data meta-analysis (including 36,498 women of reproductive age) confirmed a 6.8-fold higher risk of requiring hospital admission in pregnant women with H1N1, but did not find any evidence of an increased risk of death (27, 247).

Like Ebola, whether maternal mortality from IAV is actually disproportionately increased remains unclear, but disease is certainly more severe in pregnant women. Pregnant women appear to develop particularly severe lung injury and are also at higher risk of extrapulmonary complications. In a study reporting autopsy findings of 21 Brazilian patients who died of H1N1-related acute respiratory failure, the single pregnant woman in the cohort had the most severe pulmonary disease, with widespread necrotising bronchiolitis, diffuse alveolar damage and significant upregulation of TLR3, IFN-γ, and granzyme B-producing cells in the airway epithelium, suggesting excessive activation of the innate immune response (248).

Mouse models have shown that disrupted TLR signaling is an important component of IAV pathogenesis. Following cell entry, IAV virions are internalized into endosomes, in which the low pH induces a conformational shift in HA resulting in release of the nucleocapsid protein into the cytosol. This process is essential for viral replication but also stimulates the innate immune response: in pDC isolated from mouse spleens, endosomal sensing of IAV ssRNA by TLR7 led to massive IFN-α release, a response that was completely abrogated in $TLR7^{-/-}$ mice (129). TLR3-deficient mice also show a muted response to IAV infection, with significant reductions in pro-inflammatory cytokine expression and $CD8^{+}$ T cell recruitment to the broncho-alveolar space. However, despite higher viremic burden, this actually confers a survival advantage compared to control TLR3-competent mice (249). In purified human alveolar epithelial cells, RIG-I and TLR3 are the primary IAV sensors and trigger a type III-predominant IFN response (250). As with the other RNA viruses discussed here, IAV has a sophisticated array of innate evasion mechanisms, which are reviewed by Kikkert (251). IAV can also induce necroptosis of infected cells, leading to potent neutrophil recruitment and exacerbated lung injury. Mice deficient in MLKL, a critical mediator of necroptosis, exhibit improved survival in response to a lethal IAV challenge (252).

Pregnancy appears to suppress the systemic type I and type III IFN responses to IAV. A 2012 study showed that peripheral blood mononuclear cells from pregnant women exhibited a 10-fold reduction in the expression of protein kinase receptor, an ISG that is stimulated early in the antiviral response, compared to matched female controls in response to H1N1 IAV challenge. This effect was partially reversed by vaccination, although still not completely restored to non-pregnant levels (206). Some studies have also shown an IAV-induced upregulation of T cell costimulatory markers on pDC, hypothesizing that the virus may break the physiological attenuation of DC maturation in pregnancy and lead to exaggerated immune responses and tissue damage; conversely, others suggest that a failure to activate lung DC and recruit virus-specific CD8 T cells to the airway epithelium could contribute to the inability to control the virus in pregnancy (253, 254). Whether the severe disease observed in pregnancy results from a failure to generate robust innate responses or a harmful virus-induced disruption of the tolerogenic state remains to be clarified.

The role of hormones in modulating lung physiology and the response to IAV has been extensively studied. Progesterone, which is essential for the maintenance of pregnancy, is a muscle relaxant that causes physiological airway dilatation. It also affects the innate immune system, preventing NK cell degranulation and upregulating neutrophil-attractant chemokines in the respiratory epithelium. It interacts closely with the prostaglandins PGE2, which increases vascular permeability in the lungs and downregulates effector mechanisms in neutrophils and macrophages, and PGF2a, a potent broncho- and vasoconstrictor (86). In animal studies, female mice treated with exogenous progesterone are protected against severe IAV disease through increased expression of amphiregulin, which

promotes tissue repair in the lungs and improves survival (255). However, the immunoregulatory role of progesterone appears to be strain- and exposure-specific: in another study, pre-treatment of female mice with progesterone protected them against severe disease during initial H1N1 exposure but reduced their survival during subsequent challenge with H3N2 (256). Influenza infection in pregnant mice dysregulates the progesterone-prostaglandin axis, leading to bronchoconstriction, an alveolar influx of neutrophils and respiratory failure (86). The implications of these findings in the severity of influenza in pregnancy are discussed further in a recent review in this journal by Littauer and Skountzou (38).

Novel Coronaviruses

Coronaviruses, which were first isolated from humans in the 1960s, cause frequent mild upper respiratory tract illnesses on a large scale but are not usually associated with significant disease. However, in the last 20 years, three zoonotic novel coronaviruses capable of causing severe pneumonia have emerged: severe acute respiratory syndrome coronavirus (SARS-CoV), Middle East respiratory syndrome coronavirus (MERS-CoV) and COVID-19 (SARS-CoV-2). Global experiences of the first two, which disproportionately affected pregnant women, have caused significant apprehension about the consequences of the third, which remains subject to intensive and ongoing research (55, 257). Given that pneumonia is the leading non-obstetric infectious cause of maternal death, vigilance is required with any emerging respiratory virus and pregnant women should be prioritized in efforts to anticipate and mitigate their effects (258).

SARS-CoV caused over 8,400 cases in 29 countries in the outbreak of 2002–2003. The 916 reported deaths corresponded to a global CFR of ∼11%. Studies describing its effects in pregnant women, although small, are strongly suggestive of a higher burden of mortality and morbidity than in the general population. Wong et al. summarized outcomes in 12 pregnant women hospitalized due to confirmed SARS-CoV in Hong Kong in 2003: 50% were admitted to intensive care due to hypoxia (vs. 20% of non-pregnant patients), 33% required mechanical ventilation and 25% died. Associated complications included disseminated intravascular coagulation, renal failure and superimposed bacterial pneumonia. Of the 7 who presented in the first trimester, 5 had a spontaneous miscarriage; and of the 5 who presented beyond 24 weeks' gestation, 80% delivered preterm. Surviving neonates did not develop SARS and there was no evidence of vertical transmission, although placental histopathology did show other features indicative of maternal hypoxemia. The authors note that the use of ribavirin in 11/12 cases (following careful counseling about potential teratogenicity) may have exacerbated the high observed rate of first-trimester miscarriage (57). A case-control study, also from Hong Kong, confirmed the increased severity of disease in pregnancy by comparing 10 pregnant to 40 non-pregnant women with SARS at the same hospital: death (30 vs. 0%), intensive care admission (60 vs. 17.5%) and renal failure were all significantly more common in the pregnant group (58).

MERS-CoV, which emerged in 2012, caused 2494 confirmed cases and 858 deaths, with a CFR of 34.4%. The higher mortality rate has been attributed to both poor infection control, facilitating rapid propagation in healthcare settings, and an inherently more aggressive clinical course, with faster progression to respiratory failure. The clinical spectrum was broader than in SARS, with immunocompetent adults often reporting mild or moderate symptoms while deaths were more concentrated in those who were immunosuppressed, pregnant or had major medical comorbidities (55, 259). A 2019 synthesis of 11 pregnant women with MERS-CoV found that 54% required intensive care and 27% died. Although this was not elevated compared to the CFR in the general population, these numbers are too small to provide confident assurance that pregnant women are not at increased risk from MERS-CoV. As with SARS, no episodes of vertical transmission were documented, although umbilical cord and neonatal blood sampling were not universally performed (59).

The spread of COVID-19 (SARS-CoV-2) has vastly exceeded that of SARS and MERS, with over 21 million cases and 760,000 deaths at the time of writing (260). However, its mortality rate is considerably lower than the two previous novel coronavirus outbreaks, as is the proportion of patients who develop severe disease (pooled estimate 18.0%) (261).

In the early stages of its escalation, experience from the SARS-CoV and MERS-CoV outbreaks prompted some national health policy departments to recommend increased caution in pregnant women. However, accumulating data are reassuring on this front: a systematic review of 2,567 affected pregnancies published in July showed a 7% intensive care unit admission rate with maternal mortality of approximately 1% (262) and a population-level analysis of 427 pregnant women hospitalized with COVID-19 in the UK showed no evidence of an increased risk of severe disease compared to the general hospital population (60). In contrast to SARS-CoV and MERS-CoV, vertical transmission appears to occur in a small proportion of cases of SARS-CoV-2: in a recent meta-analysis, 3.2% of neonates tested positive on nasopharyngeal swabs and 3.7% had positive serology, based on IgM positivity (263), however, caution is required given that the validity of IgM serology tests can be compromised by cross-reactivity. These findings are supported by the recent systematic confirmation of transplacental transmission of SARS-CoV-2 following maternal infection at 35 weeks' gestation (264).

The exponential transmission of COVID-19 has prompted renewed efforts to identify integral components of the immune response to coronaviruses. While innate host defenses rely on the same cardinal mechanisms as for the other RNA viruses discussed—namely, the interferon response—coronaviruses appear to have evolved a particularly diverse repertoire of innate immune evasion strategies. Inhaled coronaviruses enter pneumocytes and macrophages in the upper respiratory tract and replicate in the cytosol, hijacking host intracellular membranes to create "replication organelles" (RO) that facilitate viral replication while simultaneously shielding the RNA within double-membrane vesicles to prevent detection by intracellular PRRs such as RIG-I (265).

Coronaviruses go to great lengths to avoid recognition: their non-structural proteins even exhibit endonuclease activity, allowing them to degrade their own RNA and forestall activation of PAMP-mediated antiviral signaling (266). As well as these evasion strategies, coronaviruses can also directly antagonize the innate immune response: the SARS-CoV non-structural protein-1 (NSP-1) binds to the 40S subunit of host ribosomes and induces "translational shutoff," bringing all cellular expression of antiviral effector proteins to a halt (267). This particularly intricate capacity of CoV to both elude and suppress innate effector mechanisms may contribute to their pandemic potential. However, no studies thus far have been specifically designed to investigate the disparities between individual coronaviruses in terms of their effects on maternal disease and vertical transmission in pregnancy.

CHALLENGES AND FUTURE DEVELOPMENTS

Determining exactly how innate immune mechanisms and corresponding viral evasion strategies contribute to the disproportionately severe disease observed in pregnancy is an ongoing problem. Challenges range from the technical and ethical (e.g., the difficulty of accessing human decidual tissue and replicating human pregnancy in animal models) to the global, with the ever-present threat of widespread outbreaks caused by novel viruses with pandemic potential. Emerging viruses, epitomized by the current COVID-19 pandemic and the ongoing Ebola outbreak in North Kivu, highlight the vulnerability and inequity in the global health infrastructure. However, there is also a considerable threat from resurgence of previously controlled viruses: the global re-emergence of measles since 2016, including in several countries where transmission had previously been eradicated, has caused thousands of deaths and poses a major risk to pregnant women (268). A 2017 UK case report described a patient who required emergency Cesarean section and extracorporeal membrane oxygenation for deteriorating respiratory failure at 32 weeks' gestation (269). Control of preventable viral diseases like measles depends on high rates of vaccination coverage, which is compromised by conflict, migration, and persistent belief in the discredited association with autism (268).

The ability to protect vulnerable groups from viral infection depends partly on public health measures such as vaccination and vector control but also on our understanding of the biological correlates of this vulnerability. The advent of technologies such as mass cytometry and single-cell RNA sequencing have already shown promise (270), offering unparalleled insights into

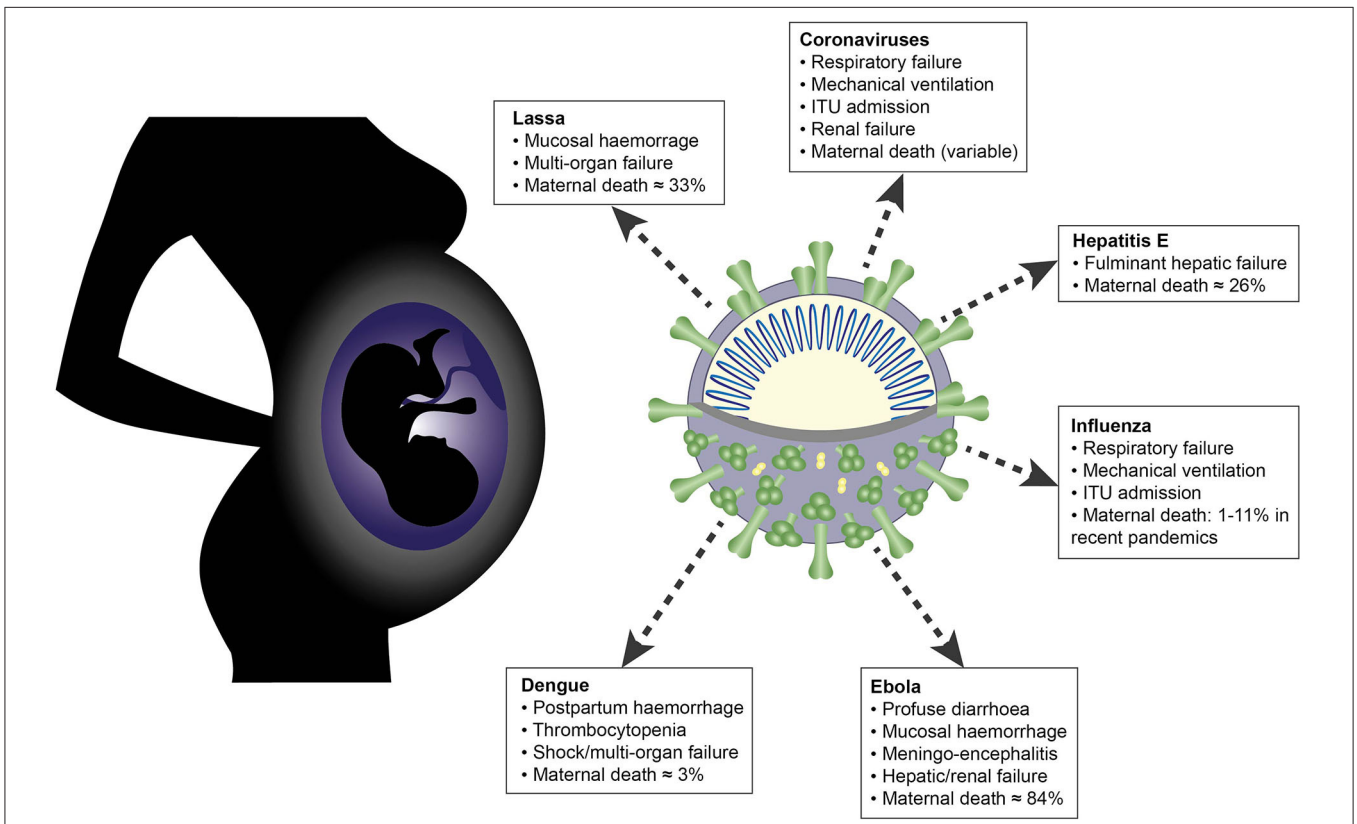

FIGURE 5 | Schematic summarizing effects of maternal infection with the following RNA viruses during pregnancy: Lassa, Ebola, dengue, hepatitis E, influenza and novel coronaviruses.

the cellular architecture of the maternal-fetal interface in early pregnancy (183, 271, 272). These, along with the recent development of human trophoblast organoids that replicate the placental secretome *in vitro,* may transform our ability to study the effects of viral infection in pregnancy (273).

CONCLUSION

The RNA viruses discussed in this article share many overlapping features in their clinical manifestations and their interactions with the maternal immune system (**Figure 5**). Common themes include placental tropism, an association with adverse obstetric outcomes and the importance of a tightly regulated interferon response, which is reflected in the evolution of diverse viral IFN evasion mechanisms. Innate immune cells are uniquely positioned at the maternal-fetal interface and orchestrate the balance between the conflicting immunological priorities of pregnancy: tolerance of the fetal allograft and defense of both mother and fetus against invasive pathogens.

The disproportionate rates of severe disease and mortality observed in pregnant women with Lassa, Ebola, dengue, hepatitis E, influenza and certain novel coronavirus infections cannot be attributed purely to immunological adaptations: social and behavioral factors also contribute (212). However, it is only through research that is tailored toward these pregnancy-specific factors that the outcomes will improve. In particular, as recently and eloquently argued by Gomes et al. (274) and Schwartz and Graham (55), the systematic exclusion of pregnant women from the design of vaccine trials for viral illnesses will perpetuate and exacerbate the problem. Addressing the gaps in our knowledge of innate immunity in pregnancy is an urgent priority.

AUTHOR CONTRIBUTIONS

EC and TM conceived the article. EC wrote the first draft of the manuscript. TM wrote sections of the manuscript, provided critical revisions, and edited the text. FÅ generated all the figures for the manuscript. DW and IF provided critical revisions of the manuscript and edited the text. All authors contributed to manuscript revision, read, and approved the submitted version.

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Cholesterol Crystals and NLRP3 Mediated Inflammation in the Uterine Wall Decidua in Normal and Preeclamptic Pregnancies

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Preeclampsia is a hypertensive and inflammatory pregnancy disorder associated with cholesterol accumulation and inflammation at the maternal-fetal interface. Preeclampsia can be complicated with fetal growth restriction (FGR) and shares risk factors and pathophysiological mechanisms with cardiovascular disease. Cholesterol crystal mediated NLRP3 inflammasome activation is central to cardiovascular disease and the pathway has been implicated in placental inflammation in preeclampsia. Direct maternal-fetal interaction occurs both in the uterine wall decidua and at the placental surface and these aligned sites constitute the maternal-fetal interface. This study aimed to investigate cholesterol crystal accumulation and NLRP3 inflammasome expression by maternal and fetal cells in the uterine wall decidua of normal and preeclamptic pregnancies. Pregnant women with normal (n = 43) and preeclamptic pregnancies with (n = 28) and without (n = 19) FGR were included at delivery. Cholesterol crystals were imaged in decidual tissue by both second harmonic generation microscopy and polarization filter reflected light microscopy. Quantitative expression analysis of NLRP3, IL-1β and cell markers was performed by immunohistochemistry and automated image processing. Functional NLRP3 activation was assessed in cultured decidual explants. Cholesterol crystals were identified in decidual tissue, both in the tissue stroma and near uterine vessels. The cholesterol crystals in decidua varied between pregnancies in distribution and cluster size. Decidual expression of the inflammasome components NLRP3 and IL-1β was located to fetal trophoblasts and maternal leukocytes and was strongest in areas of proximity between these cell types. Pathway functionality was confirmed by cholesterol crystal activation of IL-1β in cultured decidual explants. Preeclampsia without FGR was associated with increased trophoblast dependent NLRP3 and IL-1β

expression, particularly in the decidual areas of trophoblast and leukocyte proximity. Our findings suggest that decidual accumulation of cholesterol crystals may activate the NLRP3 inflammasome and contribute to decidual inflammation and that this pathway is strengthened in areas with close maternal-fetal interaction in preeclampsia without FGR.

Keywords: cholesterol crystals, decidua, fetal growth restriction, NLRP3 inflammasome, inflammation, IL-1β, placenta, preeclampsia

INTRODUCTION

Pregnancy is characterized by low-grade inflammation at the maternal-fetal interface and systemically in the mother, and this is aggravated to harmful levels in the pregnancy disorder preeclampsia (1). Preeclampsia is clinically characterized in the second half of pregnancy by new onset hypertension and proteinuria or maternal organ dysfunction and/or uteroplacental dysfunction (2, 3). The disease occurs in 4–5% of pregnancies and is a leading cause of maternal and fetal morbidity and mortality, often complicating with fetal growth restriction (FGR) (4). Preeclampsia is considered a warning sign for cardiovascular disease (CVD) later in life and common pathophysiological mechanisms are shared (5, 6). Cholesterol mediated inflammation has been suggested as a link between the disorders, a theory supported by preeclamptic pregnancies being characterized by a pro-atherogenic maternal lipid profile and cholesterol accumulation at the maternal-fetal interface (7, 8).

The uterine wall decidua and the placenta are the two aligned sites for direct maternal-fetal immunological interaction throughout pregnancy. In the decidua, specialized fetal cells, called extravillous trophoblasts, invade the tissue and establish a direct molecular dialogue with resident maternal cells such as decidual stromal cells and maternal immune cells (9). Leukocytes are key cells in modulating trophoblast behavior (10). Placental cytotrophoblasts fuse together to form a multinucleated cell layer, the syncytiotrophoblast, that covers the placenta and directly interacts with maternal blood. Preeclampsia and FGR are associated with reduced trophoblast invasion and impaired artery remodeling in the uterine wall, leading to placental oxidative stress and inflammation that increase as the fetus grows (11–13). Although significant progress has been made in understanding the central role of placental inflammation for development of preeclampsia and FGR (13–15), little is known about the involvement of inflammatory mechanisms in the decidua.

Intracellular crystallization of cholesterol is a complex process that occurs upon endocytosis of oxidized low-density lipoprotein (oxLDL) and this process has been extensively studied in the arterial wall in atherosclerosis (16, 17). The cholesterol crystals promote the development of atherosclerotic lesions by activation of the potent Nod-like receptor protein (NLRP)3 inflammasome (18). The resulting interleukin (IL)-1β production from this powerful activation may lead to extensive inflammation and tissue damage (15, 19). Trophoblasts express receptors that enable cholesterol transport and uptake of oxLDL has been shown to reduce trophoblast invasiveness (20–23), but cholesterol crystals have not been investigated at the maternal-fetal interface. Dysregulated lipid transport by reduced expression of ATP-binding cassette transporter (ABCA1) has been associated with cholesterol accumulation at the placental syncytiotrophoblast layer in preeclampsia (24) and in primary extravillous trophoblasts (25). Preeclampsia is associated with increased maternal systemic inflammatory markers including total cholesterol, oxLDL, IL-1β and soluble fms-like tyrosine kinase-1 (sFlt-1) (26–28). The NLRP3 inflammasome pathway in preeclampsia has been recently reviewed and mechanistically illustrated (29). We have previously shown that the NLRP3 inflammasome is active in the placenta and associated with preeclampsia, with a central involvement of trophoblasts (28). In the decidua, increased cholesterol accumulation in preeclampsia (7) and NLRP3 inflammasome expression in cultured cells (30) has been shown. This indicates a role for cholesterol crystal mediated NLRP3 inflammasome activation across the maternal-fetal interface, but the decidual involvement still needs to be determined.

We hypothesize that cholesterol accumulation in the decidua results in formation of cholesterol crystals, which induce decidual NLRP3 inflammasome activation and influence the important dialogue between trophoblasts and maternal immune cells. This study aimed to characterize cholesterol crystal accumulation and NLRP3 inflammasome expression by maternal and fetal cells in the uterine wall decidua of normal and preeclamptic pregnancies.

METHODS

Study Participants and Decidual Biopsies

Women with normal and preeclamptic pregnancies with and without FGR were recruited at St. Olavs and Haukeland University Hospitals during 2002-2012. Preeclampsia was defined as persistent hypertension exceeding 140/90 mmHg plus proteinuria $\geq$0.3 g/24 h or $\geq$+1 by dipstick after 20 weeks of gestation. FGR was diagnosed by serial ultrasound measurements showing reduced intrauterine growth ($n = 27$), or, for neonates small for gestational age ($n = 1$), birth weight <5th percentile of Norwegian reference curves (31) combined with clinically and sonographically suspected FGR and/or postpartum defined placental pathology. Only singleton pregnancies undergoing cesarean section with no signs of labor were included. Decidua basalis tissue was collected by vacuum suction of the placental bed during cesarean section (7, 32). Tissue samples were either

fixed in 10% neutral-buffered formalin and embedded in paraffin or snap frozen and stored at −80°C.

