UNDERSTANDING THE MATERNO-FETAL INTERFACE DURING MICROBIAL INFECTIONS

EDITED BY: Demba Sarr, Ricardo E. Fretes, Ulrike Kemmerling and Sodiomon B. Sirima

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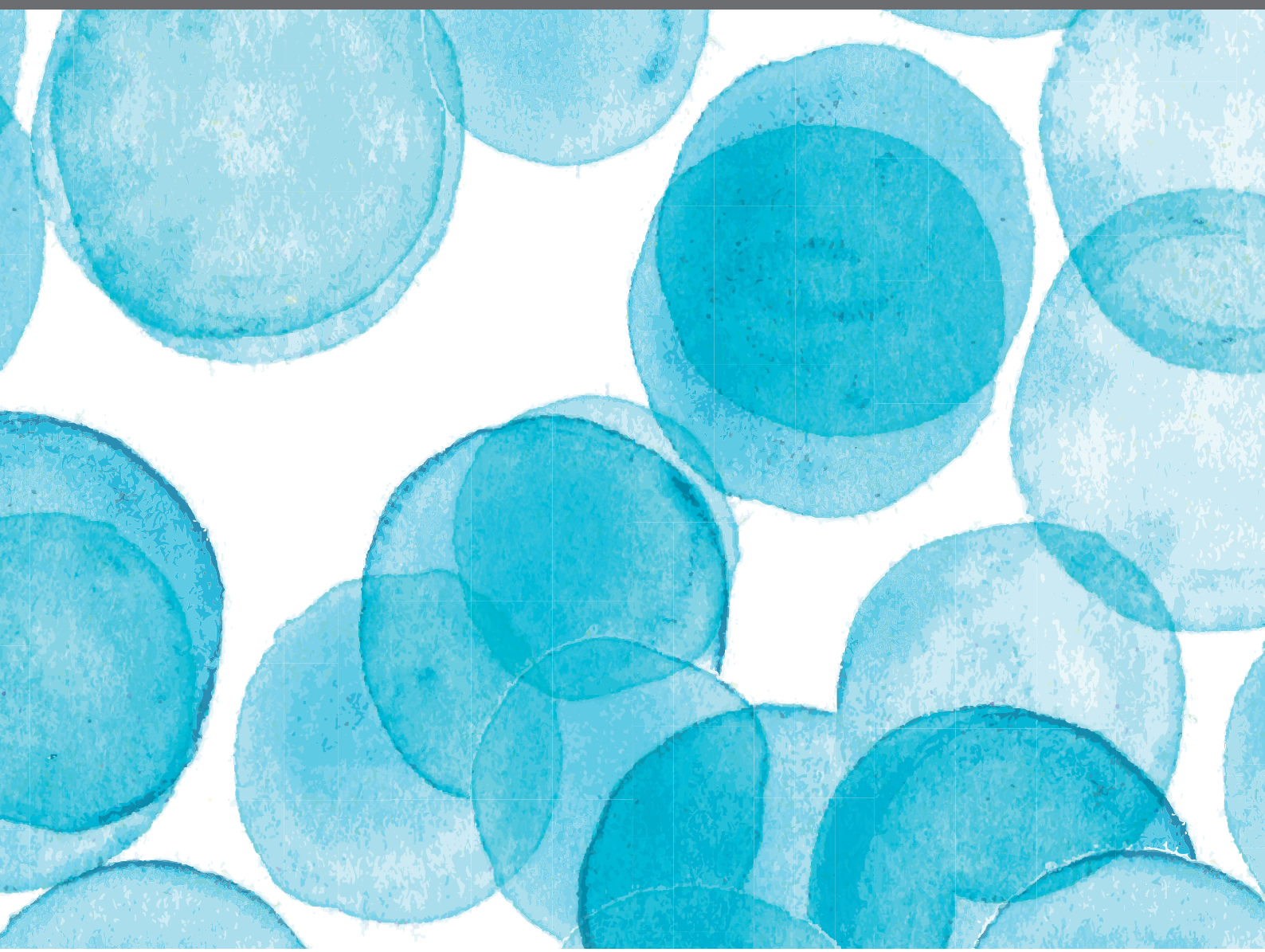

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UNDERSTANDING THE MATERNO-FETAL INTERFACE DURING MICROBIAL INFECTIONS