Placentas were collected from normal pregnancies after delivery by elective cesarean section for immediate isolation of decidual explants. The decidual tissue was dissected from the central region of the maternal side of the placenta. Samples were processed and cultured within 1.5 h after delivery.

Decidual Explants

Decidual tissue was washed in sterile phosphate-buffered saline, cut into pieces (explants with wet weight range of 15–33 mg) and distributed in the culture plate evenly between the different culture conditions (24 ± 2 mg, mean ± standard deviation). There were no significant differences in explant weight between the culture conditions. Explants were cultured in

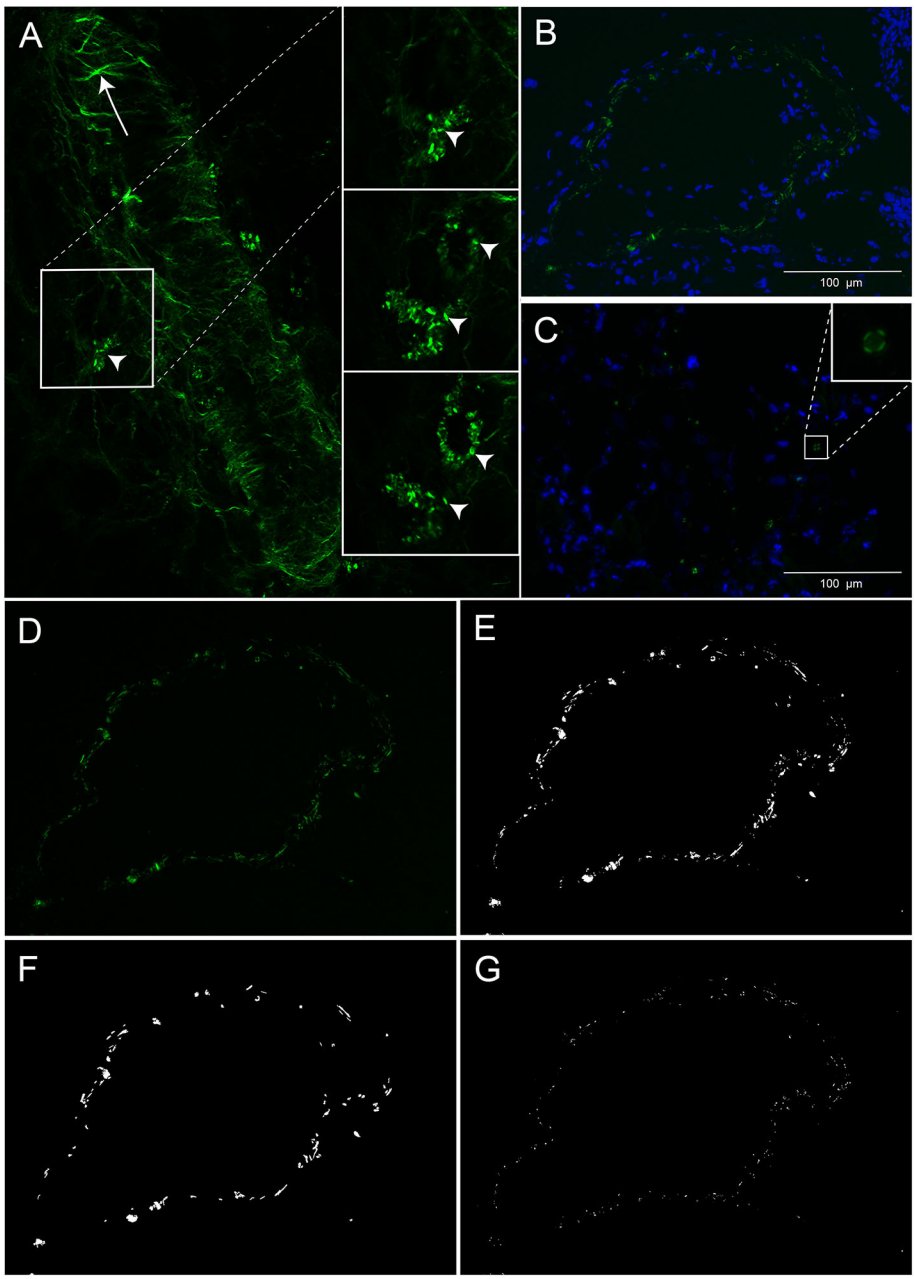

FIGURE 1 | Cholesterol crystals in decidual tissue. **(A)** Decidual tissue imaged by second harmonic generation microscopy. The arrow heads indicate cholesterol crystals and the arrow points to collagen fibers in the vessel wall. **(B,C)** Decidual tissue stained by DAPI and imaged by polarized light microscopy. Nuclei (blue) and cholesterol crystals (green) are shown near a vessel wall **(B)** and within tissue stroma **(C)**. **(D–G)** Representative images obtained by polarized light microscopy for cholesterol crystal quantification. **(D)** Unprocessed image of cholesterol crystals (green). The same image processed by MATLAB showing **(E)** total cholesterol crystals and separated into **(F)** large and **(G)** small clusters of cholesterol crystals. Scale bar 100 µM.

Ham's F12/Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum and 100 mg/mL penicillin-streptomycin (Sigma-Aldrich) and incubated for 24 h at 37°C, 8% O_2 and 5% CO_2 (33). Culture medium was then replaced by fresh culture medium with or without 500 pg/ml LPS priming (#tlrl-3pelps, InvivoGen, California, United States). After 2 h, the medium was replaced by fresh culture medium with or without stimuli; 200 or 2,000 μg/ml synthetic cholesterol crystals (#C3045, Sigma-Aldrich) or the positive control 3 mM ATP (#A7699, Sigma-Aldrich). Supernatants were collected after 24 h, centrifuged and stored at −80°C. Six technical replicates for each experimental condition were combined before analysis. Tissue viability was assessed by lactate dehydrogenase (LDH) cytotoxicity assay (#04744926001, Roche, Basel, Switzerland) (**Supplementary Figure 1**). IL-1β levels in supernatants were measured undiluted in duplicate using quantitative sandwich ELISA (#557953, BD Biosciences, New Jersey, United States).

Cholesterol Crystal Imaging and Automated Quantification

To preserve cholesterol crystals, the decidual cryosections (5 μm) were analyzed untreated. DAPI mounting medium (#F6057, Sigma-Aldrich, Missouri, United States) was used to identify cellular nuclei. Cholesterol crystals in the decidual cryosections were first assessed by second harmonic generation microscopy (25X magnification Leica SP8 confocal microscope, Wetzlar; Germany). For further analysis of cholesterol crystals, three adjacent TIFF images (2,080 × 1,544 pixels) were obtained from three different regions in the decidual section using polarization filter reflected light microscopy (20X magnification, Inverted fluorescence microscope Olympus IX71, Tokyo, Japan) and defined microscope settings. The cholesterol crystals in the nine TIFF images per decidua were quantified by a customized MATLAB script (version 2018a, the MathWorks Inc., Massachusetts, United States) developed for automatic quantification of positive pixels. The total cholesterol crystal positive pixels in each decidua was determined by the sum of the positive pixels quantified in the nine pictures. The number of positive pixels in each of the nine images was used to assess the variation in cholesterol crystal tissue distribution within each decidua. To evaluate the size of the observed cholesterol crystals, aggregation of positive pixels was determined by defining small (<50 pixels) and large (≥50 pixels) cholesterol crystal clusters (representative image in **Figures 1D–G**). To verify that the crystals dissolved in alcohol, two serial decidual cryosections (5 μm) were obtained from four decidual tissue samples. One slide from each decidual sample was treated with PBS and the other slide was immersed in alcohol at room temperature for 60 s.

Immunohistochemical Staining

Immunohistochemistry staining was performed in paraffin embedded decidua. Serial sections of 3 μm were pre-treated in PT link (#PT101, Dako, Glostrup, Denmark) using Target Retrieval Solution (#K8005 or #K8004, Dako) at 97°C for 20 min, and all sections were treated with peroxidase blocking solution (#K4007, Dako) or with dual peroxidase and alkaline phosphatase blocking solution (#K5361, Dako). Decidual tissue sections were incubated overnight at 4°C with antibodies against NLRP3 (1:100, #AG-20B-0014-C100, AdipoGen, California, United States) or IL-1β (1:200, #NB600-633, Novus, Colorado, United States); or for 45 min with cytokeratin 7 (CK7) (1:300, #M0851, Dako) or 40 min with CD45 (1:150, #M0701, Dako) at room temperature. All sections were incubated for 30 min with HRP-labeled polymer (#K4007, Dako) and for 10 min with DAB+ as chromogen (1:50, #K4007 or K5361, Dako). Decidual CK7 sections were double stained with smooth muscle actin (SMA) antibodies (1:300, #M0851, Dako) using EnVision G|2 Doublestain System Rabbit/Mouse (DAB+/Permanent Red) Kit system (K#5361, Dako). Overnight staining was performed manually and otherwise by Autostainer Plus (#S3800, Dako). Sections were counterstained with hematoxylin. Negative isotype controls were included (**Figures 3G,H**). In addition to the immunohistochemical staining, a routine staining with hematoxylin (#75290, Chemi-Teknik AS, Oslo, Norway), erythrosine 239 (#720-0179, VWR, Pennsylvania, United States) and saffron (#75100, Chemi-Teknik AS) (HES) was performed for each decidua using a Sakura Tissue-Tek © Prisma StainerTM (Sakura Finetek, Oslo, Norway).

Automated Quantification of Protein Expression

Decidual tissue slides were scanned with the EVOS™ FL Auto Imaging System (Thermo Fisher Scientific, Massachusetts, United States), using 20X magnification and defined microscope settings. The large decidual scans varied in size depending on available tissue and consisted of 4 to 81 bright field TIFF images (2,048 × 1,536 pixels) per sample slide. A customized ImageJ ($ImageJ_2$) (34, 35) script was used to perform background correction (Image calculator: Difference (img1 = |img1–img2|) and tile stitching [Grid/Collection stitching plugin (36)]. Smooth muscle tissue, placental tissue, blood vessels and tissue with poor morphology were excluded by manually defining regions of disinterest. NLRP3 and IL-1β expression was automatically quantified in the large tissue section scan for each decidua by a customized MATLAB script, and the protein expression quantified with examiners blinded to pregnancy outcomes. Cell specific staining was used to select decidual regions containing trophoblasts (CK7+) and leukocytes (CD45+). A mask of patches (1,325 × 1,325 μm) defining trophoblasts, maternal leukocytes, and maternal tissue without trophoblasts, was created for each decidual sample by using serial tissue section scans of cell-specific stained trophoblasts (CK7+) and leukocytes (CD45+). These masks were used to relate NLRP3 and IL-1β expression levels to trophoblasts and maternal leukocytes in the spatially aligned NLRP3 and IL-1β images. The *expression density* of CK7, CD45, NLRP3, and IL-1β in decidual tissue was calculated as the total number of positive stained pixels divided by the total amount of tissue pixels analyzed, to account for varying amounts of tissue between the samples. The *expression intensity* of NLRP3 and IL-1β were calculated as average staining intensity of all patches using a color deconvolution algorithm based on DAB specific RGB absorption (37).

Statistical Methods

Statistical analyses were performed in SPSS (IBM SPSS Statistics 26, Illinois, United States) and GraphPad Prism (Prism8, California, United States). For clinical data, one-way ANOVA or Kruskal-Wallis with Tukey's or Dunn's multiple comparison *post hoc* test, respectively, were used for comparisons of continuous variables, and Fisher's exact test for categorical variables. Protein measurements in supernatants were analyzed by Kruskal-Wallis with Dunn's multiple comparison *post-hoc* test.

NLRP3 and IL-1β expression levels were compared between study groups using a linear mixed model with recruitment location, study group and the trophoblast and leukocyte densities implemented as fixed effects. Subject combinations and intercept were included as random effects. The cholesterol crystal analysis was performed by a linear mixed model with recruitment location and study group as fixed effect. Correlation between variables was performed by calculating Pearson's correlation coefficient. Alpha level was set to 0.05.

RESULTS

Study Material

A total of 90 women with normal ($n = 43$) and preeclamptic pregnancies with ($n = 28$) and without FGR ($n = 19$) were included to the study (**Table 1**). The preeclamptic pregnancies with and without FGR, included more primiparas, had higher systolic and diastolic blood pressure, and their infants were delivered at earlier gestation with lower placental and birth weights, compared to normal pregnancies. The gestational age at delivery, as well as placenta and birth weights, were lower in preeclamptic pregnancies complicated with FGR compared to preeclamptic pregnancies without FGR (**Table 1**).

For the three pregnancies included in the decidual explant analysis, maternal age ranged between 31 and 38 years and gestational age between 38 and 39 weeks.

TABLE 1 | Clinical characteristics of subjects included in third trimester decidual analyses ($n = 90$).

	Normal pregnancies ($n = 43$)	Preeclampsia without FGR ($n = 19$)	Preeclampsia with FGR ($n = 28$)
Baseline characteristics			
Maternal age, years	31.2 (± 5.4)*	29.3 (± 4,9)	29.5 (± 5.3)
Primiparas, n (%)	7 (16)	12 (63)*‖	17 (61)*‖
BMI†	24.8 (± 3.9)	25.7 (± 4.6)	25.4 (± 4.1)
Characteristics at time of delivery			
Systolic BP, mmHg‡	119 (± 10)	153 (± 20)‖	148 (±18)‖
Diastolic BP, mmHg‡	72 (± 8)	100 (± 12)‖	96 (± 11)‖
Severe preeclampsia, *n* (%)	n.a.	17 (89)	20 (69)
Early onset preeclampsia <34 weeks, *n* (%)	n.a.	15 (79)	21 (79)
Placental weight, g§	638 (102)	475 (128)‖	290 (101)‖#
Fetal birth weight, g	3,409 (332)	2,224 (583)‖	1,311 (469)‖#
Fetal sex, female, *n* (%)	23 (53.5)	8 (42.1)	16 (60.7)
Gestational age, weeks	38.6 (0.6)	33.6 (2.7)‖	31.3 (3.1)‖#

FGR, fetal growth restriction; BMI, body mass index; BP, blood pressure; n.a., not applicable.

Continuous variables listed as means (± standard deviation) or median (interquartile range), assessed for differences between groups by one-way ANOVA with Tukey's post hoc test or Kruskal-Wallis with Dunn's post hoc test. Categorical variables listed as number (percent in column), assessed for differences between groups by Fisher's exact test.

**Information missing from one woman.*

† Maternal BMI in first trimester. Information is missing from five women.

‡ Blood pressure from last healthcare visit before delivery. Information missing from one woman.

§ Information missing from 12 women.

‖ $P < 0.05$ vs. normal pregnancies.

 # $P < 0.05$ vs. preeclampsia without FGR.

Cholesterol Crystals

Decidual cryosections from 76 women with normal ($n = 34$) and preeclamptic pregnancies with ($n = 27$) and without FGR ($n = 15$) were included. A marked presence of cholesterol crystals was observed dispersed in decidual tissue by both second harmonic generation (**Figure 1A**) and polarized light microscopy (**Figures 1B–D**). The crystals appeared to be localized both intra and extracellularly and cells containing cholesterol crystals were observed close to uterine vessels (**Figure 1B**) and within the tissue stroma (**Figure 1C**). The cholesterol crystals were not uniformly dispersed within the tissue stroma but were instead aggregated in distinct areas of the tissue and the distribution of such cholesterol crystal areas varied markedly between different pregnancies (**Figure 2A**). The cholesterol crystals appeared in the decidua as clusters of different size, and the ratio between large and small clusters in normal pregnancies was about 2:3 (**Figure 2B**). The cholesterol crystals detected in the decidua dissolved after alcohol treatment for 60 s, as expected (**Supplementary Figure 2**).

No significant differences in the amount, distribution and cluster size of decidual cholesterol crystals were detected between normal pregnancies and preeclamptic pregnancies with or without FGR (**Figure 2C** and data not shown).

Decidual Tissue Composition

Morphological assessment of cells and structures in the uterine wall decidua showed presence of fetal trophoblasts (CK7+), maternal leukocytes (CD45+), decidual stroma cells and uterine blood vessels (CD31+ endothelium) (**Figures 3A–D**). Trophoblasts were observed isolated or clustered in the tissue and were either apart from or in close contact with maternal leukocytes and decidual stroma cells. Both mononucleated trophoblasts and multinucleated trophoblast giant cells were identified and included in the analysis.

Decidual NLRP3 Inflammasome Expression and Function

Paraffin embedded decidual tissue sections from 85 women with normal ($n = 41$) and preeclamptic pregnancies with ($n = 26$) and without FGR ($n = 18$) were included. From these, two pregnancies were excluded from the IL-1β and four from the NLRP3 expression analysis due to methodological errors in immunostaining or image processing. NLRP3 was strongly expressed in the cytoplasm of cells in the

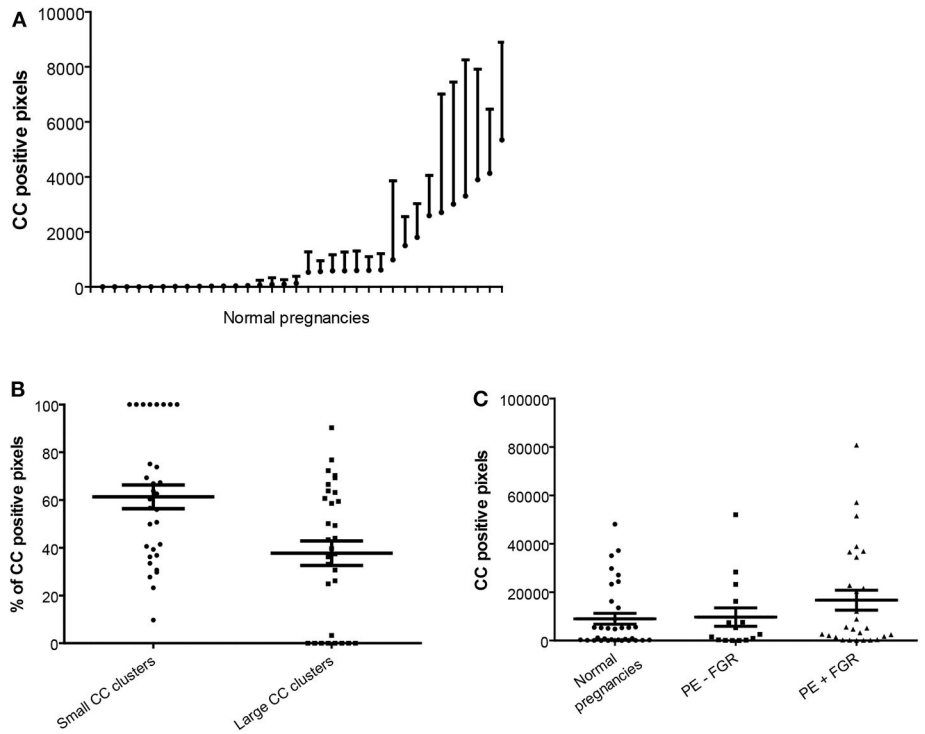

FIGURE 2 | Quantification of cholesterol crystal in decidual tissue. Cholesterol crystal (CC) quantification was performed in nine pictures per decidual cryosection (5 μm). **(A)** Descriptive statistics of the cholesterol crystal positive pixels, shown as estimated means with standard error of mean, per normal pregnancy. **(B)** Percentages of the total cholesterol crystal positive pixels corresponding to small CC clusters (<50 pixels) and large CC clusters (≥50 pixels), in normal pregnancies. **(C)** Total cholesterol crystal positive pixels were quantified in decidual cryosections obtained from normal ($n = 34$) and preeclamptic pregnancies with ($n = 27$) and without fetal growth restriction (FGR) ($n = 15$).

decidual tissue, including trophoblasts, maternal leukocytes, decidual stromal cells and endothelial cells (**Figure 3E**). The cell specific expression pattern of IL-1β in decidua was comparable to NLRP3 (**Figure 3F**). The cellular distribution and overall expression of NLRP3 and IL-1β in decidua of preeclamptic pregnancies appeared comparable to normal pregnancies (**Supplementary Figure 3**). The expression intensity (**Figure 4**, **Supplementary Table 1**) and density (**Supplementary Table 2**) of decidual NLRP3 and IL-1β were quantified. Preeclamptic pregnancies with normal fetal growth showed higher decidual expression intensity (**Figures 4A,B**) and density (**Supplementary Table 2**) of both NLRP3 and IL-1β compared to normal pregnancies (NLRP3 intensity $P = 0.028$ and density $P = 0.006$; IL-1β intensity $P = 0.044$, and density $P = 0.010$) and preeclamptic pregnancies complicated with FGR (NLRP3 intensity $P = 0.372$ and density $P = 0.057$; IL-1β intensity $P = 0.024$ and density $P = 0.065$, respectively). The increased decidual NLRP3 and IL-1β expression associated with preeclampsia with normal fetal growth could not be explained by differences in decidual leukocyte and trophoblast density (**Supplementary Table 2**). Preeclamptic pregnancies with FGR were associated with higher density of leukocytes, but not trophoblasts, compared to both preeclampsia with normal fetal growth and normal pregnancies (**Supplementary Table 2**).

A significant positive correlation between the decidual expression intensity of NLRP3 and IL-1β was observed in normal pregnancies ($R = 0.516$, $P = 0.01$) and pregnancies complicated with preeclampsia without FGR ($R = 0.499$, $P = 0.05$), but not in preeclamptic pregnancies with FGR ($R = 0.323$, $P = 0.116$). We have previously reported placental NLRP3 and IL-1β expression in 21 of the pregnancies included in this study (28). In this subgroup, we found no correlation between the decidual and placental expression intensity, neither in preeclampsia with ($n = 5$, NLRP3 $R = -0.329$, $P = 0.588$; IL-1β $R = -0.579$, $P = 0.306$) or without FGR ($n = 10$, NLRP3 $R = 0.096$, $P = 0.791$; IL-1β $R = -0.251$, $P = 0.484$), nor in normal pregnancies ($n = 6$, NLRP3 $R = -0.718$, $P = 0.108$; IL-1β $R = -0.059$, $P = 0.912$).

Synthetic cholesterol crystals induced NLRP3 inflammasome activation in LPS primed decidual tissue explants ($n = 3$) from normal pregnancies by significantly increasing the release of IL-1β (**Figure 5**). LDH cytotoxicity assay confirmed that the stimuli had no toxic effect on tissue viability (**Supplementary Figure 1**).

Cellular NLRP3 Inflammasome Expression in Decidua

A significant positive correlation was found between the density of trophoblasts and the expression intensity of both NLRP3 ($R = 0.244$, $P = 0.01$) and IL-1β ($R = 0.289$, $P = 0.01$)

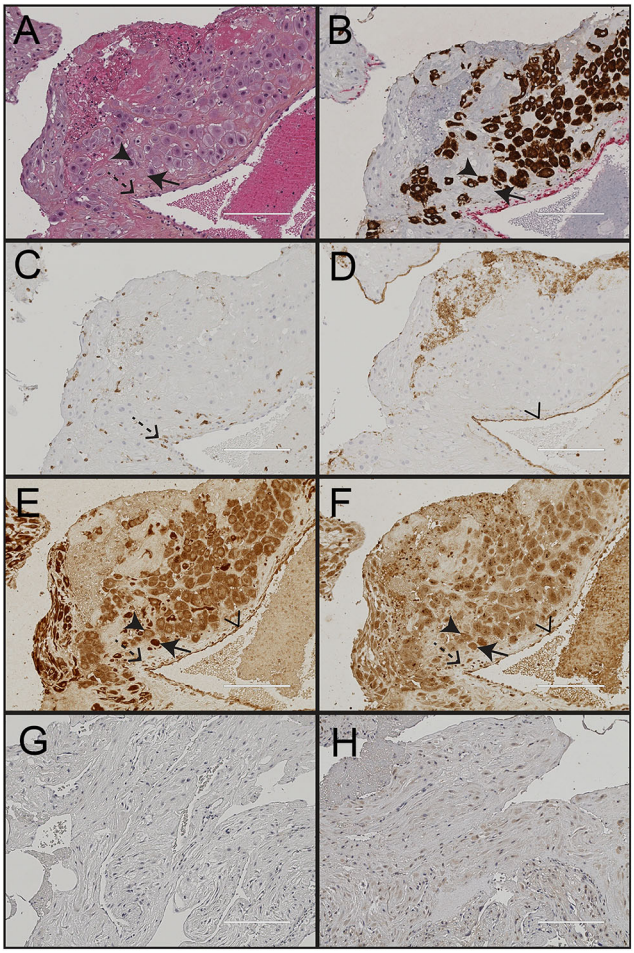

FIGURE 3 | Immunohistochemical staining of decidua from a normal pregnancy. Representative images of decidual tissue from a normal pregnancy at gestational age 40 weeks. **(A)** HES; **(B)** the trophoblast marker cytokeratin 7 (CK7); **(C)** the endothelium marker CD31; **(D)** the leukocyte marker CD45; **(E)** nod-like receptor protein (NLRP)3; and **(F)** interleukin (IL)-1β. Negative isotype control shown for **(G)** NLRP3; and **(H)** IL-1β. Black arrowheads indicate trophoblasts, black arrows indicate maternal decidual stroma cells, dashed arrows indicate maternal leukocytes and transparent arrowhead indicate endothelial cells. Scale bar 200 μM.

in decidua. In decidual areas containing trophoblasts, both the NLRP3 and IL-1β expression intensity was significantly higher in preeclamptic pregnancies with normal fetal growth compared to normal pregnancies (NLRP3 $P = 0.019$; IL-1β $P = 0.023$) (**Figures 4C,D**). Further, the IL-1β expression intensity in trophoblast-containing decidual areas was significantly higher in preeclampsia without FGR compared to preeclampsia with FGR ($P = 0.021$) (**Figure 4D**). Excluding trophoblasts from analysis abolished the significant differences between the study groups (**Supplementary Table 1**).

The maternal leukocyte density in the decidua correlated weakly with the decidual expression intensity of NLRP3 ($R = 0.044$, $P = 0.01$) and IL-1β ($R = 0.040$, $P = 0.01$). Decidual tissue containing leukocytes showed higher expression intensity of NLRP3, but not IL-1β, in preeclamptic pregnancies with normal fetal growth compared to normal pregnancies (NLRP3 $P = 0.025$ and IL-1β $P = 0.052$) (**Supplementary Table 1**).

A closer look at decidual tissue with trophoblast and maternal leukocytes in proximity showed that the expression intensity of NLRP3 and IL-1β was significantly increased in preeclampsia without FGR compared to normal pregnancies (NLRP3 $P = 0.017$ and IL-1β $P = 0.031$) (**Figures 4E,F**). A comparison between trophoblast containing areas was made to assess the influence of leukocyte presence (**Figure 6**). Significantly higher expression intensity of both NLRP3 and IL-1β expression was observed in areas containing trophoblast and maternal leukocytes in proximity, compared to areas with trophoblasts and no leukocytes, and this dependence on maternal-fetal cell proximity was apparent in all study groups (**Figure 6**).

DISCUSSION

This study is the first to reveal the presence of cholesterol crystals at the maternal-fetal interface in the uterine wall decidua, and the crystals were shown to be markedly present in both normal and preeclamptic pregnancies. The cholesterol crystal responsive NLRP3 inflammasome and IL-1β were expressed by both fetal trophoblasts and maternal leukocytes in the decidua. Pathway functionality was confirmed by cholesterol crystal mediated activation of IL-1β production in decidual explants. The expression intensity levels of NLRP3 and IL-1β correlated within the decidua but not between the two sites of the maternal-fetal interface; decidua and placenta. Preeclampsia with normal fetal growth was associated with increased expression of NLRP3 and IL-1β, particularly in decidual areas of close maternal-fetal interaction.

Normal pregnancy is characterized by elevated maternal serum cholesterol and uric acid levels (7, 28, 38), and the correlation with increased serum levels of C-reactive protein (CRP) and sFlt-1 (28) indicates a potential contribution to the elevated maternal inflammatory state of normal pregnancies. The increased serum cholesterol may contribute to accumulation of cholesterol at the maternal-fetal interface (7), eventually leading to formation of cholesterol crystals in decidual tissue, as shown in the present study. Both trophoblasts and leukocytes are equipped with receptors that enable cholesterol uptake, such as the scavenger receptor CD36 (16, 21, 22, 39). Another aspect of how cholesterol crystals may contribute to decidual inflammation is linked to atherosis formation in uterine arteries, a vascular malformation resembling early stage atherosclerosis (40). CD36 is involved in macrophage foam cell formation and atherosclerosis progression by mediating endocytosis and conversion of oxLDL into cholesterol crystals, thus promoting complement and NLRP3 inflammasome activation (16, 41–43). Oxidative stress in the decidua may induce accumulation of oxLDL and cholesterol crystal formation. A similar role for cholesterol crystals in decidual macrophage foam cell accumulation and atherosis formation is supported by our observation of cholesterol crystals around the wall of uterine vessels, but further investigation is needed. We found that

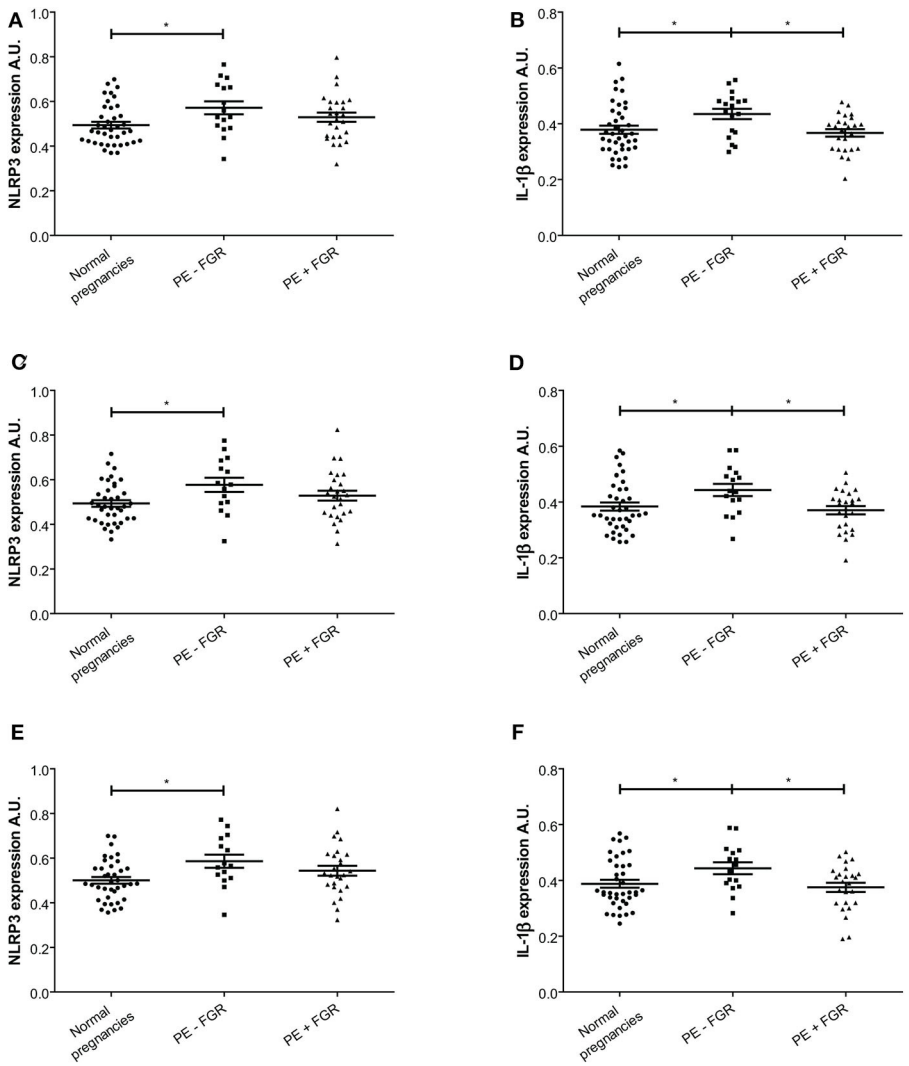

FIGURE 4 | Expression intensity levels of nod-like receptor protein (NLRP)3 and interleukin (IL)-1β in decidual tissue. **(A,C,E)** NLRP3 expression intensity in decidual tissue from normal ($n = 39$) and preeclamptic pregnancies with ($n = 26$) and without fetal growth restriction (FGR) ($n = 16$); and **(B,D,F)** IL-1β expression intensity in normal ($n = 40$) and preeclamptic pregnancies with ($n = 25$) and without FGR ($n = 18$). Expression intensity is shown for **(A,B)** decidual tissue; **(C,D)** decidual areas containing trophoblasts; and **(E,F)** decidual areas containing trophoblast and leukocytes. Data were analyzed by a linear mixed model and expression levels are shown as estimated means with standard error of mean. *$P < 0.05$. A.U. indicates arbitrary units.

cells containing cholesterol crystals appeared aggregated within decidual tissue, rather than randomly scattered, suggesting that cholesterol crystal mediated inflammation may be localized to specific regions in the decidua. Previous studies have suggested that the size of cholesterol crystals and their clusters may be an important factor in the inflammatory potential (44, 45), but this hypothesis was not substantiated for decidual inflammation in the current study. The amount and size of cholesterol crystals in the decidua did not differ between normal and preeclamptic pregnancies. Further assessment of cell specific involvement in the decidual uptake and formation of cholesterol crystals, as well as involvement of relevant priming signals, such as complement factors (46), is warranted to fully understand the inflammatory potential of cholesterol crystals at the maternal-fetal interface. It must also be determined how different pathological processes in decidua interact since oxidative stress may promote the accumulation of oxLDL and cholesterol crystal formation (16, 17). The use of established advanced microscopy methodology for cholesterol imaging learned from atherosclerosis and hepatocytes lipid droplets (47–49), combined with removal of the crystals by alcohol treatment (50), strongly support that the imaged crystals in decidua are crystalline cholesterol. Still, further verification of the chemical identity of the crystals may be performed by other advanced microscopy techniques, such as coherent anti-Stokes Raman scattering (CARS) imaging.

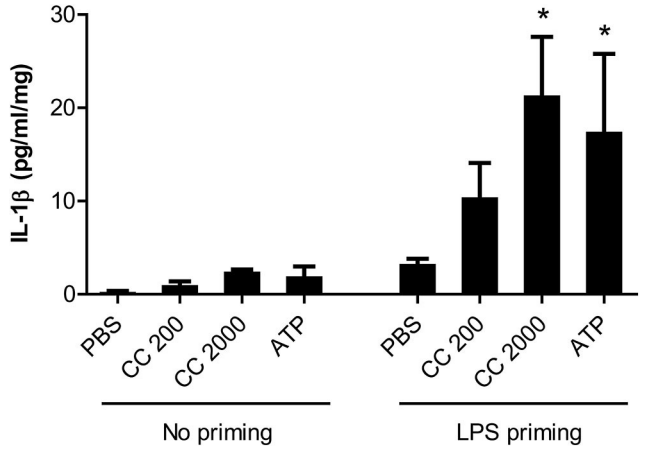

FIGURE 5 | Interleukin (IL)-1β response following cholesterol crystal stimulation of decidual explants. Decidual explants from normal pregnancies ($n = 3$) were primed for 2 h with or without lipopolysaccharide (LPS), before adding cholesterol crystals (CC, 200 or 2,000 μg/ml) or adenosine triphosphate (ATP) for 24 h. Six technical replicates were included for each experimental condition. Release of IL-1β to the supernatant was measured by ELISA and presented as mean with standard error of mean, relative to explant weight. Data were analyzed using Kruskal-Wallis test with Dunn's multiple comparison *post-hoc* test. *$P < 0.05$. PBS, phosphate-buffered saline.

This is the first demonstration of NLRP3 and IL-1β protein expression in maternal and fetal cells in the uterine wall decidua. A report of gene expression of NLRP3 inflammasome components in isolated first trimester decidual stromal cells partly supports our findings (30). Combined with our previous discovery of NLRP3 and IL-1β expression in placental trophoblasts of early and late pregnancies (28), this study clearly supports a role for NLRP3 inflammasome activation at both sites of the maternal-fetal interface throughout pregnancy. In addition, the positive correlation between decidual expression of NLRP3 and the responsive cytokine IL-1β substantiates the functionality of the NLRP3 inflammasome in decidua. Extravillous trophoblasts were here shown to be central for the decidual NLRP3 inflammasome response. This means that the characterization of placental trophoblasts and trophoblast cell lines as immunocompetent cells by their functional pattern recognition receptors (PRR) by us (28, 51, 52) and others (9, 53), has now been extended to extravillous trophoblasts in the decidua. This indicates importance for immunomodulating trophoblast activity at the maternal side of the maternal-fetal interface in the final stages of pregnancy, with dependence on the proximity and possible direct interaction between fetal and maternal cells. Supporting such maternal-fetal communication is that maternal leukocytes and trophoblasts in the decidua express complementary ligands and receptors (9, 10) and that leukocytes are key cells in modulating trophoblast behavior (54). In addition to leukocytes and trophoblasts, decidual stromal cells markedly expressed NLRP3 and IL-1β and are considered potential responders to cholesterol crystals, but further studies focused on this cell type are needed to address their inflammatory role.

Preeclampsia without FGR was associated with increased decidual expression of NLRP3 and IL-1β, suggesting that the NLRP3 inflammasome aggravates the inflammatory response and substantiates the reported shift to a pro-inflammatory profile and cell type distribution at the maternal-fetal interface in preeclampsia (9, 14, 29). The increased decidual inflammasome expression was trophoblast dependent and strongest in areas where trophoblast and leukocytes are in proximity, suggesting that NLRP3 mediated inflammation may disturb maternal-fetal communication. The novel identification of decidual cholesterol crystals combined with increased NLRP3 inflammasome expression presents a novel link between the pathophysiology of CVD and preeclampsia. NLRP3 inflammasome response may lead to extensive tissue damage and cell death and is associated with formation and progression of atherosclerotic lesions (18, 19). Correspondingly, increased formation of decidual atherosis and inflammation at the maternal-fetal interface are pathophysiological features of preeclampsia (40, 55). The NLRP3 inflammasome expression pattern in decidua further points to interesting pathophysiological differences between preeclampsia subgroups. We have previously demonstrated a placental role for the NLRP3 inflammasome in preeclampsia combined with FGR (28), while the decidual contribution presented here was apparent in preeclampsia without FGR. This points to divergent NLRP3 inflammasome activation in preeclampsia subgroups. Supporting such divergent regulation is the lack of correlation between decidual and placental expression levels of NLRP3 and IL-1β, indicating that activators in the maternal serum affect placental and decidual tissue differentially and that the local inflammatory responses in the decidua and placenta are not directly coordinated. We hypothesize that the placental tissue may be more influenced by placental dysfunction and fetal complications. FGR has been shown associated to a fetal pro-atherogenic lipid profile and placental cholesterol accumulation due to abnormal cholesterol transport (24, 56). This may lead to cholesterol accumulation and crystallization in the placenta and activation of the NLRP3 inflammasome in a process that may not influence decidual tissue. In the present study, increased decidual NLRP3 inflammasome response was observed in preeclampsia without FGR, and this could indicate that the decidual tissue may respond more to the increased maternal danger signals, including pro-atherogenic lipid profile, circulating levels of inflammatory mediators, such as HMGB1 and uric acid, and predisposition to inflammation.

Therapeutic and preventive approaches in preeclampsia are limited. A large prospective study showed limited preventive effect of administration of low-dose anti-inflammatory and antiplatelet agent aspirin on preeclampsia development (57). Pravastatin, used for treatment of dyslipidemia and prevention of CVD, is a suggested candidate for treatment and prevention of preeclampsia (58). In addition to reducing hypercholesterolemia, statins may ameliorate major pathological responses involved in preeclampsia, including inhibition of sFlt-1 release and reduction of inflammation and oxidative stress (59). Importantly, statins may inhibit formation and improve solubility of cholesterol crystals in atherosclerotic plaques (60), adding to the beneficial effects of these cholesterol-lowering drugs. Further investigation is needed to demonstrate whether a positive effect of pravastatin in preeclampsia may involve removal of cholesterol crystals at the

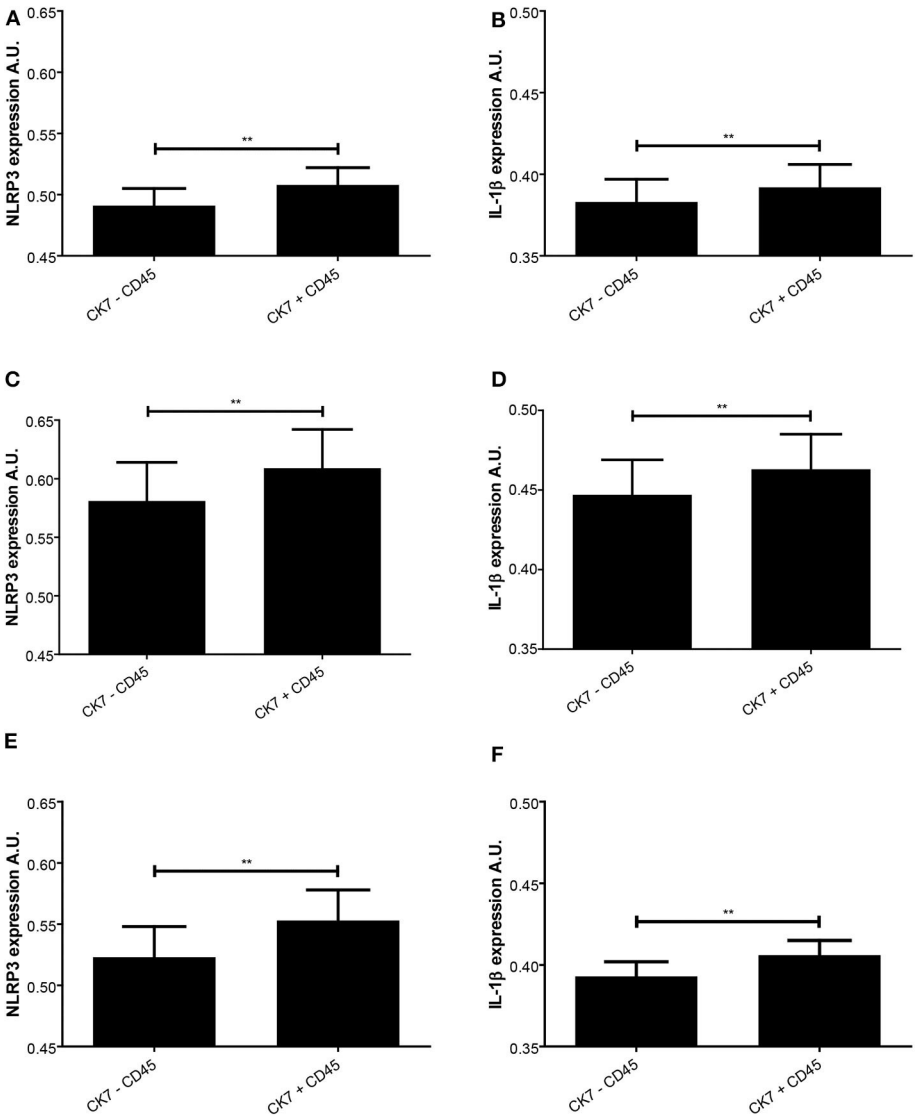

FIGURE 6 | Expression intensity levels of nod-like receptor protein (NLRP)3 and interleukin (IL)-1β in decidual tissue related to presence of trophoblast and leukocytes. NLRP3 **(A,C,E)** and IL-1β **(B,D,F)** expression was measured in normal pregnancies **(A,B)** and preeclamptic pregnancies without **(C,D)** or with **(E,F)** fetal growth restriction (FGR). The expression levels were assessed in trophoblast containing areas with (CK7 + CD45) or without (CK7 – CD45) presence of leukocytes. $^{**}P < 0.01$. A.U. indicates arbitrary units.

maternal-fetal interface. Growing evidence supports the clinical benefits and anti-inflammatory effects of drugs targeting the NLRP3 inflammasome and IL-1β pathway in several diseases (61), but whether they are pregnancy-safe and effective in preeclampsia needs to be determined.