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Pregnancy is a physiologically and immunologically challenging health state. Immunological and physiological changes throughout the course of pregnancy make pregnant women usually susceptible to infection with microbial agents. Infections with pathogens during pregnancy can have devastating consequences to both the fetus and his/her mother. These infections are linked with adverse pregnancy outcomes. Infections with parasites, viruses, or bacteria can be associated with maternal anemia, abortion, intrauterine growth retardation, preterm delivery, fetal morbidity and high risk of mortality during the first years of life. Despite these significant consequences and complications associated with infections by microbial pathogens during the course of gestation, very little is known about the underlying mechanisms of the pathogenesis and immunopathology of infections during pregnancy.

The Research Topic proposed here in, will focus on microbial infections during pregnancy. Studies and review papers addressing the pregnant host/fetus/pathogen interactions, the host/fetus immunological response against infections during gestation, trans-placental transfer of infections during pregnancy are welcome. Topics related to model systems used to mirror the biology in human, the pathogenesis and molecular pathways as well as the mechanisms of the disease at the maternofetal interface including the placenta, the amniotic fluid, and the fetal membranes will be considered making the scope and interest of the topic relatively broad.

There is a growing number of pathogens associated with pregnancy. In most cases, women are more susceptible to infections with these pathogens when they become pregnant in comparison to their non-pregnant counterparts. Unfortunately, vertical transmission occurs in most cases but the underlying mechanisms are still unknown. The placenta has always been considered as a barrier against congenital infections but studies have indicated that microbial pathogens breach this barrier. The amniotic fluid, and the fetal membranes are also important components of vertical transmission because of their non-sterile state even in most healthy pregnancies. During pregnancy, infections by malaria or toxoplasmosis as well as other viral or bacterial pathogens lead to an uncontrolled inflammatory response recognized as a significant cause for preterm delivery and intra uterine growth retardation leading to low birth weight, a risk factor to infant morbidity and mortality. To successfully prevent, treat, eradicate or educate about microbial infections during pregnancy,

we must understand the molecular mechanisms by which they cause poor birth outcomes including how vertical transmission occurs at the maternofetal interface.

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Transfusion-Transmitted Zika Virus Infection in Pregnant Mice Leads to Broad Tissue Tropism With Severe Placental Damage and Fetal Demise

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Zika virus (ZIKV) infection during pregnancy can cause significant problems, particularly congenital Zika syndrome. Nevertheless, the potential deleterious consequences and associated mechanisms of transfusion-transmitted ZIKV infection on pregnant individuals and their fetuses and babies have not been investigated. Here we examined transmissibility of ZIKV through blood transfusion in ZIKV-susceptible pregnant A129 mice. Our data showed that transfused-transmitted ZIKV at the early infection stage led to significant viremia and broad tissue tropism in the pregnant recipient mice, which were not seen in those transfused with ZIKV-positive ($ZIKV^+$) plasma at later infection stages. Importantly, pregnant mice transfused with early-stage, but not later stages, $ZIKV^+$ plasma also exhibited severe placental infection with vascular damage and apoptosis, fetal infection and fetal damage, accompanied by fetal and pup death. Overall, this study suggests that transfusion-related transmission of ZIKV during initial stage of infection, which harbors high plasma viral titers, can cause serious adverse complications in the pregnant recipients and their fetuses and babies.

Keywords: Zika virus, blood transmissibility, broad tissue tropism, placental infection, fetal demise

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INTRODUCTION

Zika virus (ZIKV), a flavivirus in the *Flaviviridae* family, was first identified in rhesus monkeys in 1947 (Dick et al., 1952; Wikan and Smith, 2016). Similar to other flaviviruses, including dengue virus, yellow fever virus, West Nile virus, and Japanese encephalitis virus, ZIKV is a positive-stranded RNA virus, whose genome encodes structural proteins, including capsid, pre-membrane (prM)/membrane (M), and envelope (E), as well as seven non-structural proteins (Dai et al., 2016; Sirohi et al., 2016; Zhao et al., 2016). ZIKV E protein is a major protein in receptor binding and fusion, and it consists of ectodomain, e.g., domain I (EDI), II (EDII), and III (EDIII), stem region, and transmembrane region (Dai et al., 2016; Kostyuchenko et al., 2016).

Most people infected with ZIKV have no clinical symptoms or only exhibit mild symptoms, without requiring hospitalization. However, ZIKV infection during pregnancy can cause significant problems, particularly congenital Zika syndrome, which involves congenital brain abnormalities, microcephaly at birth, and motor anomalies and epilepsy in infants, as well as other malformations

(Melo et al., 2016; Mlakar et al., 2016; Cui et al., 2017; Krauer et al., 2017; Zorrilla et al., 2017; Chimelli et al., 2018; Pessoa et al., 2018). In addition, ZIKV infection is linked to Guillain-Barre syndrome (GBS), a severe neurological disease (Cao-Lormeau et al., 2016; Roze et al., 2017; Salinas et al., 2017; Simon et al., 2018). The association of ZIKV infection with these unexpected diseases has thus brought worldwide attention to study this virus and its pathogenic mechanisms.

Several mouse models are developed to study ZIKV infection and associated pathology, and to evaluate the efficacy of ZIKV vaccines and therapeutics. We and others have shown the susceptibility of type I interferon receptor (IFNAR)-deficient A129 mice, as well as type I and II interferon receptor (IFNAR/IFNGR)-deficient AG129 and AG6 mice, to ZIKV, and identified candidate vaccines and therapeutic agents that protect against ZIKV infection in these mouse models (Aliota et al., 2016; Cui et al., 2017; Du et al., 2017; Wu et al., 2017; Sumathy et al., 2017; Yu et al., 2017; Espinosa et al., 2018; Tai et al., 2018; Jiang and Du, 2019).

As seen with other mosquito-borne flaviviruses (Harrington et al., 2003; Iwamoto et al., 2003; Wilder-Smith et al., 2009; Aubry et al., 2015), ZIKV has acquired the ability to infect humans via blood or blood components (Barjas-Castro et al., 2016; Motta et al., 2016; Musso et al., 2016; Benjamin, 2017; Bloch et al., 2018). ZIKV RNA was identified in blood donations, and the risk of ZIKV transmission by blood donations does exist, even in the regions with low disease circulation (Galel et al., 2017; Williamson et al., 2017; Magnus et al., 2018). Nevertheless, the transmissibility of ZIKV through blood transfusion and associated pathogenic mechanisms, particularly in pregnant women and their fetuses or babies, are poorly understood. The present study aimed to answer these questions in ZIKV-susceptible pregnant A129 mice. Our data have demonstrated that transfusion with plasma from high-dose, early-stage ZIKV infection led to significant viremia and broad tissue tropism, particularly brain, in pregnant mice. In addition, such transfusion-transmitted ZIKV infection also resulted in severe placental vascular damage, apoptosis, and inflammation, as well as fetal damage and fetal and pup demise. In contrast, transfusion with ZIKV-positive ($ZIKV^+$) plasma at later stages of ZIKV infection, which contained low-titer or no ZIKV, did not enable significant ZIKV replication and/or congenital Zika infection.

MATERIALS AND METHODS

Ethics Statement

Adult (6–8-week-old) and pregnant [8–12-week-old, embryonic day (E7–9 and E10–12)] A129 mice were used in the study. The animal studies were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocols were approved by the Committee on the Ethics of Animal Experiments of the New York Blood Center (Permit Numbers: 344.00 and 345.01).

Virus and Plasma at Different Stages After ZIKV Infection

To understand transmissibility of ZIKV through blood transfusion and determine which stage(s) of ZIKV infection after $ZIKV^+$ plasma transfusion that will not cause severe consequences to the pregnant recipients, plasma from early, middle, and late stages of ZIKV infection was prepared (**Figure 1**). A contemporary ZIKV strain, R103451, which has demonstrated effective infectivity in A129 mice (Tai et al., 2018), was used in the study. To set up $ZIKV^+$ plasma transfusion and eliminate potential effects of other factors, such as preexisting antibodies, in the plasma to be transfused, plasma from naive A129 mice was manually added with a non-lethal, low-dose ZIKV (∼90 plaque forming unit: PFU), and transfused into adult A129 mice (200 μl/mouse, 18 mice/group) via retro-orbital injection. Here a non-lethal ZIKV dose was used to maintain mouse survival during the long-term observation period. Plasma was collected from the mice at defined time points after transfusion, and pooled at early, middle, and late stages of infection for subsequent transfusion experiments. To prepare plasma at early-stage ZIKV infection containing a high ZIKV titer (early stage: high dose), adult A129 mice was transfused with plasma (200 μl/mouse, 18 mice/group) with manually added ZIKV (∼10^4 PFU), and plasma was collected on days 3 and 5 after transfusion. Plasma from A129 mice transfused with normal plasma (200 μl/mouse, 12 mice/group) was collected for control experiments.