This study identified cholesterol crystal mediated NLRP3 inflammasome response as an inflammatory mechanism associated with maternal-fetal interaction in the uterine wall decidua in pregnancy. Cholesterol crystals were detected in considerable amounts in decidua and the expression of the NLRP3 inflammatory pathway showed importance for close interaction between fetal trophoblasts and maternal leukocytes. The increased NLRP3 inflammasome expression in preeclampsia with normal fetal growth suggests that an enhanced pro-inflammatory profile in the decidua negatively affects maternal-fetal communication and plays a role at late stages of preeclampsia pathology, possibly by intercommunication with the maternal systemic inflammatory response. The identification of decidual cholesterol crystals and increased decidual NLRP3 inflammasome expression in preeclampsia with normal fetal growth further substantiates the pathophysiological link between preeclampsia and CVD. This study showed that combined investigation of cell specific pathological mechanisms at the two sites of maternal-fetal interface may provide more comprehensive knowledge of the regulation and importance of maternal-fetal communication.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

The Norwegian Regional Committee for Medical and Health Research Ethics (REC) approved the study (REC 2012/1040 and 2009/03) and written informed consent was obtained from participants. Procedures were in accordance with institutional guidelines. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

GBS, LG, and A-CI designed the study and all authors contributed with valuable inputs. GBS, LT, and LB collected the clinical material and information. GBS, LG, JR, SM, AT, ME, and A-CI developed the methods for image processing and automated quantification. GBS and GSS performed the decidual explant experiments. GBS, LG, and A-CI interpreted the data and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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CD206+ M2-Like Macrophages Are Essential for Successful Implantation

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Macrophages (MΦs) play important roles in implantation. Depletion of CD11b+ pan-MΦs in CD11b-diphtheria-toxin-receptor (DTR) mice is reported to cause implantation failure due to decreased progesterone production in the corpus luteum. However, of the M1 and M2, the type of MΦs that is important for implantation is unknown. In this study, we investigated the role of M2 MΦ in implantation using CD206-DTR mice. To deplete M2-MΦ, female CD206-DTR C57/BL6 mice were injected with DT before implantation. These M2-MΦ depleted mice (M2(-)) were naturally mated with Balb/C mice. As the control group, female C57/BL6 wild type (WT) mice injected with DT were mated with male Balb/C mice. The number of implantation sites and plasma progesterone levels at implantation were examined. Implantation-related molecule expression was determined using quantitative-PCR and immunohistochemistry of uterine tissues. The mRNA expression in the endometrial tissues of 38 patients with implantation failure was examined during the implantation window. In WT mice, CD206+M2-like MΦs accumulated in the endometrium at the implantation period, on embryonic (E) 4.5. In M2(-), the implantation number was significantly lower than that in control ($p < 0.001$, 7.8 ± 0.8 vs. 0.2 ± 0.4), although the plasma progesterone levels were not changed. Leukemia inhibitory factor (LIF) and CD206 mRNA expression was significantly reduced ($p < 0.01$), whereas the levels of TNFα were increased on E4.5 ($p < 0.05$). In M2(-), the number of Ki-67+ epithelial cells was higher than that in control at the pre-implantation period. Accelerated epithelial cell proliferation was confirmed by significantly upregulated uterine fibroblast growth factor (FGF)18 mRNA ($P < 0.05$), and strong FGF18 protein expression in M2(-) endometrial epithelial cells. Further, M2(-) showed upregulated uterine Wnt/β-catenin signals at the mRNA and protein levels. In the non-pregnant group, the proportion of M2-like MΦ to pan MΦ, CD206/CD68, was significantly reduced ($p < 0.05$) and the TNFα mRNA expression was significantly increased ($p < 0.05$) in the endometrial tissues compared to those in the pregnant group. CD206+ M2-like MΦs may be essential for embryo implantation through the regulation of endometrial proliferation via Wnt/β-catenin signaling.

Keywords: CD206, diphtheria-toxin receptor mouse, fibroblast growth factor, implantation, M2 macrophage, Wnt/β-catenin signal

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INTRODUCTION

Macrophages (MΦ) are a crucial player in the generation and execution of immune responses through various functions, including phagocytosis, antigen presentation, and secretion of a variety of cytokines and growth factors (1–3). Recently, MΦs have been reported to play an essential role in tissue development and homeostasis through increased angiogenesis and vascular remodeling (1, 4, 5). MΦs also have attracted significant interest in human diseases as they play crucial roles in many diseases associated with chronic inflammation such as atherosclerosis, obesity, diabetes, cancer, skin diseases, and neurodegenerative diseases (6, 7). Implantation is a vital process of the first feto-maternal encounter in the uterus, leading to pregnancy. Good coordination between a blastocyst and receptive uterus is essential for successful implantation (8, 9). Although implantation is an important phenomenon in pregnancy, its precise mechanism is not fully understood due to its complexity involving multi-factors. Animal studies using different kinds of genetically altered mice have been undertaken to elucidate the mechanism of implantation (10–13). Although few studies have examined the relationship between MΦ and implantation, Care et al. first reported that MΦ plays an important role in the implantation process in CD11b-DTR mice (14). They showed that depletion of CD11b+ MΦs resulted in the implantation failure due to decreased progesterone production in the corpus luteum (14). MΦs are classified into two subtypes, M1 and M2 MΦs. M1 MΦs, or classically activated MΦs, are pro-inflammatory and play a central role in host defense against infection, whereas M2 MΦs, or alternatively activated MΦs, are associated with responses to anti-inflammatory reactions and tissue remodeling (15).

The precise role of MΦs in the uterus at the implantation period is unclear in implantation period.

MΦs demonstrate plasticity and polarize to the M1 or M2 type according to their surrounding microenvironment and stimuli (2, 16) and skewness to M1 or M2 MΦs has been reported in various diseases (4). But it is not clear which type of MΦs mostly contributes to the implantation. In the present study, we investigated the role of CD206+ M2-like MΦ in implantation using CD206-diphtheria-toxin (DT)-receptor transgenic mice (17–19), in which M2-like MΦs can be specifically depleted.

RESULTS

CD206+M2-Like MΦs Are Located in the Uterus at the Implantation Period

Most MΦs in non-pregnant mice are known to be present in the uterine stroma, but are reported to exist in the lumen and glands during the implantation period (20). To examine the localization of M2-like MΦs in the uterus at the implantation period on embryonic day 4.5 (E4.5), we performed CD206 immunohistochemistry in wild type (WT) mice. At the implantation period, we found that CD206+ M2-like MΦs were located in the uterine stromal region as well as close to the lumen and glands. Immunofluorescence analysis revealed that CD206+ cells were found in WT with DT and TG with PBS group, while these were completely depleted in TG with DT mice (**Figure 1A**-a). To examine the change of M2-like MΦs in the uterus at the implantation period, we compared CD206 mRNA expressions between non-pregnancy and implantation periods. The mRNA expressions of uterine CD206 was significantly increased during implantation period, peaking at embryonic (E) 3.5, compared to non-pregnancy (**Figure 1A**-b).

Implantation Was Impaired in the M2(-) Group

To investigate the role of CD206+ M2-like MΦ in implantation, we set the protocol of an implantation model using CD206 DTR mice (**Figure 1B**). We naturally mated C57/B6 female mice with Balb/c male mice as controls, or mated CD206 female DTR mice with Balb/c mice, defined as M2(-). DT was intraperitoneally administered to the control and M2(-) mice before the implantation period. The depletion of CD206+ M2-like MΦs was checked by qPCR. We compared the number of implantation sites between the two groups at E4.5 after in Chicago Blue dye administration. The implantation sites in M2(-) mice were significantly fewer compared to those in the control mice ($P < 0.001$, 7.8 ± 0.8 vs. 0.2 ± 0.4) (**Figures 2A,B**). As leukemia inhibitory factor (LIF)-Stat signaling is known to be essential for implantation (21), LIF mRNA expression was examined in the uterine tissues in M2(-) mice and was found to be significantly decreased compared to that in control ($p < 0.05$, **Figure 3A**). Immunostaining for phosphorylated Stat3 was also found in uterine epithelial cells of control mice but not in M2(-) mice (**Figure 3B**). The proportion of phosphorilated (p) STAT3-positive epithelial/total epithelial cells was significantly reduced in the M2(-) group compared to control (mean ± SD, $38.5 \pm 13.2\%$ vs. 0%; $p < 0.01$, **Figure 3C**).

The Accelerated Proliferation of Epithelial Cells Was Found in M2(-) Mice

We examined the morphological changes in the just after implantation period (E 5.5). In M2(-) mice, cell proliferation in the stromal region was impaired, and epithelial cells were proliferative compared to control (**Figure 4A**). We then examined the cell proliferation at pre-implantation period (E3.5). In M2(-) mice, the number of Ki-67-positive epithelial cells was higher compared to that in control at the pre-implantation period (E3.5). However, there were no histological differences in the corpus luteum and the plasma P4 concentration between both groups (**Figures 4B,C**). These suggest that endometrial epithelial cells had not transformed to become receptive to embryo implantation (**Figure 5**).

Uterine Wnt/β-Catenin Signaling Is Upregulated in M2(-) Mice at the Pre-implantation Period (E3.5)

Uterine Wnt/β-catenin signals regulate the production of fibroblast growth factor (FGF), and proper modification of these signals is essential for implantation (22). In M2(-) mice, at the implantation period, the mRNA expression of Wnt 4A, Wnt 7B, and β-catenin, was significantly increased ($p < 0.05$) compared

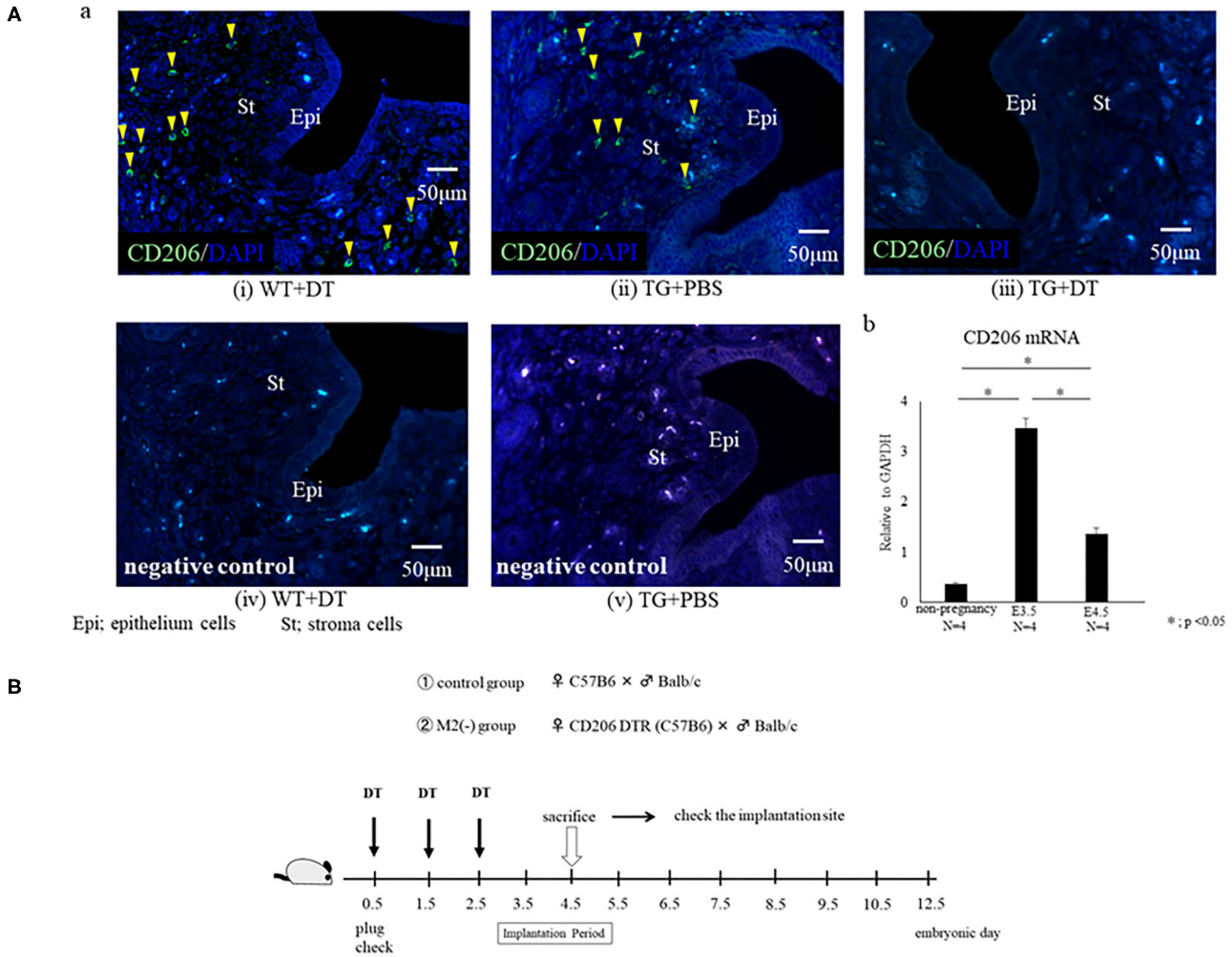

FIGURE 1 | (A) Localization of CD206+ M2-like macrophages (MΦ) in the uterus. Immunofluorescence for CD206 was performed for the uterus at embryonic day 4.5 (E4.5) in wild type (WT) and CD206 diphtheria toxin receptor transgenic mice (TG). The data of WT+DT(i), TG+PBS (ii), TG+DT (iii), WT+DT (iv), and TG+PBS(v) were shown. Anti- CD 206 antibody (i, ii, and iii) or control rabbit IgG (iv and v) were used for primary antibody. CD206-positive cells are in green, and nucleus were stained in blue. Yellow arrow heads show CD206+ M2-like MΦ. The uterine mRNA expression of CD206 at non pregnancy, E3.5 and E4.5 are shown in (b). Data were normalized to GAPDH mRNA levels to determine the relative abundance and are shown as the mean ± SEM. *$p < 0.05$. **(B)** Depletion protocol for CD206+ M2-like macrophages (MΦs) in the implantation model using CD206 DTR mouse. After checking the plug, diphtheria-toxin (DT) was intra-peritoneally injected prior to the implantation period (embryonic day; E0.5, E1.5, E2.5) in CD206-DTR female mice naturally mated with Balb/C male mice, defined as the M2(-) group. DT was also injected to C57/B6BL wild type (WT) female mice mated with Balb/C male mice, and were defined as the control group. The number of implantation sites and plasma progesterone levels at the implantation period (E4.5) were examined.

to the control, and endometrial epithelial cells exhibited strong staining for active β-catenin (**Figure 6A**). In detail, the basal site of uterine epithelial cells was strongly stained with β-catenin in M2(-) mice (**Figure 6B**). As expected, the mRNA expression of FGF18, downstream of the Wnt/β-catenin signal, was significantly upregulated ($P < 0.05$) compared to that in control; further, FGF18 protein was also strongly stained in the endometrial epithelial cells of M2(-) mice (**Figure 6C**).

Uterine Wnt/β-Catenin Signaling Was Enhanced by Inflammatory M1-Like MΦ

Wnt signaling is reported to be increased by TNFα in gastric tumor cells (23). We also found upregulated expression of TNFα, iNOS, and CD11c mRNAs produced by M1-like MΦs (4) in the uterus of M2(-) mice compared to control ($p < 0.05$) (**Figure 7**).

The Proportion of CD206+ M2-Like MΦs Among Total MΦs in Uterine Tissues Was Significantly Reduced in Patients With Infertility

We performed uterine endometrial biopsy in cohort of 38 infertility patients at the time of the implantation window. Implantation failure was diagnosed as the infertility factor for all these patients. After the endometrial biopsy, 19 patients got pregnant with assisted reproductive technology. The median age of the non-pregnant and the pregnant group was 40

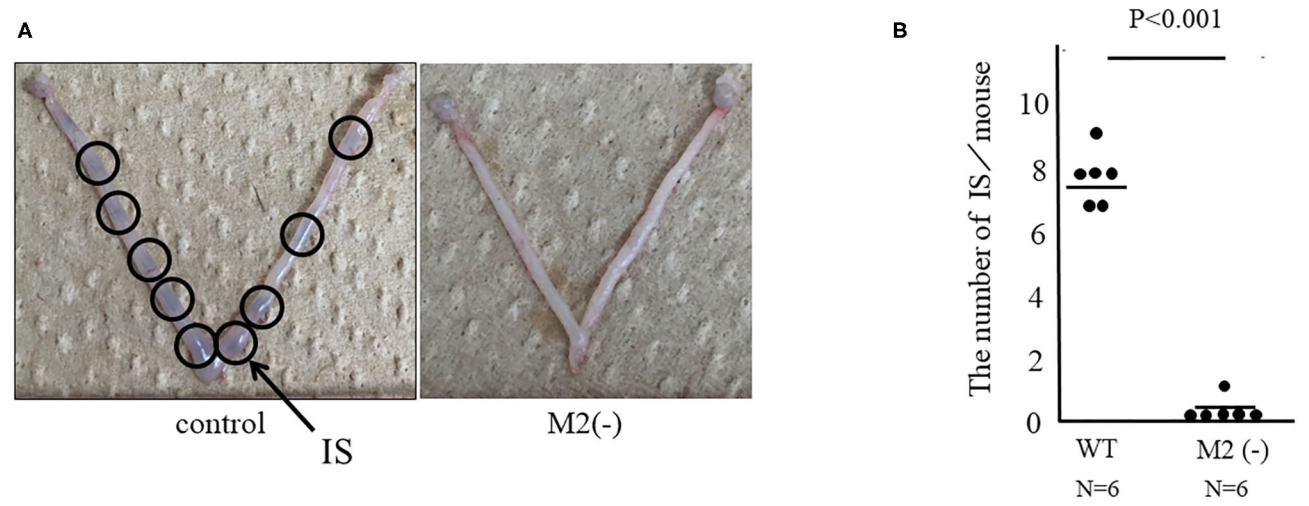

FIGURE 2 | Assessment of implantation site (IS). Representative appearances of the IS in CD206 DTR with DT (M2(-)) and control. ISs were stained with Chicago blue dye for facilitating their detection. ISs are shown (arrows) **(A)** and the number of ISs are shown as dots and the mean **(B)**.

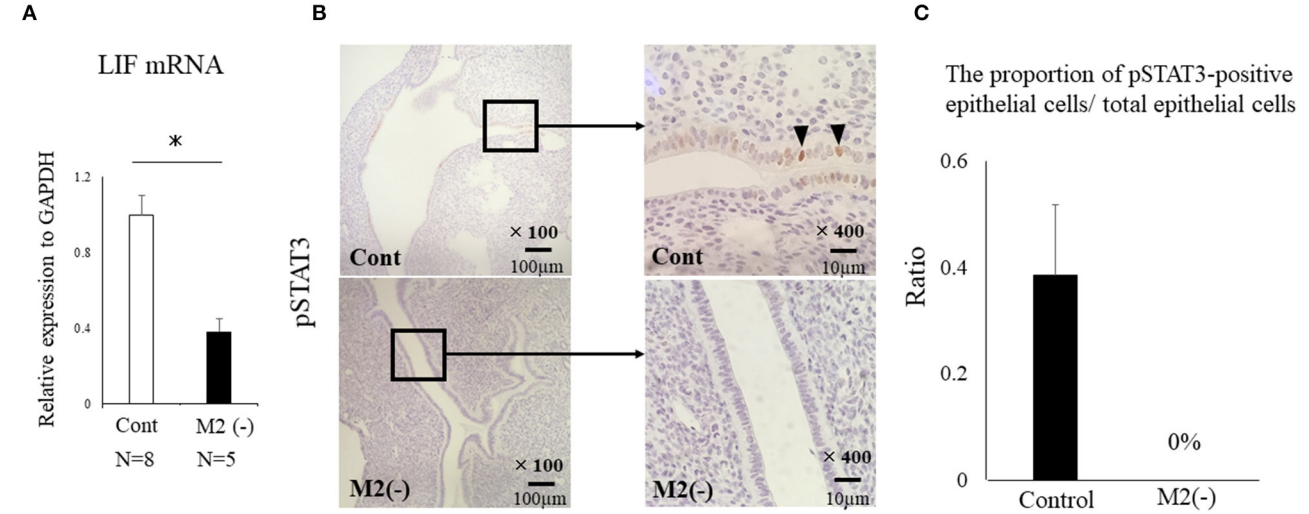

FIGURE 3 | LIF-STAT signal in the uterus at the implantation period. LIF mRNA expression in the uterus was examined. Data were normalized to GAPDH mRNA levels to determine the relative abundance and shown as **(A)**. *$p < 0.05$. Immunohistochemistry was performed for phosphorylated (p) STAT3 in the control and M2(-) mice **(B)**. We randomly selected six different sites on the section of immunostaining and counted the number of phosphorylation (p) STAT3-positive epithelial cells, which were divided by the total number of epithelial cells between both groups. The proportion of pSTAT3-positive epithelial cells/ total epithelial cells was examined **(C)**.

(29–44) years and 37.5 (33–43) years old, respectively, which was comparable between two groups. We then compared the proportion of uterine CD206+ M2-like MΦs to pan MΦs at the mRNA levels of CD206/CD68, between pregnancy and non-pregnant groups. The relative ratio of M2-like MΦ to total MΦ was significantly reduced ($P < 0.05$) in non-pregnant group compared to that in the pregnant group, while upregulation of TNFα mRNA expression was observed in the non-pregnant group ($p < 0.05$) (**Figure 8**).

DISCUSSION

This is the first report investigating the role of M2 MΦs in the uterus at the implantation period in the mouse model. Previous reports showed that MΦs are important regulator of implantation and their depletion disrupts luteal vasculature resulting in reduced progesterone production from corpus luteum, which cause implantation failure (14, 24). These data suggest that depletion of pan MΦs during the implantation period causes implantation failure not owing to defects in the uterus but attributed to the ovary. Plaks et al. used the CD11c DTR mouse model to report that uterine dendric cells (DCs) are essential for embryonic implantation (25). However, as CD11c-positive cells include both MΦs and DCs, there was a limitation to determining the effect of each cell on implantation when CD11c cells were depleted. In the present study, we showed for the first time that CD206+ M2-like MΦs are essential to implantation by using CD206 DTR mice.

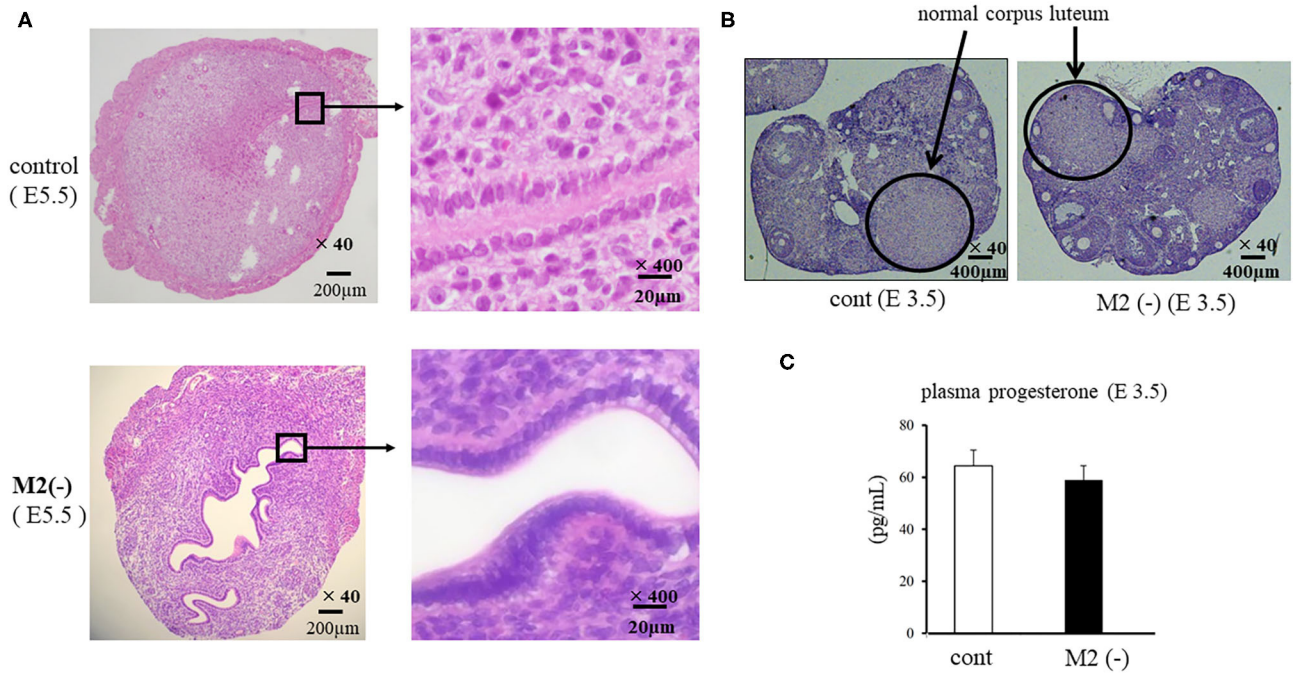

FIGURE 4 | Cross section of uterus and ovary. The cross sections of the implantation site (IS) are stained by hematoxylin and eosin (HE). The representative HE sections are shown at lower (x40) and higher (x400) magnification in the control and M2(-) at E5.5 **(A)**. The corpus luteum [**(B)**, hematoxylin and eosin stain] and the plasma progesterone levels **(C)** in control and M2(-) at E 3.5 are shown.