Plasma Transfusion, Sample Collection and Evaluation

Three experiments were designed for evaluating potential consequences of transfusion with plasma containing ZIKV at different infection stages to pregnant mice. First, the aforementioned plasma collected from mice at early, middle, and late stages of ZIKV infection with calculated ZIKV titers was transfused into pregnant (E10–12) A129 mice (200 μl/mouse, 6 mice/group) via retro-orbital injection. Six days later, plasma, tissues, placentas, fetuses, and amniotic fluid were collected for detecting viral replication and for subsequent experiments described below. Second, pregnant (E7–9 and E10–12) A129 mice were transfused with early-stage (high dose) $ZIKV^+$ plasma (200 μl/mouse, 6 mice/group), and uteri and placentas were collected 6 days post-transfusion to compare for placental morphology and fetal changes. Third, pregnant (E10–12) A129 mice transfused with early-stage (high dose) $ZIKV^+$ plasma (200 μl/mouse, 6 mice/group) were counted for newborn pups with or without survival. A129 mice transfused with normal plasma (200 μl/mouse, 6 mice/group) were used as controls.

Quantitative Reverse Transcriptase PCR (qRT-PCR)

ZIKV RNA copies in the collected samples were detected by qRT-PCR (Tai et al., 2018). Briefly, RNA was extracted using QIAamp MinElute Virus Spin Kit (for plasma and amniotic fluid) (Qiagen)