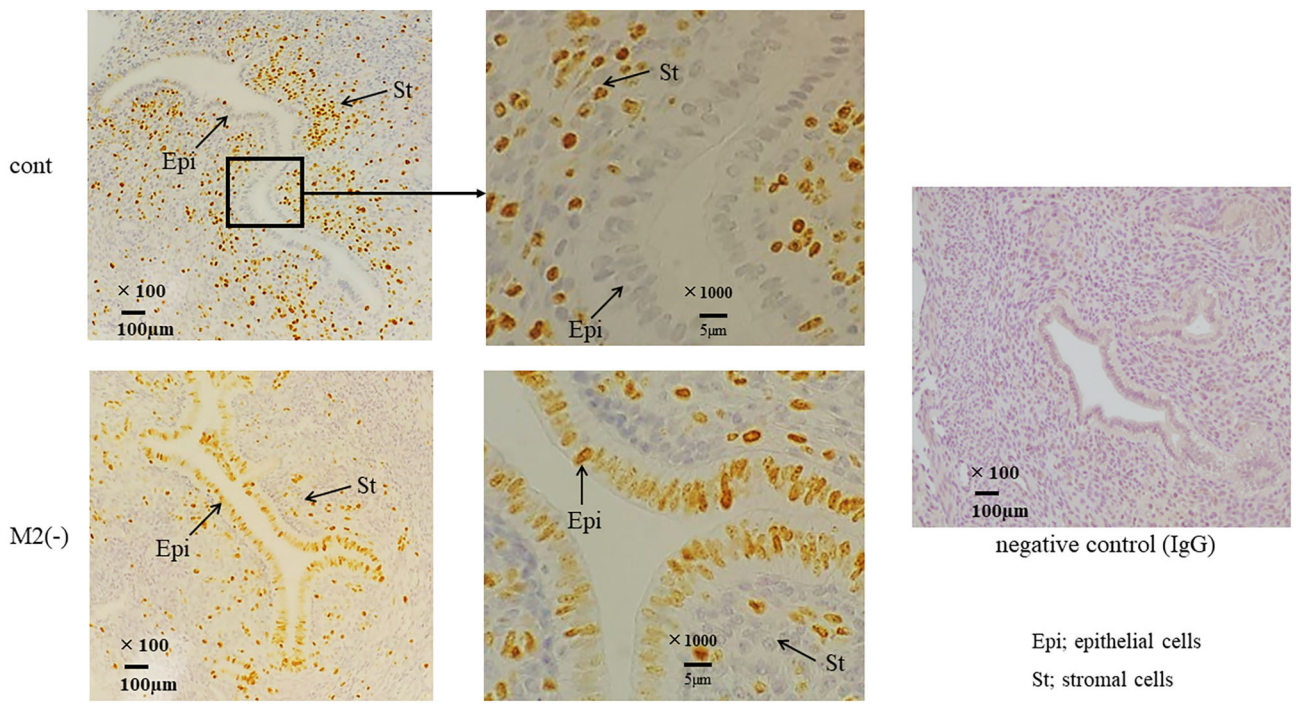

FIGURE 5 | Ki-67 immunostaining in the uterus at the pre-implantation period. Immunostaining for Ki-67, a proliferation marker, was performed in control and M2(-) mice. The arrows indicate the endometrial epithelial and stromal cells at E3.5. The representative sections are shown at lower (x100) and higher (x1000) magnification. Rabbit IgG was used for negative control.

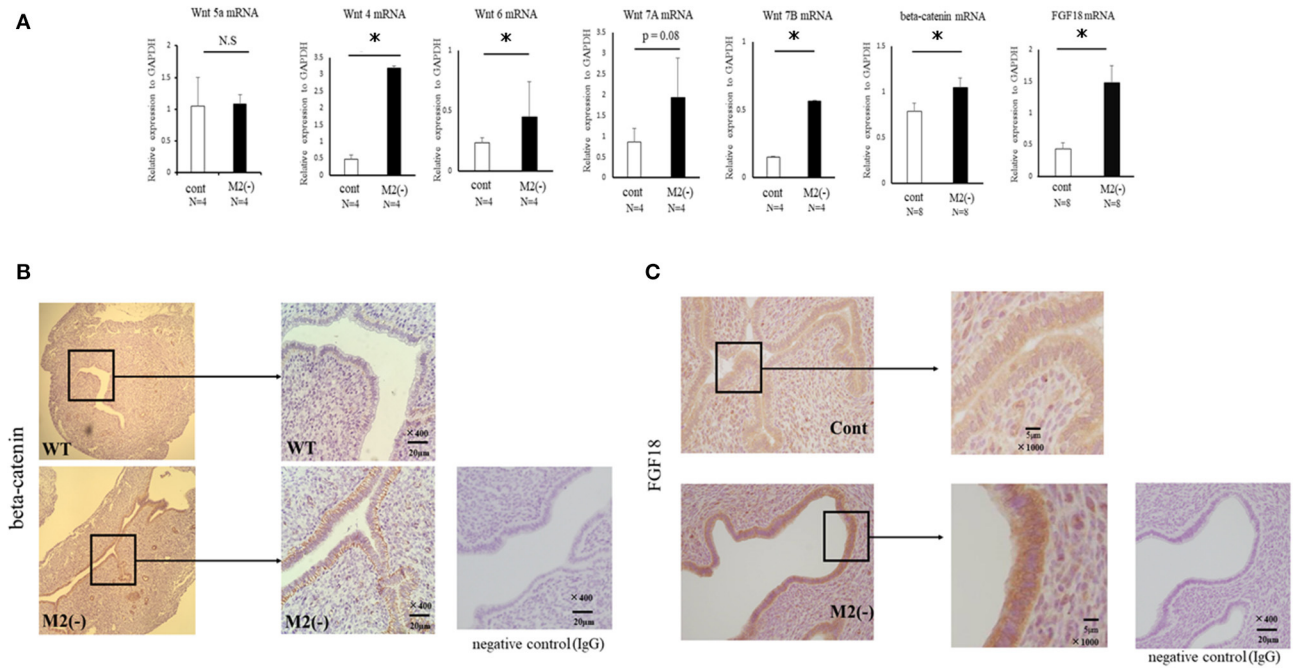

FIGURE 6 | Wnt/β-catenin signaling in the uterus at the pre-implantation period. The mRNA expression of Wnt4, Wnt5a, Wnt6, Wnt7A, Wnt7B, β-catenin, and FGF18 in the uterus at E 3.5 was examined by quantitative-PCR in the control and M2(-) mice **(A)**. Data were normalized to GAPDH mRNA levels to determine the relative abundance. The expression of β-catenin and FGF18 protein in the uterus at the pre-implantation period in control and M2(-) mice at E3.5 was examined by immunohistochemistry. Rabbit IgG was used for negative control **(B,C)**. Data are shown as the mean ± SEM. $^*p < 0.05$.

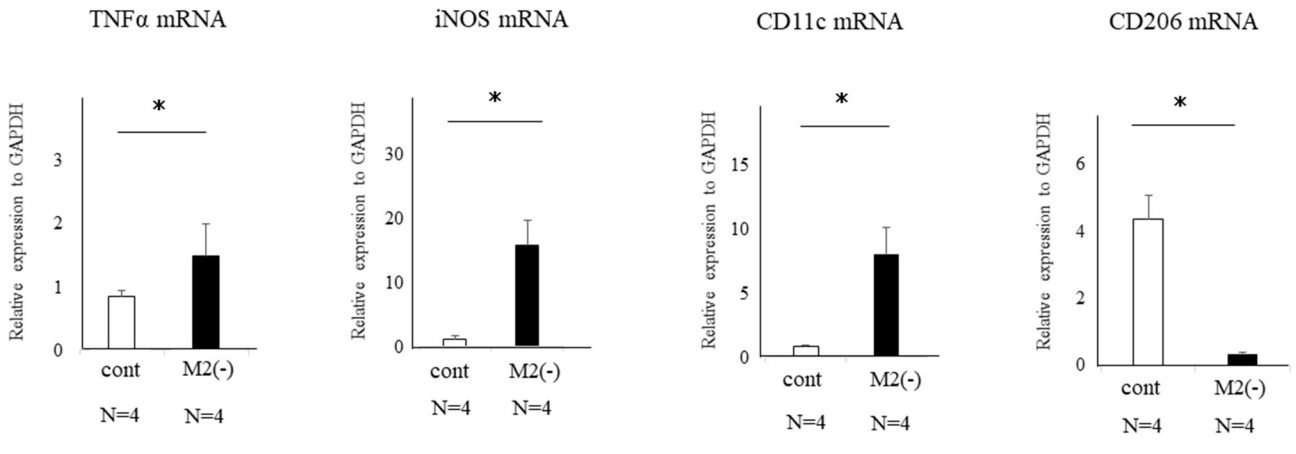

FIGURE 7 | Expression of TNFα, iNOS, CD11c, and CD206-mRNAs in the uterus of control and M2(-) mice at the pre-implantation period (E3.5). The mRNA expression of TNFα, iNOS, CD11c as the M1 MΦ marker, and CD206 in the uterus at the pre-implantation period in control and M2(-) mice was examined. Data were normalized to GAPDH mRNA levels to determine the relative abundance and are shown as the mean ± SEM. $^*p < 0.05$.

In the CD206 DTR mouse model, implantation failure occurred exclusively by the depletion of CD206+ M2-like MΦs. As we have reported previously (19), we also found that the histological structure of the corpus luteum in M2(-) was not different from that of the control mice, and the plasma P4 levels were not changed, suggesting that the ovarian function at the implantation period was maintained in the absence of M2-like MΦs. Reduced plasma progesterone level by luteal dysfunction in depletion of pan MΦs mice model might be caused by the depletion of M1 MΦs. Therefore, the implantation failure may be attributed to the abnormal interaction between the embryo and uterus. In our previous study, we examined the effects of oocytes and embryos quality derived from M2MΦ depletion mouse on fertilization and implantation (19). In detail, after inducing superovulation in wild type (WT) and CD206+M2-like MΦ depleted mice, oocytes obtained from the fallopian tubes

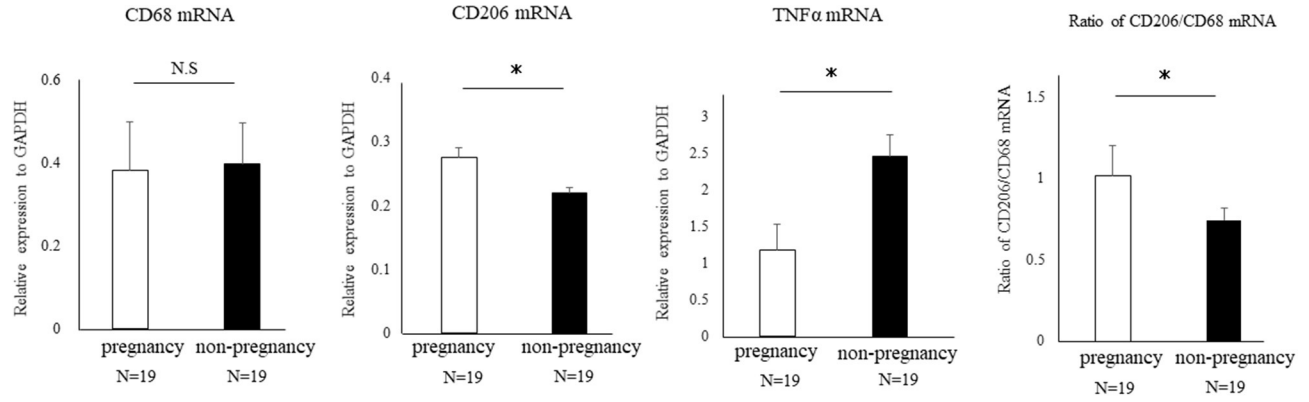

FIGURE 8 | The proportion of uterine CD206+ M2-like MΦ in human cases of pregnancy success and failure. A cohort of 38 patients with implantation failure were subjected to uterine endometrial biopsy at the timing of the implantation window. The mRNA expression of CD68 (pan-MΦ), CD206, and TNFα in uterus were examined between the pregnant and non-pregnant group after endometrial biopsy. Data were normalized to GAPDH mRNA levels to demonstrate the relative abundance. Also, the proportion of uterine CD206+ M2 MΦ to pan-MΦs at the mRNA level between two groups is shown. Data are shown as the mean ± SEM. $^{*}p < 0.05$.

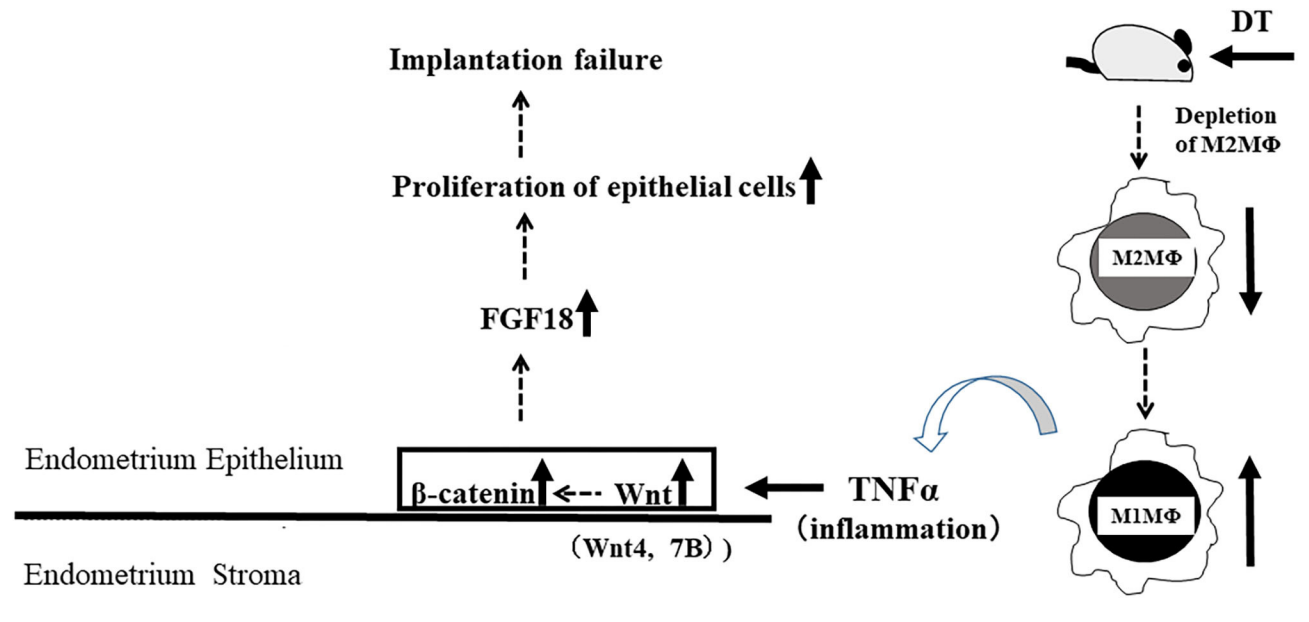

FIGURE 9 | Schematic of the study.

of these mice were *in vitro* fertilized, followed by transferring to pseudo pregnant WT mice. The fertilization rate, blastocyst formation rate, and pregnancy rate of CD206 DTR-mice derived oocytes were comparable to that of WT-mice derived oocytes, suggesting that oocytes derived from CD206+M2-like MΦ-depleted mice did not affect fertilization and implantation (19). In the present study, the structure of the corpus luteum and the plasma progesterone level was maintained during the implantation period. We detected morphological abnormality only in the uterus, so decreased uterine MΦs were considered the cause of implantation failure. In addition, at the pre-implantation period (E3.5), embryos obtained by flushing the uterine cavity with saline in both WT and CD206 DTR mice exhibited no morphological differences (data not shown). These data suggest that the depletion of M2MΦ *in vivo* did not affect the embryo quality and hormonal milieu. Therefore, the cause of implantation failure in M2(-) mice as observed in the present study, was the uterus and was not due to the abnormalities in the ovary or the embryo. Subsequently, we focused on the role of M2 MΦs in the endometrium during implantation. Elevated P4 concentrations after ovulation dramatically change the state of endometrial cell proliferation and render the uterus receptive to the embryo as a normal uterine morphological change that occurs during pre-implantation (26). In normal conditions,

luminal epithelial cells are known to cease proliferation for implantation (26, 27); however, in M2(-) mice, the number of Ki-67-positive endometrial epithelial cells was higher compared to the control at the preimplantation period (E3.5), suggesting that the endometrial epithelial cells did not undergo the required change for receiving the embryo (**Figure 5**).

Nallasamy et al. have reported that targeted mutation of the homeobox transcription factors, Msx1 and Msx2, which control organogenesis and tissue interactions during embryonic development, in both the uterine epithelium and stroma, results in implantation failure. Based on gene expression profiling of the uterine epithelium and stroma from Msx1/2d/d mice, elevation of Wnt/β-catenin signaling leads to an increase in fibroblast growth factor (FGF) production in the uterine stroma (28). Moreover, upregulated FGFs act in a paracrine manner on the uterine epithelium to promote epithelial proliferation, which prevents endometrial differentiation and creates a non-receptive uterus for the embryo (28). This indicates that an excessive increase in Wnt/β-catenin signaling leads to an unreceptive uterus, which is refractory to implantation due to its inability to control epithelial proliferation, though moderately balanced uterine Wnt/β-catenin signaling is reported to be necessary for implantation (22). In the present study, the accelerated proliferation of epithelial cells in M2(-) mice might be due to a higher expression of FGF-18 in endometrial epithelial cells.

Aberrant expression of TNFα has been reported as one of the causes of enhanced Wnt/β-catenin signaling (23). In the present M2(-) mouse model, we found that the expression of TNFα and M1-like MΦ markers such as inducible nitric oxide synthase (iNOs) and CD11c (10), were significantly increased at the mRNA level ($P < 0.05$). This upregulation of M1-like MΦ related molecules might be due to a relative increase in M1-like MΦs owing to the depletion of CD206+ M2-like MΦs. Kambara et al. also reported that the expression of pro-inflammatory cytokines such as TNFα, IL-1β, IL-6, and MCP-1 were significantly upregulated in the lung tissues of CD206 DTR mice in response to DT treatment (17). Collectively, we hypothesized that upregulation of TNFα secreted by M1-like MΦs after depletion of CD206+ M2-like MΦ might accelerate the uterine Wnt/β-catenin signal in endometrial epithelial cells. Further, in epithelial cells, FGF18 expression was increased aberrantly, resulting in the proliferation of epithelial cells, which caused implantation failure (**Figure 9**). These results indicate that the balance of M1 and M2 MΦs may be critical for embryonic implantation. Additionally, LIF, essential for implantation, is also known to be produced by MΦ (29) and MΦ derived LIF is identified as a potential factor mediating MΦ-epithelial signaling (30). Therefore, the decrease in LIF expression (**Figure 3**) might be involved in the implantation failure. In our analysis of human samples, the proportion of uterine CD206+ M2-like MΦ, based on the CD206 mRNA expression compared to the total MΦ marker CD68, was significantly reduced ($P < 0.05$) in non-pregnant patients at the implantation period compared to that in the pregnant patients. And, in non-pregnant patients, the TNFα mRNA expression was significantly increased compared to that in the pregnant patients. Thus, dysregulation of M1/M2 MΦs may be one of the causes of implantation failure in humans.

In conclusion, we showed that the depletion of M2 MΦ led to implantation failure. Further studies are needed to clarify whether the mechanism of implantation failure is due to change in balance of M1/M2 MΦs, or decrease in number of M2 MΦs.

MATERIALS AND METHODS

Reagents and Materials

Roswell Park Memorial Institute (RPMI)-1640 medium and Diphtheria Toxin (DT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Life Technologies (Minato-ku Tokyo, Japan). Antibiotics (a mixture of penicillin, streptomycin, and amphotericin B) were purchased from Wako Pure Chemical Industries (Chuo-ku, Osaka, Japan).

Immunohistochemistry

Paraffin-embedded tissues were cut into 5-μm-thick sections and mounted on slides. The mouse uterine and ovarian sections were deparaffinized in xylene, rehydrated through a graded series of ethanol, and washed in water. Antigen retrieval was performed in 10 mM sodium citrate buffer (pH 6.0) in a microwave for 10 min and then cooling to room temperature. Rabbit IgG was used as a negative control. Slide staining with the first and second antibodies was performed according to the manufacturer's instructions. Immunostaining was performed using antibodies specific to Ki-67 (Abcam, Tokyo, Japan, Cat# 15580, 1:100 dilution), β-catenin (Abcam, Tokyo, Japan, Cat# 138378, 1:100 dilution), phosphorilated-STAT3 (Cell Signaling Technology, Massachusetts, USA, Cat #9145, 1:100 dilution), and FGF18 (Abcam, Cat# ab169615, 1:100 dilution). An immunofluorescence analysis of implantation site (uterus) was performed using rabbit anti-mannose receptor (CD206) (Abcam, Cat# 64693, 1:100 dilution). And the primary antibody was incubated overnight at 4°C. As a second antibody, the rat anti-rabbit antibody was used. 4′,6-diamidino-2-phenylindole (DAPI; 1:500) was used to detect nuclei. Rabbit IgG were used instead of the primary antibody for negative control.

Mice and Diphtheria Toxin Administration

Female, CD206 DTR mice (17, 18), aged 12 to 20-weeks old were used. The mice were housed in a specific pathogen free (SPF) animal facility with a controlled environment of 22–24°C and 60–70% relative humidity, on a 12 h light/12 h dark cycle with food and water provided *ad libitum*. DT was diluted with sterile phosphate buffered saline (PBS) to the desired concentration and was intra-peritoneally injected to mice to deplete the CD206 positive cells. According to BioGPS, a complete resource for learning about gene and protein function (http://biogps.org/), CD206 seems to be expressed in mouse uterus more than other M2MΦ markers. In our preliminary experiment, in each organ, CD206 mRNA seemed to be more expressed than other M2MΦ markers (data not shown). Decidual MΦs are reported to be show higher expression of CD206 (31, 32). Wang et al. (33) reported that the CD206 expression in MΦs could be a marker for spontaneous abortion. From these results, CD206 is considered to be a valid marker for uterine M2 MΦ. The experiments

and procedures were performed at 48 h after the final DT administration, as previously reported by Nawaz et al. (18). The final DT injection was administered at E 2.5 before implantation. The depletion of CD206 positive cells was confirmed at mRNA levels by qPCR every experiment.

Assessment of the Implantation Site (IS)

We prepared control group by mating C57/B6 female WT mice with Balb/c male mice, and M2(-) group by mating CD206-DTR female mice with Balb/c mice (**Figure 1B**). To deplete M2-like MΦs at the implantation period, DT was administered to each mouse at a dose of 30 ng/gram body weight before implantation. We then checked the implantation sites between two groups. To identify implantation sites on embryonic day 4.5 (E4.5), mice were anesthetized using Avertin (2% tribromoethanol, 15 μl/g i.p.; Sigma-Aldrich), administered the Chicago blue dye solution (0.4% in PBS i.v.; Sigma-Aldrich) and then analyzed after 10 min. Uteri were dissected and assessed for clearly delineated blue bands as evidence of early implantation sites. In other mice, uterine paraffin sections from control and M2(-) mice were collected on E3.5 and E4.5 and stained with H&E to assess the implantation sites.

Patients With Implantation Failure

Uterine endometrial biopsy as performed at the time of the implantation window in 38 patients with implantation failure who visited the outpatient department of obstetrics and gynecology at the University of Tokyo. After the endometrial biopsy, we compared the proportion of uterine M2-like MΦ to the pan-MΦ based on the mRNA levels of CD206 and CD68 between the pregnancy and non-pregnant group.

Reverse Transcription (RT) and Quantitative Real-Time Polymerase Chain Reaction (PCR) Analysis

Total RNA was extracted from the mouse endometrial region from the peritoneal cavity, using the ISOGEN-II (NIPPON GENE, Tokyo, Japan). RT was performed using Rever Tra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO, Tokyo, Japan). About 1.0 μg of total RNA was reverse-transcribed in a 20-μL volume. For the quantification of various mRNA levels, real-time PCR was performed using the Mx3000P Real-Time PCR System (Agilent Technologies, CA, USA) according to the manufacturer's instructions. The PCR primers used with the SYBR Green protocol were selected from different exons of the corresponding genes to discriminate the PCR products that might arise from possible chromosomal DNA contaminants. The SYBR Green thermal cycling conditions were as follows: 1 cycle of 95°C for 30 s, and cycles of 95°C for 10 s, 60°C for 10 s and 72°C for 10 s. The primer sequences used were as follows: 3-phosphate dehydrogenase (GAPDH, NM_002046: 628–648 and 1079–1060), mouse CD206 (NM_000710.3: 326–347 and 495–473), mouse TNFα (NM_000623.3: 432–453 and 605–584), mouse IL-10 (NM_010548.2: 390-412 and 464-443), mouse CD11c (NM_001363985.1: 82-103 and 194-175), mouse iNOS (NM_001313922.1: 2363-2382 and 2489-2470), β-catenin (NM_000623.3: 432–453 and 605–584), Fibroblast Growth Factor 18 (NM_000623.3: 432–453 and 605–584), Wnt 4 (NM_009523.2: 318-337 and 426-409), Wnt 5A (NM_009524.4: 565-583 and 668-650), Wnt 6 (XM_006495889.2: 670-688 and 796-779), Wnt 7A (NM_001363757.1: 501-518 and 578-558), and Wnt 7B (NM_009528.3: 421-440 and 492-472). The relative mRNA levels were calculated using the standard curve method and were normalized to the mRNA levels of GAPDH (forward, 5′-AATGTGTCCGTCGTGGATCTGA-3′ and reverse, 5′-GATGCCTGCTTCACCACCTTCT-3′) (**Supplementary Data 1**).

Measurement of Estradiol (E2) and Progesterone (P4) Levels

Mouse blood samples were collected during the analysis. Plasma levels of E2 and P4 were measured in duplicate using the specific EIA kits (Cayman, USA).

Statistical Analysis

Data were evaluated by Mann Whitney test using Jump version 10. $P < 0.05$ was accepted as statistically significant.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the committee of the University of Tokyo (10991). The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the committee of University of Toyama (A 2015med-55).

AUTHOR CONTRIBUTIONS

SS, OY, KT, YH, and YOn: conception and design. YOn, ANak and AU: acquiring and processing samples. YOn, ANaw, YF, AU, OY, and ES: execution of the experiment. YOn and OY: analysis of data. SS, OY, ANaw, TH, ANak, and YOs: interpretation of data. YOn and OY: drafting the manuscript. SS, OY, YOn, and SW: revision of the manuscript for important intellectual content. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.557184/full#supplementary-material

Supplementary Data 1 | Primer sequences of mouse and human.

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Innate Lymphoid Cells in Human Pregnancy

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Innate lymphoid cells (ILCs) are a new set of cells considered to be a part of the innate immune system. ILCs are classified into five subsets (according to their transcription factors and cytokine profile) as natural killer cells (NK cells), group 1 ILCs, group 2 ILCs, group 3 ILCs, and lymphoid tissue inducers (LTi). Functionally, these cells resemble the T helper population but lack the expression of recombinant genes, which is essential for the formation of T cell receptors. In this work, the authors address the distinction between peripheral and decidual NK cells, highlighting their diversity in ILC biology and its relevance to human pregnancy. ILCs are effector cells that are important in promoting immunity, inflammation, and tissue repair. Recent studies have directed their attention to ILC actions in pregnancy. Dysregulation or expansion of pro-inflammatory ILC populations as well as abnormal tolerogenic responses may directly interfere with pregnancy, ultimately resulting in pregnancy loss or adverse outcomes. In this review, we characterize these cells, considering recent findings and addressing knowledge gaps in perinatal medicine in the context of ILC biology. Moreover, we discuss the relevance of these cells not only to the process of immune tolerance, but also in disease.

Keywords: innate lymphoid cells, innate immune response, inflammation, pregnancy, preterm birth

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INTRODUCTION

From the immunology point of view, we may consider the fetus as a semi-allograft concept initially put forward in 1953 by Sir Peter Medawar (1). Accordingly, taken from the knowledge attained in transplantation science, the trophoblast carrying paternal antigens must invade the maternal uterine mucosa, in a process called implantation, while escaping immune defense mechanisms against the alloantigen. This is the first paradox in the biology of pregnancy (2).

Indeed, there are three major phases in pregnancy, involving the immunologic response: implantation of the trophoblast, widely regarded as an inflammatory process; followed by a protective anti-inflammatory milieu, needed throughout the whole development of the fetus; and finally, labor itself, which is also regarded as an inflammatory event (3–9). During pregnancy, differences in inflammatory states relate to different cytokine profiles. Evidence in the literature

supports a shift from a T helper 1 (Th1) cytokine profile to a Th2 profile favoring a humoral response (10). However, other cell types contribute to the immune regulation of pregnancy, such as T regulatory cells, IL-17-producing cells, and tissue resident cells (11, 12).

The majority of scientific investigation has focused on the T cell repertoire and on the balance between Th1, Th2, and Th17 cytokines (13–16); nonetheless, it is evident that the innate component of the immune system has a preponderant role in pregnancy (17, 18). Moreover, the classic Th1/Th2 paradigm fails to explain the immunomodulatory actions of locally secreted cytokines.

In this review, we focus on innate immune responses during pregnancy, considering a recently categorized set of cells called innate lymphoid cells (ILCs).

INNATE LYMPHOID CELLS

ILCs are a group of cells that share a common lymphoid progenitor. ILCs are characterized by the absence of recombination-activating gene (RAG)-dependent rearranged antigen receptors and the lack of myeloid and dendritic cell phenotypical markers, hence denominated as lineage negative (Lin^-). ILC1s, ILC2s, and ILC3s are dependent on transcription factors T-bet, GATA-3, and RORγt, respectively. Moreover, natural killer (NK) cells are dependent on the transcription factors eomesodermin (Eomes) and T-bet for their development. These cells share the expression of a common γ chain, IL-7Rα (CD127), except for tonsil and intraepithelial ILC1 (19). In addition, ILC2 is characterized by the expression IL-2Rα (CD25), a receptor that is also present in $CD56^{bright}$ NK cells but has a lower expression in ILC1 and ILC3 (19, 20). They are functionally diverse and belong to the innate component of the immune system (20). A summarized diagram of ILCs is presented in **Figure 1**.

ILCs were initially classified as NK cells in 1975 (21); afterward, in 1997, another cell type was added, named lymphoid tissue inducer (LTi) (22). Although NK cells represent cytotoxic ILCs capable of killing virus-infected or tumor cells and releasing pro-inflammatory cytokines (23), LTi are critical for the development of secondary lymphoid organs during embryogenesis (24). However, in the context of pregnancy, there are significant differences regarding NK function, which we address further on in this review.

ILCs play an essential role in tissue homeostasis, defense against infection and inflammation, and tissue repair (25). ILCs are mainly tissue-resident cells found in the mucosal surfaces (26) as well as in the decidua of pregnant women (27).

ILCs were classified based on their relative cytokine profiles, centered on effector phenotypes that mirror T helper cells. Over the years, the classification of ILCs has been the subject of great debate, mainly due to their heterogeneity. However, the nomenclature approved by the International Union of Immunological Societies (IUIS) considers five distinct groups: NK cells known to produce IFN-γ; group 1 (ILC1), also known to produce IFN-γ, a Th1-like cytokine; group 2 (ILC2), which are characterized by the expression of transcription factor Gata3 and the ability to produce Th2-like cytokines; group 3 (ILC3), known to produce IL-22 and IL-17; and LTis, important in secondary lymphoid organ formation (19, 25).

Moreover, it has become evident that ILCs have great plasticity. Their effector characteristics are highly dependent on their microenvironment, mainly on the cytokines secreted by tissue-resident cells, and other cells from the innate immune system (28). Due to the ability of some ILCs to produce pro-inflammatory cytokines and to the fact that ILCs express MHCII molecules, their importance in the regulation of labor is rational.

Immune tolerance and controlled inflammation are key processes in a successful pregnancy. Dysregulated inflammatory reactions often lead to complications, such as spontaneous abortion, preterm labor (PTL), preeclampsia, and intrauterine growth restriction (10, 29).

NK Cells and ILC1

The importance of NK cells in pregnancy is paramount, not only because these cells belong to the innate immune system, but also because NK cells play an important role in placentation, remodeling of the spinal arteries, and control of trophoblast invasion (30–33). Decidual NK (dNK) cells differ substantially from peripheral NK cells: peripheral NK cells are predominantly $CD56^{dim}$ $CD16^+$, and dNK cells are $CD56^{bright}$ $CD16^-$ (23, 34). This phenotype is accompanied by functional differences because $CD56^{dim}$ $CD16^+$ cells have a strong cytolytic activity, and dNK cells are predominately cytokine-producing cells.

One key feature of dNK cells is their inability to lyse trophoblastic cells despite the expression of activating receptors (NKp46, NKp30, NKG2D, and DNAM-1) as well as their perforin and granzyme content. Instead, they produce IL-8, stromal cell–derived factor 1 (SDF-1), vascular endothelial growth factor (VEGF), and interferon gamma-induced protein 10 (IP-10), all with important roles in tissue remodeling (30, 35–37). Even though NK cells were discovered many years ago, it is only more recently that these cells were included in the ILC group. Recent work by Vento-Tormo et al. proposed three main dNK subsets: dNK1, dNK2, and dNK3 cells. This classification has been further confirmed by Huhn et al. (38, 39). Also, previous work by Yudanin et al., conducted in tissues other than uterine origin, highlights the overlapping characteristics of NK cells with ILC1, a fact also reported by Huhn et al., and this raises the question, are dNK3 subsets in fact ILC1 (40)? The nature and consequent nomenclature of the different dNK subsets and ILC1 are still a matter of great dispute.

Classically, uterine ILC1 are characterized by the expression of T-bet and Eomes and produce IFN-γ. ILC1 do not express perforin and have the inability to produce Th2- and Th17-type cytokines (27, 41). ILC1 have diverse regulatory actions dependent on the cell type and on the stimuli received (42–44).

ILC1 can be further characterized by their surface markers CD56-, CD94-, CD127+, CD117-, and they have been identified in low numbers in human decidua (27), suggesting a minor role in pregnancy (45).

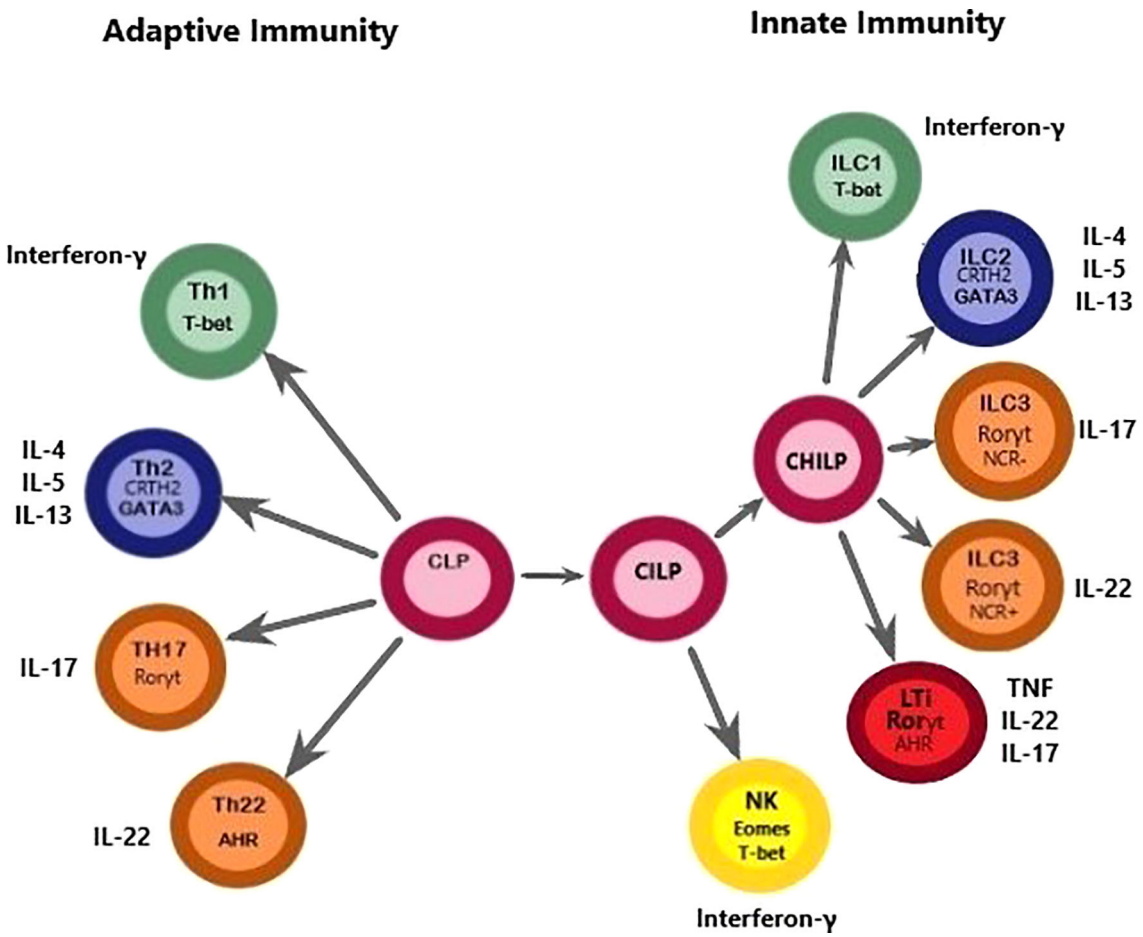

FIGURE 1 | This figure depicts a proposed model for the differentiation paths of ILCs, highlighting the similarities with Th cells regarding common transcription factors. A common lymphoid progenitor (CLP), originated from a hematopoietic stem cell (HSC) can give rise to adaptive and innate lymphocytes. However, it should be noted that this figure was simplified to convey the message that Th cells and ILCs are of lymphoid origin because Th cell populations do not differentiate directly from CLPs. Downstream of the CLP, a common ILC precursor (CILP) then divides into 1) a branch that differentiates into NK cells and 2) a branch that generates a common helper-ILC precursor (CHILP). The CHILP further differentiates toward a different branch of the ILC family, namely ILC1, ILC2, and ILC3, and generates an LTi population. This figure highlights similarities between ILCs and Th cells. The classification of ILCs is based on functional criteria. ILCs functionally resemble adaptive lymphocytes with the distinction that ILCs lack antigen-specific receptors. Instead, ILCs are known to exert their effects through the production of cytokines and cell surface molecules with important consequences for tissue homeostasis, inflammation, and disease. Dysregulation or expansion of pro-inflammatory ILC populations may directly promote disease through production of pro-inflammatory cytokines, which seems to be important in the pathogenesis of PTL. T-bet, T-box transcription factor 21; Eomes, eomesodermin; CRTH2, Chemoattractant receptor-homologous molecule expressed on TH2 cells; GATA3, GATA binding protein 3; AHR, aryl hydrocarbon receptor; ROR, Retinoic acid–related orphan receptor; IFNγ, interferon-γ; IL, interleukin; LTi, lymphoid tissue inducer; NCR, natural cytotoxicity receptor; NK, natural killer; PTL, Preterm labor.

Group 2 ILCs

Group 2 ILCs are phenotypically characterized by the surface markers $CD56^-$, $CD127^+$, and $CD161^+$ and chemoattractant receptor homologous molecules expressed on Th2 cells (CRTH2) (27, 43). ILC2 are dependent on GATA binding protein 3 and transcription factor retinoid-related orphan receptor alpha (RORα) for their development (20, 46). ILC2 produce type 2 cytokines (IL-4, IL-5, and IL-13) under the control of IL-25 and IL-33, which is important in extracellular parasitic infections and allergic responses (19). The expression of CRTH2 is of great interest for labor because it is a G protein–coupled receptor for prostaglandin D2, which promotes ILC2 differentiation and type 2 pro-inflammatory responses (47). Another important feature of the ILC2 population, found in a study conducted in a mouse model, is the expression of major histocompatibility complex class II (MHCII) as well as the co-stimulatory molecules CD80 and CD86 (48). In this study conducted by Oliphant et al., it is shown that ILC2 can not only perform endocytosis, but also process and present antigens (48). These characteristics allow ILC2 to present antigens to T $CD4^+$ cells and induce proliferation toward a Th2 phenotype in an IL-2-dependent manner (48). These data, albeit conducted in mouse models, reinforce the notion of cross-talk between the innate and the adaptive immune systems (49–51).

Group 3 ILCs

Group 3 ILCs are characterized by the expression of the surface marker CD117 and the transcription factor RORγt. In a mouse model, it was proven that ILC3 express MHC class II, and they are also shown to promote T cell–mediated responses (52). Two different studies suggest that ILC3 might promote neutrophil activation with pro-angiogenic abilities, contributing to the inflammatory phase needed for implantation (53, 54). ILC3 can be further divided based on the presence of the natural cytotoxic receptor (NCR) NKp44. ILC3 NCR$^+$ produce IL-22, and ILC3 NCR$^-$ produce IL-17 (55, 56); both subsets have been found in human decidua (27). NCR is also present in activated peripheral NK cells and dNK. In NK cells, NCRs mediate cytotoxic (57) and antitumor responses (58); however, when present in uterine NK cells, NCR receptors have an important role in placentation through the production of IL-8, VEGF, IP-10, and SDF-1 (36).

The ability of ILC3 to act as pro-inflammatory agents (through the secretion of IL-17) suggests a preponderant role in pregnancy, which both favors embryo implantation and has an antimicrobial effect. In fact, decidual ILC3 seem to be important to pregnancy maintenance through innate defenses and tissue remodeling (27). Nevertheless, the inappropriate release of pro-inflammatory cytokines during the quiescent phase of pregnancy may prompt complications, mainly the precocious activation of the normal mechanism of labor.

ILCS IN UTERINE AND FETAL COMPARTMENTS

Male et al. first made the distinction between uterine NK cells and ILC subsets in humans. In this work, ILCs were first considered precursors of uterine NK cells; however, these cells showed differences in function and phenotype through the expression of RAR related orphan receptor C (RORC), Lymphotoxin α, and IL2 genes (59), which were later attributed to ILC3 and LTi subsets. Subsequent studies identified ILC1 (60), ILC2 (61), and ILC3 (60, 61) in human endometrium and decidua based on evidence that ILCs share a common lymphoid progenitor.

ILC1 can be found in the endometrium and decidua of pregnant women as early as 9–12 weeks of gestation (27), representing an important source of IFN-γ (60) and implying a relevant role in the immune response against intracellular pathogens. In addition, the expression of CD103, an adhesion molecule that promotes the communication between lymphocytes and epithelial cells, suggests an epithelial localization of ILC1 in the endometrium and decidua (27).

Xu et al. show that, in term pregnancies, ILC2 is the most abundant population in the human decidua, and it is capable of producing Th2-type cytokines, such as IL-4, IL-5, and IL-13. In this study, the authors suggest that the pro-inflammatory qualities of ILC2 might underlie the pathological process prompting PTL (45). Specifically, Xu et al. argue that ILC populations dynamically change throughout pregnancy. In fact, they also detected ILC3 in the decidua *parietalis* that are capable of producing IL-17 and IL-22, suggesting that these cells may be responsible for inflammation-driven PTL.

ILC3 were also initially described as a subset of NK cells in the human endometrium, expressing CD127, CD161, RORC, and IL-22 (59). Later, work by Vacca et al. confirmed the ILC3 phenotype and their presence in the human endometrium and decidua during pregnancy and further divided them into two subgroups: ILC3 NKp44$^+$ and ILC3 NKp44$^-$ (27, 60). It is shown that, similarly to Th cells, ILCs display some degree of plasticity in response to their microenvironment. Studies conducted in mouse models show that, in response to IL-12 and IL-18, ILC3 reveal an increased expression of T-bet and decreased expression of RORγt, which results in IFN-γ production and loss of their capacity to produce IL-17 and IL-22 (62, 63). These data may explain, in part, the low numbers of ILC1 found by Xu et al. in late gestation due to overlapping functions with ILC3 phenotypes.

Amniotic fluid surrounds the embryo and fetus, protecting it mechanically during development in the event of the maternal abdomen being subject to trauma. Amniotic fluid also protects the fetus from infectious agents due to its inherent antibacterial properties (64). Amniotic fluid provides the fetus with a reservoir of fluid, nutrients, and growth factors that allow normal development and growth of fetal organs (64). The main population identified in this compartment is ILC3 of fetal origin, expressing CD127, CD117, CD161, and CD56 (64). Indeed, ILC3 are abundant in the amniotic fluid until the second trimester (64), when their numbers start to decay as gestation progresses (65). In this context, the ability of ILC3 to produce IL17 suggests a role in regulating intra-amniotic infection (64).

Fetal ILCs have been identified in the liver, secondary lymphoid organs (SLOs), intestine, lungs, and cord blood (66, 67). In the liver, ILCs assume a preponderant role because it is in this organ that hematopoiesis takes place (68, 69) and where ILC precursors (ILCPs) originate (66). In their work, Lim et al. suggest that circulating ILCP can migrate to different tissues, where they differentiate according to fetal development needs and organogenesis (66). Moreover, studies from animal models suggest that the presence of LTi cells in the fetus is essential for the successful formation of SLOs, such as the spleen, mesenteric lymph nodes, and Peyer's patches (70–73).

Previous work has demonstrated that NK, ILC1, ILC2, and ILC3 subsets can be readily identified in the human fetal intestine (55, 64, 74, 75). It is shown that intestinal ILC2 produce IL-13 (74), and ILC3 and LTi-like cells produce IL-17A and IL-22 (55).

Mjösberg et al. report the presence of ILC2 in the fetal lung (74), and Marquardt et al. have detected increased numbers of ILC3 in the second trimester when compared to the first trimester (64).

Most of the information available regarding ILCs comes from animal models. However considering the great degree of similarity between mouse and human ILC ontology (19), we attempted a reasonable extrapolation to human biology.

The ubiquity of ILCs present in the uterine and fetal compartment denotes the importance of the innate immune

system in pregnancy. Not only do ILCs take part in organ formation, but they also act as key mediators in protecting the fetus against infection and pathogens. The main findings that are the object of this review are summarized in **Table 1**.

INNATE LYMPHOID CELLS AND THE INDUCTION OF TOLERANCE

In order to escape the maternal immune system, trophoblast cells only express human leukocyte antigen (HLA) HLA-C, the nonclassical HLA-E, HLA-F, and HLA-G molecules (77–80).

In pregnancy, one key mechanism regulating induction of tolerance is through the actions of HLA-G molecules. The HLA-G gene is located at chromosome 6 within the class I gene cluster of MHC. HLA-G belongs to the nonclassical HLA-class I (or class Ib) genes; it is expressed mainly in the fetal–maternal interface on the extravillous cytotrophoblast (81), amnion (82), and thymus (83), and its soluble form is detectable in peripheral blood (84).

HLA-G exerts its effects by modulating antigen-presenting cells (85), suppressing proliferation of $CD4^+$ T lymphocytes (86, 87), and inhibiting NK cells' actions. In fact, HLA-G inhibits NK cells' (84) cytolytic actions, upregulates NK inhibitory receptors (88), and is essential for implantation (89).

Also, in this perspective, there is evidence that progesterone, a key immunomodulatory steroid hormone, contributes to a pregnancy protective milieu by promoting HLA-G expression (90) and regulating NK activity (91) (92).

Tolerance is widely regarded as an adaptive response. Accordingly, it is a process that involves antigen presentation, clonal expansion, and the formation of memory cells; the expression of HLA class II molecules in ILC2 and ILC3 populations suggests that these cells might also have a role in pregnancy by presenting paternal antigens to the mother's immune system. Although ILC2 seem capable of eliciting Th proliferation, Hepworth et al. reported, in animal models, that ILC3 lack classical costimulatory molecules, such as CD40, CD80, and CD86. If this is the case, ILC3 antigen presentation may, in fact,

TABLE 1 | Main findings in the literature regarding human ILCs in uterine and fetal compartments.

Resident ILC population	Species	Tissue	Gestation	Main Findings	Reference
ILC1/ILC3	Human	Decidua	1st Trimester	Decidual ILC3 have a frequency comparable, if not higher, with that of tonsil ILC3.Results from this study indicate that NCR^+ ILC3 and LTi-like cells present in decidua can produce pro-inflammatory cytokines including IL-8, IL-22, IL-17A, TNF, and IFN-γ.	Vacca et al (27).
ILC3	Human	PBMCs	3rd Trimester	Increased IL-17 levels observed in patients with preeclampsia, gestational diabetes, and chronic diabetes are associated with ILC3.	Barnie et al (76).
ILC1/ILC2/ ILC3	Mouse/ Human	Endometrium/ Decidua	1stTrimester	$CD127^+$ ILC1 are absent in human endometrium or decidua. ILC2 are found deep in the uterine wall and not in human or murine decidua, nor in human endometrium. NCR^+ ILC3 and LTi-like ILC3 are present in both human endometrium and decidua.	Doisne et al (61).
ILC3	Human	Decidua	1st Trimester	NCR^+ ILC3 are present in decidual tissue, where they produce CXCL8 and GM -CSF, suggesting that they may have a role in neutrophil recruitment and survival.NCR^+ ILC3-derived GM-CSF induces the expression of both heparin-binding EGF-like growth factor and IL1ra in neutrophils, important in angiogenesis and trophoblast growth/invasion.	Croxatto et al. (54)
ILC3	Human	Amniotic fluid (AF)/1st and 2nd trimester fetal tissue	1st Trimester	CD45+ cells in AF contained very low frequencies of T cells, B cells, and monocytes.Fetal CD103+ ILC3s in AF are functional and produce high levels of IL-17 and TNF. A similar subset was identified in second trimester fetal gut and lung, suggesting that CD103+ ILC3s develop in fetal tissues and subsequently egress to the AF.	Marquardt et al (64).
ILC1/ILC2/ ILC3	Human	Decidua	Term and Preterm Pregnancies	The proportion of total ILCs was increased in the decidua parietalis of women with preterm labor.ILC1s were a minor subset of decidual ILCs during preterm and term gestations; ILC2s were the most abundant ILC subset in the decidua during preterm and term gestations. The proportion of ILC2s was increased in the decidua basalis of women with preterm labor. The proportion of ILC3s was increased in the decidua parietalis of women with preterm labor; during preterm labor, ILC3s had higher expression of IL-22, IL-17A, IL-13, and IFN-γ compared to ILC2s in the decidua.	Xu et al (47).
ILC2	Human	lung and gut	–	In fetal gut, ILC2 expressed IL-13 but not IL-17 or IL-22.	Mjösberg et al (74).
ILC3	Human	Amniotic fluidIntestineLung	15 to 16Weeks	ILC3 are the main ILC population in the amniotic fluid, producing high levels of IL-17 and TNF. ILC3 are abundant in fetal intestine and lung.	Marquard et al (64).
ILC1/ILC2/ ILC3	Human	Umbilical cord blood, Fetal liver	14 to 20 weeks	Human ILCPs robustly generate all ILC subsets *in vitro* and *in vivo*. This study identified unipotent ILCPs that could give rise to IFN-g^+ ILC1s, IL-13^+ ILC2s, or IL-$17A^+$ and/or IL-22^+ ILC3s.	Lim et al (66).
ILC1/ILC2/ ILC3	Human	Liver	6 to 10weeks	In this study, the authors identified that fetal liver harbored almost exclusively NKp44– ILC3s, with ILC1s, ILC2s, and NKp44+ ILC3s being detectable only at later gestational age. Also, NKp44– ILC3s in the fetal liver were different from the corresponding population in the adult since fetal ILC3s expressed NRP1.	Forkel M. et al (67).
ILC1/ILC2/ ILC3	Human	Gut	16 to 22 weeks	The study applied mass cytometry to analyze ILCs in the human fetal intestine, distinguished 34 distinct clusters and identified a previously unknown intermediate innate subset that can differentiate into ILC3 and NK cells.	Li N. et al (75).

limit T cell responses by negatively regulating CD4+ T cell responses *in vivo* through T cell anergy (49, 93).

Whether ILCs are on the forefront in establishing tolerance toward the fetus is a matter that requires further research.

INNATE LYMPHOID CELLS IN DISEASE

Studies in NK cell biology corroborate the involvement of the innate immune system in preterm birth (PTB), preeclampsia, fetal growth restriction, and morbidly adherent placentation as well as spontaneous abortion (94–99). Dysregulation or expansion of pro-inflammatory ILC populations may directly promote disease through production of pro-inflammatory cytokines, namely IL-17, which are considered important in the pathogenesis of preeclampsia and PTB (76). Moreover, high levels of IL-18 and IFN-γ have been associated with preeclampsia (100), and in PTB, there is evidence for an inadequate inflammatory response (101).

Progesterone has been known to play an important role in reproductive health for the initiation and maintenance of pregnancy with good results in the prevention of spontaneous abortion and recently in PTL (102–104).

The immunosuppressive effects of progesterone have been recognized for a long time. Despite its mode of action remaining largely unknown, progesterone has been widely adopted by clinicians around the world for prevention of PTB. Our group has already demonstrated that progesterone modulates the human T regulatory cell population during pregnancy (13, 102, 105, 106). There is also evidence, conducted in a small sample of T cell clones, suggesting that progesterone favors Th2 while dampening Th1 and Th17 responses and, thus, participates in the establishment of a favorable environment for pregnancy by its effects on T cells (107). Work from Henderson et al. shows that NK cells do not express progesterone receptors (108); however, the expression of CRTH2 in ILC2 suggests that ILCs are subject to hormonal regulation. Also, work done by Gibson et al. (109) shows that uNK cells are regulated by membrane estradiol receptors (E46), highlighting the relevance of hormone regulation in NK activity during pregnancy.

CONCLUSION

Human ILCs are mainly tissue resident with relevant roles in mediating infection, inflammation, and tissue repair. Namely, ILC1 are known to promote immunity to intracellular pathogens and are associated with inflammatory bowel disease (110). ILC2 support antiparasite immunity and play an important role in airway inflammation (111). ILC3 are essential in immunity against extracellular pathogens and skin inflammation (112) and mediating graft-versus-host (113). In the past 10 years, we have witnessed a growing interest in ILC biology and their role in pregnancy. Future work, focusing on endocrine and environmental factors influencing ILC phenotype, will contribute to answer unsolved questions in clinical practice.

AUTHOR CONTRIBUTIONS

JM: Scientific analysis, manuscript writing and editing. Areia: Writing and supervision of scientific content PR-S: Manuscript writing and supervising all the scientific analysis. MS-R: Writing and supervision of scientific content. AM-P: Coordination of research group, supervision of scientific content. All authors contributed to the article and approved the submitted version.

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Activation of the Complement System in the Lower Genital Tract During Pregnancy and Delivery

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Background: Human pregnancy alters profoundly the immune system. The local involvement and mechanisms of activation of the complement system in the cervicovaginal milieu during pregnancy and delivery remain unexplored.

Objectives: To determine whether normal pregnancy and delivery are associated with local activation of complement or changes in the immunoglobulin profile in the cervix.

Study Design: This study was designed to assess IgA, IgG, and complement activation in the cervicovaginal area in three groups of patients: i) 49 pregnant women (week 41+3–42+0) not in active labor, ii) 24 women in active labor (38+4–42+2), and iii) a control group of nonpregnant women (n=23) at child-bearing age. We collected mucosal samples from the lateral fornix of the vagina and external cervix during routine visits and delivery. The Western blot technique was used to detect complement C3 and its activation products. For semiquantitative analysis, the bands of the electrophoresed proteins in gels were digitized on a flatbed photo scanner and analyzed. IgA and IgG were analyzed by Western blotting and quantified by ELISA. One-way ANOVA and Tukey's Multiple Comparison tests were used for statistical comparisons.

Results: A higher abundance but lower activation level of C3 in both the external cervix ($P<0.001$) and lateral fornix of the vagina ($P<0.001$) was observed during delivery (58 ± 22, n= 24) in comparison to the groups of nonpregnant (72 ± 13%; mean ± SD, n=23) and pregnant women (78 ± 22%, n=49). Complement activating IgG was detected in higher abundance than IgA in the cervicovaginal secretions of pregnant women. In a small proportion samples also C3-IgG complexes were detected.

Conclusions: Our results reveal an unexpectedly strong activation of the complement system and the presence IgG immunoglobulins in the cervicovaginal area during pregnancy, active labor, and among nonpregnant women. In contrast to the higher amounts of C3 in the cervicovaginal secretions during labor, its activation level was lower. Complement activating IgG was detected in higher concentrations than IgA in the mucosal secretions during pregnancy and labor. Taken together our results imply the presence a locally operating humoral immune system in the cervicovaginal mucosa.

Keywords: uterine cervix, vaginal mucosa, IgG, IgA, C3, parturition, inflammation, delivery

INTRODUCTION

A unique phenomenon of successful coexistence of the maternal immune system and the semi-allograft fetoplacental unit is seen in pregnant women (1–3). Growing knowledge about the immunological microenvironment within the female genital tract has increased the interest in understanding the immunological processes and their role in parturition (4–6). Reports describing chemotactic recruitment and activation of inflammatory neutrophils and macrophages into the uterus, decidua, fetal membranes and cervix during labor point towards local rather than systemic inflammatory events (7–10). The innate immune system has been linked to these processes and to the generation of a sterile pro-inflammatory state that will pave the way to labor and delivery of the baby (11, 12).

The complement (C) system is part of innate immunity (13, 14). The proteolytic activation cascades of C comprise about 50 proteins (15). Complement can become activated through three distinct pathways: the classical, lectin and alternative pathways (16). These pathways each converge at the central step of the C system, i.e. the cleavage of C3 by C3 convertases (16). Complement functions in antimicrobial defense and as an opsonophagocytic clean-up system of the body together with phagocytes. Activation products generated include e.g. the anaphylatoxins C3a and C5a that can induce major physiological changes, like contracting smooth muscle and increasing vascular permeability (17). Pregnancy and parturition are associated with increased levels of C components and with C activation in the blood (18–26). The presence of C components in tissues is a result of their diffusion from blood plasma and local production by different cell types including macrophages, fibroblasts, and endothelial cells. Information about the local role of C in the cervicovaginal area in humans is scarce.

Under normal circumstances C activation is well regulated and only minimal deposition of its activated components, including C1q, C4b, C3b/iC3b, or the membrane attack complex (MAC), occurs in the mucosa. However, in a variety of adverse pregnancy outcomes dysregulation of C has been demonstrated. These include hypertensive diseases of pregnancy (27, 28), antiphospholipid antibody syndrome–associated fetal loss (29), recurrent miscarriage and preterm birth (30–33). To our knowledge, local activation of the C system in the cervicovaginal mucosa in humans and its relation to timing of parturition have not yet been examined.

We hypothesized that, because of e.g. a strong microbial exposure in the lower genital tract, the C system is constantly active, but should be tightly regulated at the time of parturition in order to protect the mother and fetus from an immune attack. Our present study aimed at investigating whether and to what extent C is activated in the cervicovaginal area, and whether local C3 activation is related to the parturition process.

MATERIALS AND METHODS

Study Subjects and Samples

To address the role of complement in parturition, a cohort of samples was collected to determine C activation in the cervicovaginal area. We recruited three groups of study subjects: i) pregnant women (n=49) with pregnancy duration of 41+3 to 42 +0 weeks in whom labor had not yet become initiated, ii) women in active labor (n=24) (38+4 - 42+2 weeks), and iii) non-pregnant women (n=23) in child-bearing age, who had arrived for routine out-patient visits in the clinic. All pregnancies were singleton pregnancies with intact fetal membranes at the time of sampling. None of the women had been previously treated for cervical precancerous lesion. All women were generally healthy with no chronic disease or diagnosed immune deficiency, except for one with IgA deficiency in the pregnant women group. None of the study subjects had used any type of corticosteroids for at least 6 months before sampling. None of the women recruited had had unprotected sexual intercourse for at least 48 h before sampling. All pregnant individuals were screened for gestational diabetes in mid-pregnancy. Altogether 17% tested positive. They were treated conservatively and monitored at the maternity clinic with no need for antidiabetic drugs. Demographic and clinical characteristics of the pregnant study subjects are presented in **Table 1**.

All subjects (n=96) were in child-bearing age (17–40 years old). The median BMI value before pregnancy was 23 ± 2.4 (median ± range, n=73). In the pregnancy, delivery and control groups, systemic or local vaginal usage of antibiotics less than 6 months from sampling was recorded in 22%, 21%, and 20% of the women, respectively (**Table 1**). In the pregnancy group 53% (26/49) and the labor group 33% (8/24) of the women were primiparas ($p<0.05$).

All samples were collected at the Helsinki University Hospital between October 2015 and March 2017. The study was approved by the Helsinki University Hospital's Ethical Committee (91/13/03/03/

TABLE 1 | Patient characteristics.

	Non pregnant	Pregnant patients	Patients in labor
	n = 23	n = 49	n = 24
Age (mean, SD)	30 ± 5	32.3 ± 4.3	30.2 ± 5
First delivery (%)		53	33
Median prepregnancy BMI		23	22
Smoking (%)	30	4.1	4.2
Antibiotic consumption in the past 6 months (%)		22	21
Pregnancy length in days (median)		294	282
Range of pregnancy length (days)		289-296	270-92
Average baby weight (g)		3700	3574
Positive for vaginal group B streptococcus (%)		33	35
Artificially induced delivery (%)		61	0
Gestational diabetes (%)		15	18

2015). All participating women provided written informed consents and were requested to fill up an exploratory questionnaire. Serial samples were collected by two experienced physicians from the lateral fornix of the vagina (LF) and external cervix (EC) using the Rovers Viba-Brush tool (Rovers Medical Devices, Oss, The Netherlands). Ten subjects provided two additional swabs from the EC and LF directly into 10 mM EDTA to control for possible *ex vivo* C activation. The additional group of 23 non-pregnant subjects were sampled from the LF. These samples were taken at a colposcopy clinic. Many of the patients came because they may have cervical changes. Thus, samples from the external cervix would not have been representative. All samples were inserted immediately into Eppendorf tubes containing 20 µl phosphate-buffered saline, pH 7.4 (PBS) and were frozen into -80°C within 30 min of sampling. No blood-contaminated samples were included in the study.

Analysis of IgG and IgA

IgG and IgA in cervicovaginal samples were analyzed by immunoblotting and quantified by an ELISA assay. Twenty µl portions of appropriately diluted samples were loaded onto 4%–12% SDS-PAGE gels under reducing conditions. After transferring the proteins to a nitrocellulose filter, nonspecific binding sites were blocked by incubation with 5% milk in phosphate-buffered saline (PBS) containing 0.05% Tween 20 detergent. The membranes were then incubated for 1 h at RT with HRP-conjugated rabbit-anti-human IgG or IgA antibody (Dako; final dilutions 1:10,000 and 1:5,000, respectively, in milk/PBS/Tween). Protein bands were visualized by an in-house protocol for electrochemiluminescence. After washing with PBS+0.05% Tween 20 an enhanced chemiluminescence solution (WesternBright ECL, Advansta, San Jose, CA) that includes hydrogen peroxide was added and films developed at different exposure times.

To quantify levels of IgG and IgA in the cervicovaginal mucosal samples standardized ELISA assays (Bethyl Laboratories, Inc. USA, Catalog no. E101-104 and Catalog no. E100-102, respectively) were used. Human sera with known IgG and IgA concentrations were used as controls. In this assay the intra- and inter-assay coefficients of variations were less than 5%. Mucosal samples and sera were diluted 1:1000, 1:3000, and 1:10000 for IgG and 1:10, 1:30, 1:100, and 1:300 for IgA and analyzed in duplicate. After incubating the samples for 2 h on the plate wells they were washed with a buffer (TBS) containing 0.05% Tween 20 using an automated plate washer (EL*x*50 Washer, BioTek). Thereafter, HRP-anti human IgG and HRP-anti-human IgA (both from Dako), diluted 1:5000 and 1:2000 respectively in PBS were added and incubated for another 2 h at 22°C. After washing with TBS, 0.05% Tween 20, substrate (OPD) was added. The reaction was stopped with 0.5M H_2SO_4 and a microplate spectrophotometer (SpectraMax, Bio-strategy) was used to measure the optical density of samples at a wave-length of 492 mm.

Analysis of C3 Cleavage in Vaginal Lateral Fornix and External Cervix Samples

Western blot analyses were performed using in-house protocols (34). The samples were thawn on ice, centrifuged 12,000 × *g* for 3 min and diluted 1:10 in sterile PBS. 20 µl portions of a dilution series of samples (in PBS) in SDS and 5% mercaptoethanol containing sample buffer were loaded onto 4%–12% SDS-PAGE gels and run under reducing conditions. A normal human serum (NHS) pool was obtained from healthy laboratory personnel after a written informed consent and used as a reference. The proteins from the gel were electrotransferred to a nitrocellulose filter. To prevent nonspecific binding the nitrocellulose membranes were incubated in 5% milk in PBS/Tween 0.05% for 1 h. The membranes were then incubated with rabbit anti-human C3c antibody (Dako; final dilution: 1:10,000 in milk/PBS/Tween) overnight at +4°C. For additional detection of C3-IgG complexes, also rabbit antibodies against C3d (Dako) were used similarly as anti-C3c antibodies. The membranes were washed with PBS/Tween and incubated for 1 h at RT with HRP-goat-anti-rabbit IgG antibody (Jackson ImmunoResearch; 1:10,000 in milk/PBS/Tween). Finally, the membranes were washed, and protein bands were visualized by electrochemiluminescence.

Quantification of C3 Activation

For quantitative determination of the C3 bands the films were digitized on a flatbed photo scanner and quantified using ImageJ/Fiji win-64 software. Activation of C3 results in several split

products (**Figure 2**), including C3b, iC3b, C3c, C3dg, and C3d. The level of C3 activation was determined by assessment of the relative level of intensity of the C3α-chain split products (α'-chain fragments in the split products) from total C3 reactivity. The respective α'-chains of these split products can be visualized by Western blotting and may be quantified in relation to the amount of total C3 α/α'-chain reactivity (native plus activated C3) found in the same specimen. The β chain is not cleaved, thus its amount remains the same regardless of the level of activation. Calculation of the total percentage of C3 activation was done by calculating the intensity of C3α' split products [x 100%] and dividing the result by [intensity of native C3α + C3α' split products].

Results are shown as mean ± SD values. For comparing the significances of differences between the study groups the two-tailed Student's t-test or one-way ANOVA with Tukey´s multiple comparison test were used.

RESULTS

Immunoglobulins IgA and IgG in the Cervicovaginal Samples

Since IgG is known to activate complement, but IgA is not, we wanted to analyze the relative proportions of these immunoglobulins in the cervical secretions. **Figures 1A, B** show two representative Western blots for analysis of the IgG and IgA heavy chains, γ and α, respectively. In the majority of samples (n=96) the IgA heavy chains were intact. For IgG, however, in approximately half of the samples, especially in those taken during delivery, additional bands with a lower molecular weight than that for the intact 50 kDa heavy chain were observed in the blots. This suggests proteolytic cleavage. The intensities of the IgG heavy chains were stronger than those for IgA suggesting that IgG is abundantly present in all the mucosal samples. In one case an apparent IgA deficiency was detected (**Figure 1B**).

More accurate quantification of IgG and IgA was done by ELISA (**Figures 1C, D**). On the average, higher concentrations of IgG than IgA were observed in samples from pregnant women (both in those in late pregnancy or in labor; P<0.001: one-way ANOVA and Tukey´s multiple comparison test). In contrast, in nonpregnant women the IgA levels (1.0±0.4, n=19; mean ± SD) were higher than IgG levels (0.5±0.3, n=19, P<0.001, Student's t-test). The IgG levels were higher in samples taken during labor (EC: 1.7±0.7, n= 22; LF: 1.6±0.9, n=21) or late pregnancy (EC: 1.2 ± 0.6 mg/ml, n=46; LF: 1.3±0.7, n= 30) than in those from nonpregnant women (0.5±0.3, n=19, P<0.01; Tukey´s multiple comparison test). Values during labor (both in EC and LF) were slightly higher than in late pregnancy (P<0.01). No significant difference was detected between the study groups in IgA concentrations.

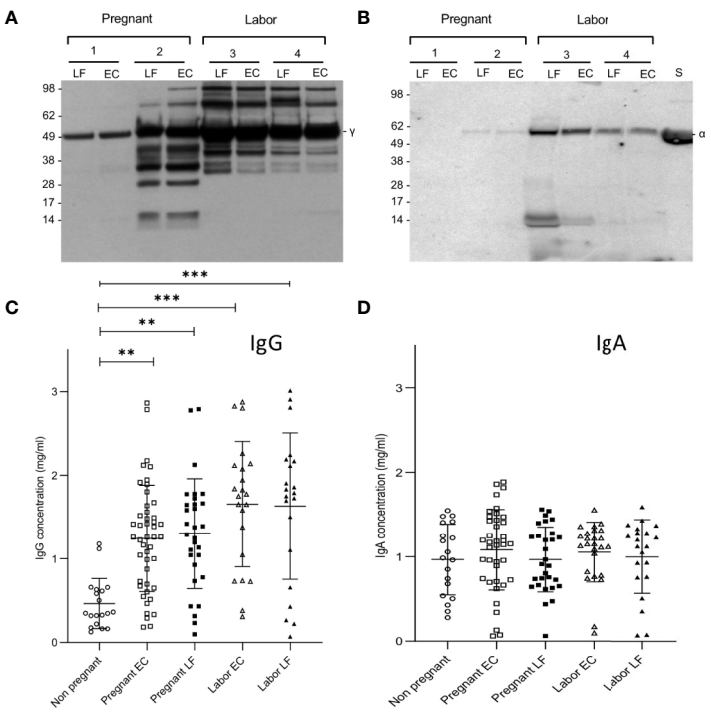

FIGURE 1 | Immunoblotting analysis of IgG and IgA heavy chains and levels of IgG and IgA in EC and LF during pregnancy and labor. Samples 1 and 2 were from pregnant women and samples 3 and 4 from women in labor. As shown in **(A)**, IgG is abundant in all samples. More quantitative variation exists for IgA **(B)**. In the selected samples from pregnant women patient number 1 was found to be IgA-deficient. Antibodies used were specific for the γ (IgG) and α (IgA) heavy chains, both approximately 50 kDa in size. Quantification of IgG **(C)** and IgA **(D)** was done by ELISA. Higher concentrations of IgG were found in the EC and LF during pregnancy and labor in comparison to IgA. IgG concentration is significantly higher during pregnancy and during labor both in the EC and the LF in comparison to the nonpregnant controls (**P < 0.01, ***P < 0.001; one-way ANOVA and Tukey´s multiple comparison test).

Local C3 Activation in Non-Pregnant and Pregnant Women and During Labor

Our next aim was to study the presence and activation of C3 locally in the cervicovaginal area. Native complement C3 consists of one α and one β chain linked together *via* a disulfide bond. The activation of C3 is schematically described in **Figure 2**. The band identities in the immunoblots were determined by comparison to C3 activation fragments in zymosan-activated serum and by inactivating purified C3b with factors H and I (not shown). During activation, the C3 β-chain remains intact in the cleavages and thus reflects the total original amount of C3 in the sample. In addition to the α-chain the Western blots traced the α'-chain and its cleavage fragments, which were quantified by using the "ImageJ/Fiji win-64 software J". As shown in **Figure 3**, C3 in the cervicovaginal samples becomes extensively activated and degraded into multiple cleavage fragments. Occasionally, in the blots a band above the C3 beta-chain was observed (**Figure 3**). It is likely an autolytic cleavage product of the C3 alpha-chain, and thus not an activation product.

The average C3 activation level was 72 ± 13% (mean ± SD, n=23) in the non-pregnant control group, 78 ± 22% (n=49) in the pregnant group and 58 ± 22% (n=24) during active labor (**Figure 4**). Differences were significant between the pregnant group and the labor group in EC samples (P<0.001), and between the non-pregnant group and the labor group (p<0.01) (**Figure 4**). The results indicate that C3 in this local environment is in a continuous state of activation. In ten additional swabs immersed directly into 10 mM EDTA for control of possible *ex vivo* C activation the percentages of cleaved C3 in the PBS/EDTA-containing buffer were 62 ± 23% (mean ± SD), and 70 ± 18% in the EC and LF samples, respectively. In the non-EDTA containing samples the respective percentages of C3 activation were 62 ± 21% s and 87 ± 22% in the EC and LF samples. This indicated that, unlike in EC samples, in the LF samples, some C activation continued still *ex vivo*.

C3 Activation in Lateral Fornix Compared to External Cervix

In LF and EC samples from pregnant women and women in labor large amounts of C3 activation fragments indicated strong local activation of C3. A higher level of C3 activation was observed during pregnancy in LF: 79 ± 20% (n=49) in comparison to EC: 57 ± 30% (n=49) (P<0.001) (**Figure 4**). No significant difference was seen in values in samples from different locations during labor.

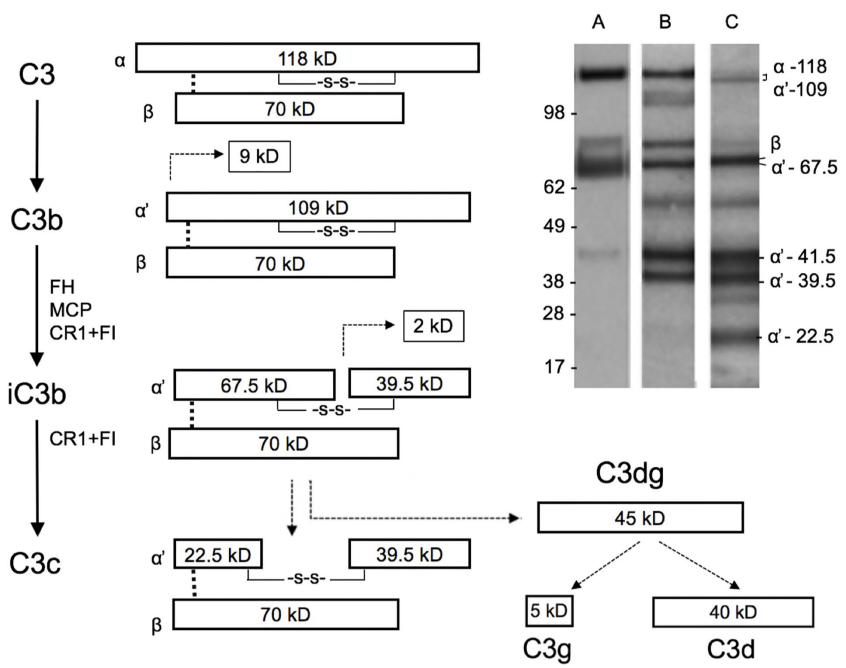

FIGURE 2 | Schematic structure of C3 and its cleavage. The three distinct complement pathways will converge at C3 and promote activation of C3 to C3a and C3b by C3 convertases. The 109 kDa alpha´-chain in C3b becomes cleaved by factor I and a cofactor, e.g. factor H, to smaller 67.5 and 39.5 kDa fragments that are then part of iC3b. iC3b will subsequently get further cleaved to C3c and C3dg. The β chain remains uncleaved as a 70 kDa band. Examples of the C3 cleavage patterns (determined by rabbit anti-human C3c antibody) in our samples are shown in the upper right corner. Lanes A, B, and C show representative samples of different levels of C3 breakdown in the patient samples. Lanes A, B, and C represent samples containing C3b (A), iC3b (B), and a mixture of iC3b and C3c (C), respectively. In lane B, and faintly in lane C, the band at 118–109 kDa represents alpha/alpha-chains, which have remained uncleaved. The identity of the ≈55 kDa band is not known, but could represent a proteolytic cleavage fragment of the 67.5 kDa band. Such a fragment could be generated e.g. by plasmin, which is likely present in the mucosal fluids. The fragment above the 70 kDa beta-band could represent an autolytic cleavage product of the alpha-chain, where C3a (9kDa) has remained bound to the alpha'-67.5 kDa fragment.

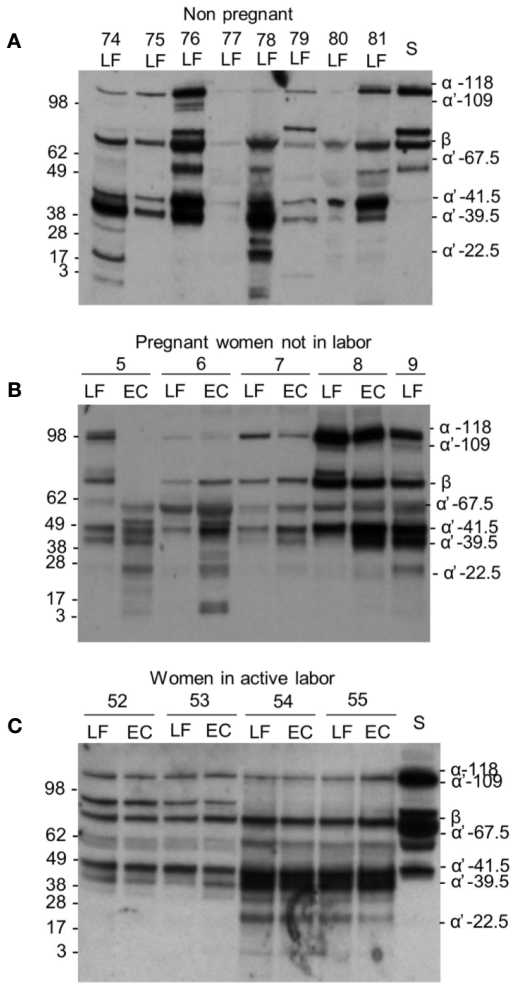

FIGURE 3 | C3 cleavage in the cervicovaginal area. Examples from the three different sample groups are shown. Samples were from: **(A)** women in the nonpregnant group; **(B)** pregnant women, who are not in labor and **(C)** women in active labor but with intact membranes. The cleavage was studied by running the samples in SDS-PAGE under reducing conditions and using Western blotting with an anti-C3c antibody. Sample numbers and types within each group are indicated on the top of the blot. As shown, a wide variation in the extent of C3 cleavage occurs in the samples. The β chain and the key α´-chain fragments are indicated on the right. Molecular weight markers (kDa) are indicated on the left. S, serum control.

Complement Activation by IgG

As possible evidence for complement activation by IgG we detected in some samples by Western blotting high molecular weight bands that stained both for the IgG heavy chain and C3 (**Figure 5**). These bands could present SDS- and reduction-resistant covalent complexes between the IgG heavy chain (50 kDa) and C3 activation fragments. The C3-IgG complexes were observed in 10% of the pregnant women, in 33% of women at delivery and in 12% of control women. These complexes could indicate local complex formation between IgG and C3b and thus C3 activation by the IgG antibodies. No similar bands were detected for IgA.

DISCUSSION

We observed robust local complement activation in the cervicovaginal milieu. C3 activation was seen in women in all the three different groups: when not pregnant, during pregnancy and during labor. High amounts of C3 cleavage products suggest that the complement system at this site is under continuous state of activation. During labor the complement C3 amounts in samples from the cervicovaginal space appeared as the highest, but the activation percentages were the lowest. IgG was more abundantly present in the reproductive secretions during pregnancy and labor in comparison to the nonpregnant state. In contrast, IgA levels did not differ between the study groups.

Unlike usually assumed for mucosal surfaces, IgG is detected in the cervicovaginal area in addition to IgA (35). We observed higher levels of IgG than IgA in samples from women, who were pregnant or in labor. The opposite, higher levels of mucosal IgA than IgG, was true for nonpregnant women. In body fluids containing IgG and complement, a potential for immune complex formation and complement activation exists. IgG and generation of immune complexes are needed for antibacterial and antiviral defense (36). When comparing the Western blots of C3 activation fragments and IgG from the same samples, in some samples, similar high molecular weight bands (>100 kDa) were observed (**Figure 5**) suggesting the presence of covalent complexes between the IgG heavy chain and one or more C3 fragments that remain covalently bound on structures that activated complement. The C3-IgG complexes were more commonly present in samples taken during pregnancy (33%) than in the other samples (10% and 12%). This is a further indication that local complement activation has taken place. It also suggests that local IgG-containing immune complexes could be one of the complement-activating factors. Apart from the obvious factors from the local microbial flora, the nature of local antigens and the relevance of the immune complexes during pregnancy and delivery are unknown. No similar bands were observed for IgA, which is compatible with the fact that IgA does not activate complement.

In recent years, understanding the role of sterile inflammation during pregnancy and labor has increased significantly. Although the complement system has traditionally and evolutionarily been known as part of the host defense against microbes and in causing inflammation, C also maintains homeostasis by controlling the elimination nonviable tissue components (37–39). Previous studies have suggested that the complement system is involved in pregnancy and parturition, participating in tissue remodeling, recognizing injured cells and enhancing phagocytosis (10, 21, 40). C3 is abundantly produced by the uterus, and levels of C3 increase in blood towards the end of pregnancy (26). Also, additional synthesis may occur in the local mucosa (41). Higher amounts, but a lower level, of C3 activation in the cervicovaginal interphase during labor may be related to the dilation and effacement of the cervix, enabling migration of C3 from plasma. On the basis of our results we cannot say to which extent C3 is locally produced and how much is diffusing from blood. This likely depends on the situation (e.g. more C3 from blood during delivery) and varies from patient to

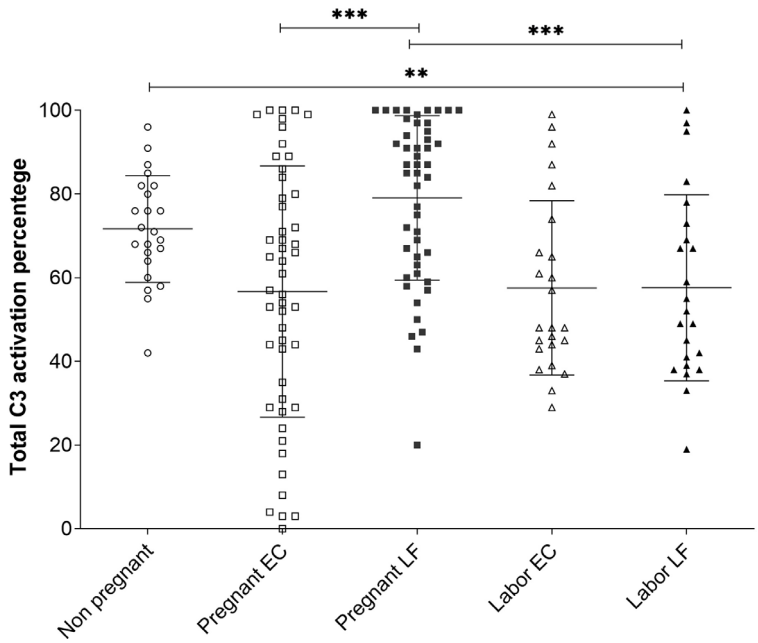

FIGURE 4 | Comparison of C3 activation levels in samples from the EC and the LF in the 3 different groups. The percentages of C3 activation in the three groups were determined from the Western blot analysis of C3 split products using the "ImageJ" program. The mean ± SD values for the different groups were: nonpregnant women 72 ± 13% (n=23), pregnant women EC 57±30%, LF 79 ± 20% (n=49) and women in labor 58 ± 22% (n=24) in both the EC and LF. The differences were significant between women at labor vs. pregnant women (***, P < 0.001; for LF samples) or nonpregnant women (**, P<0.01) (Tukey's Multiple Comparison Test). The C3 activation percentages in samples taken during pregnancy were significantly higher in the LF (79 ± 20%; n=49) than in the EC (57 ± 30%; n=49); (***, P < 0.001). No difference between LF and EC in samples taken at labor were seen.

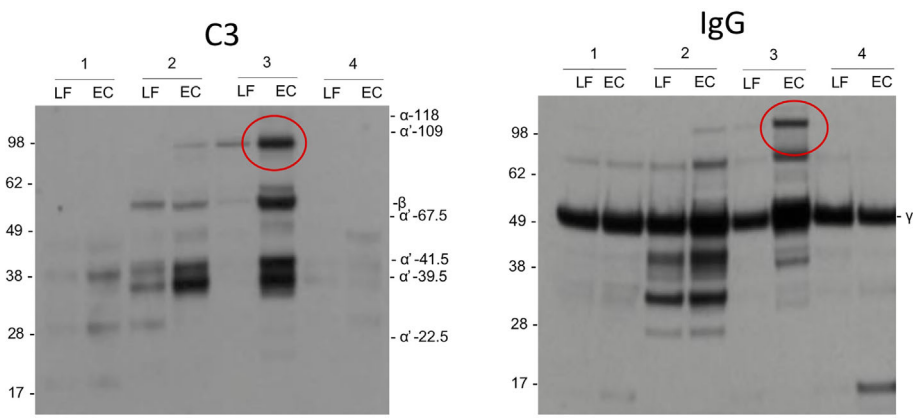

FIGURE 5 | Complement activation by IgG. As evidence for complement activation by IgG we detected in some samples by Western blotting high m.w. bands (circled) that stained both for IgG heavy chain and C3. These bands may present SDS- and reduction-resistant covalent complexes between the IgG heavy chain (50 kDa) and a C3 activation fragment. The C3-IgG complexes could indicate local complex formation between IgG and C3b and thus C3 activation by the IgG antibodies.

patient. Blood-contaminated samples (visible red color) were excluded from the analyses. By measuring absorbances at 539 nm we observed that the highest level of blood contamination in the tested samples was 3.8%. Thus, not all C3 in the samples can originate from contaminating blood.

Our results indicated that complement activation continued to some extent in the LF samples *ex vivo*, because a difference was seen between samples taken to EDTA or buffer only. In both situations (presence or absence of EDTA) the C3 activation level was higher in the LF samples. A similar difference (stronger

activation in LF samples; section *Local C3 Activation in Non-Pregnant and Pregnant Women and During Labor*) was seen in pregnant women, who were not in labor, but not in women at labor. Thus, although we observed some additional C3 activation in LF samples *ex vivo*, it did not influence the general conclusions of the study.

The higher activation percentage of C3 in the LF during pregnancy could result from differences in mucosal secretions and microbiome environments that may affect C3 activation. The lack of difference in C3 activation between the two sites during labor might be explained by the changed anatomical and physiological conditions, like in the fluid and blood inflow during cervical effacement. The lower level of C3 activation during labor suggests that the C system is in this situation better controlled than other times at this local site. This could also explain the lack of difference between EC and LF samples during labor. Whether the better control of C activation in the cervicovaginal space during delivery is due to greater amounts of complement regulating proteins, possibly diffusing from circulation, remains to be studied.

Knowledge about the level of local activation and regulation of the complement system during the physiological inflammation process related to delivery is scarce. In general, the activation level of C3 in the cervicovaginal samples was exceptionally high. As examples, an earlier study on local complement activation in otitis media in children reported an up to 40% level of C3 activation (34) and in dense deposit disease a nearly 100% level of C3 activation in blood plasma could be reached (42). Like in the middle ear space in otitis media, also in vagina many potential factors could contribute to C activation and C3 cleavage. They include microbes, sperm cells, damaged host cells, migrating leukocytes and proteolytic enzymes, like plasmin or matrix metalloproteinases. The cervicovaginal area is thus a true interphase, where a homeostatic balance must persist. The lower genital tract epithelium consists of multiple cell layers of stratified squamous epithelial cells that lack tight junctions. Therefore, it can allow the movement of small molecules, including complement components, through the cell layers. The high level of estrogen at the end of pregnancy increases estradiol receptor expression in cells of the reproductive tract (epithelial cells, macrophages, stromal cells, and lymphocytes). This affects the lower genital tract immunity and promotes complement C3 synthesis (43). What we observed, could thus partially be due to locally produced C3. While complement activation likely has a beneficial function, it may also be harmful. Maternal systemic complement overactivity may result in fetal damage, coagulation disorders, or excessive bleeding during delivery and postpartum. Therefore, complement needs to be carefully controlled. Complement activation can be regulated by local inhibitors or by those coming from circulation. The regulation of complement in this local environment merits more studies.

Quantification of IgG suggested that like for C3, IgG was more abundant in the samples from women in labor than in the other groups. Previous studies suggest that most mucosal IgG originates from blood plasma (7). Our results suggest that IgG is naturally present in the cervicovaginal mucosa. Part of it could derive from the local immune tissue. We have previously demonstrated the presence and activation of local lymphoid tissue including B cells in the vulvovaginal area (44). The presence and activities of IgG in promoting both complement activation and opsonophagocytosis can protect the cervicovaginal space from infections. However, IgG may also make this region vulnerable to inflammation, e.g. by activation of complement and leukocytes, which can generate reactive oxygen metabolites and other mediators of inflammation. Apparently, the balance between these two phenomena varies according to the different physiological challenges, including pregnancy and delivery. It may also depend on the expression of cellular receptors for both activated complement components, like CR1, CR3, C5aR type 1 and for IgG, i.e. the Fc receptors. The different types of Fc receptors can bind different subclasses of IgG and be linked either to activating or inhibiting submembranous domains. The net effects of receptor interactions could thus range from proinflammatory activation to more homeostatic clearance responses. During and after delivery, the need for repair processes is naturally very high. Activation of C3 to C3b (for CR1), and especially for iC3b (for CR3) thus probably plays an essential role in promoting clearance of damaged tissue components and healing at the end. In addition, antigen-bound C3dg and C3d could, *via* their receptor CR2 (CD21), favor activation of B cells and antibody synthesis in the local immune tissue. C5a in turn would promote inflammation through its phlogistic activities via C5aR1.

Hereby we have demonstrated a key activity, activation of complement C3, in the local cervicovaginal immune system. What are the beneficial functions and what are the potential risks need to be analyzed in further studies. Also, the present study did not address the complement inhibitors, which could operate to avoid a full-blown complement activation before the baby enters the birth canal. The presence of local, mucosal IgG implies that this area is an immunologically intermediate region, where both mucosal IgA-based immunity and parenteral IgG-based active immunity coexist. Both may be needed for an active, yet not too excessive inflammatory immune response against potential pathogens. Whether there also is a more direct effect on successful pregnancy or delivery remains an important topic for further studies.

The main strength of the study is the unique sample material obtained from volunteers. The samples were precisely timed, localized and in all cases taken by two experienced doctors. Pregnant women were sampled in the "late term" stage, during which, for unknown reasons, the labor had not yet started. Whether delayed delivery is related in any way to complement dysfunction is not known. Multiple activities of complement in inflammation, in regulating blood flow, cell migration, and activation suggest that complement could have a role in the induction of labor.

In conclusion, our study demonstrates a robust local activation of the complement system and the presence of IgG, in addition to IgA, in the cervicovaginal interface both under normal circumstances, during pregnancy and during delivery. This indicates the presence of an active humoral immune system in this important area. The physiological and potential

pathophysiological consequences of this phenomenon remain to be worked out in further studies.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Helsinki University Hospital's Ethical Committee (91/13/03/03/2015). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SL and JH-E collected samples and. performed the measurements, HJ, IL, and IK were involved in planning and supervised the work, SL, HJ, IK, and SM processed the experimental data, performed the analysis, drafted the manuscript and designed the figures. SL, HJ, IK, IL PN, and SM aided in interpreting the results and worked on the manuscript. All authors contributed to the article and approved the submitted version.

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