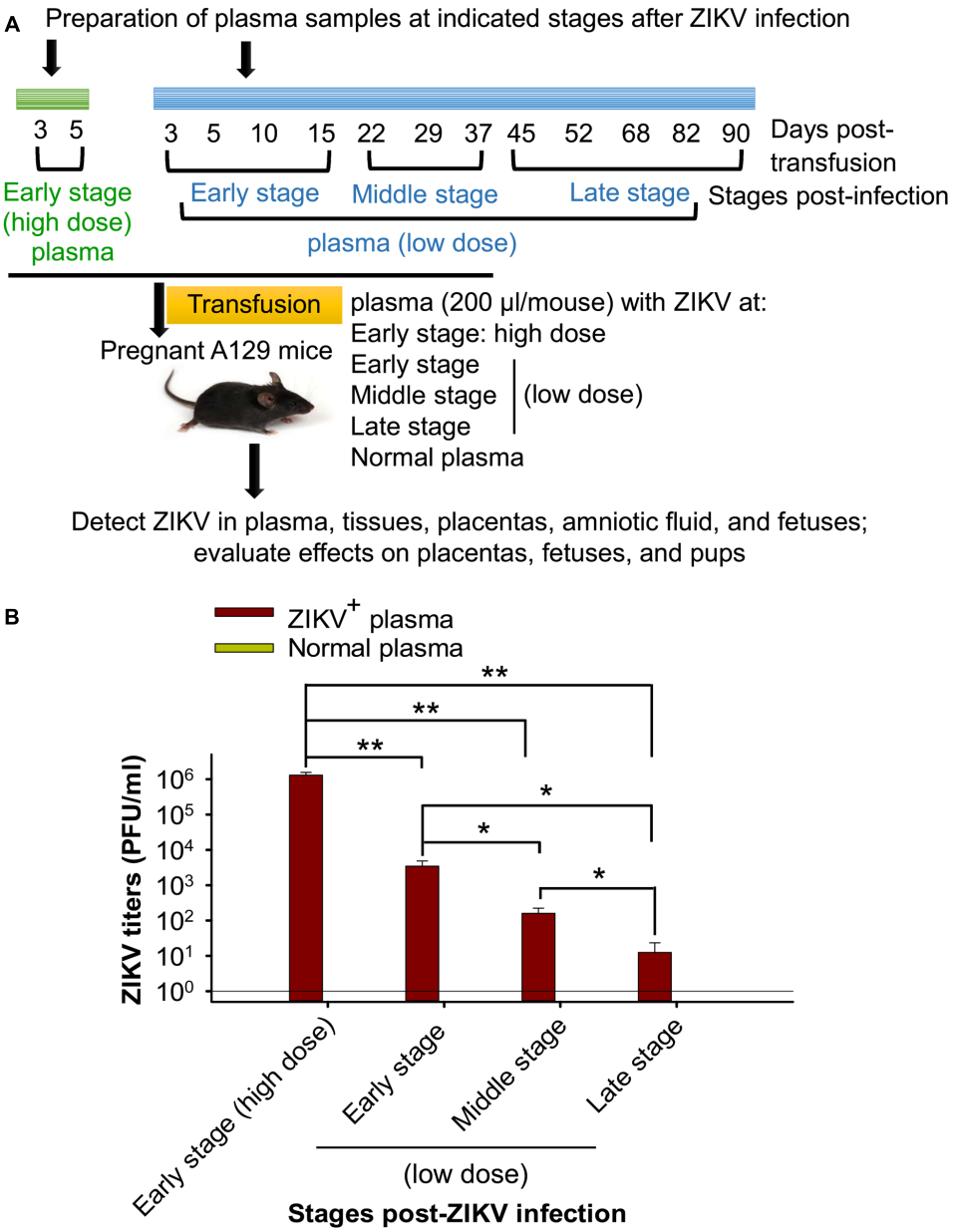

FIGURE 1 | Sample preparation and experimental design. **(A)** Schematic diagram of plasma preparation and experimental procedure. Adult A129 mice were transfused with naive mouse plasma inoculated with or without a non-lethal, low-dose of ZIKV (~90 PFU). Blood was then collected at the indicated time points to prepare for the pooled plasma at early, middle, and late stages post-ZIKV infection (low dose, shown in blue). In addition, adult A129 mice were transfused with naive mouse plasma with or without ZIKV (~10^4 PFU), and blood was collected on days 3 and 5 post-transfusion for preparation of pooled plasma containing high-titer ZIKV at early infection stage (early stage: high dose, shown in green). The above pooled plasma at different stages post-ZIKV infection was transfused to pregnant A129 mice (200 µl/mouse, 6 mice/group) for subsequent experiments. **(B)** Detection of ZIKV titers of above pooled plasma from different infection stages by plaque assay. The ZIKV titers are expressed as PFU/ml, based on which each mouse receiving plasma containing specific ZIKV titer at early stage (high dose), early-, middle-, and late-stages (low dose) was calculated. * and ** indicate significant differences between early stage (high dose) and other stages, between low dose of early stage and middle or late stage, or between low dose of middle and late stages, of ZIKV infection. The data are presented as means ± s.e.m of duplicate wells. The experiments were repeated twice, and similar results were obtained. Normal plasma: control plasma from A129 mice transfused with normal plasma without ZIKV (0 PFU).

and RNeasy Mini Kit (for tissues) (Qiagen), and quantified using one-step qRT-PCR in the presence of Power SYBR Green PCR Master Mix, MultiScribe Reverse Transcriptase, and Ambion™ RNase Inhibitor (Thermo Fisher Scientific) in ViiA 7 Master Cycler PCR System (Thermo Fisher Scientific). The forward (5′-TTGGTCATGATACTGCTGATTGC-3′) and reverse (5′-CCTTCCACAAAGTCCCTATTGC-3′) primers were used for the amplification. To establish a standard curve

of qRT-PCR, the M-E genes of ZIKV (strain R103451, 141–1800 bp) were amplified and cloned into pCR2.1-TOPO vector (Thermo Fisher Scientific) to construct a recombinant plasmid. This plasmid was serially diluted at 10-fold, and a linear standard curve at 10^2 to 10^{10} RNA copies (correlation coefficient R^2 value > 0.99; detection limit: $\sim 10^2$ RNA copies) was selected to calculate ZIKV RNA in the plasma-transfused mouse samples. ZIKV RNA was purified from about 40 μl (for plasma or amniotic fluid) or 40 mg (for tissues) of transfused samples, so the detection limit per milliliter (ml) or gram (g) was about 2.5×10^3 RNA copies.

Measurement of ZIKV Titers

Plaque-forming assay was carried out to measure ZIKV titers in collected samples (Wu et al., 2017; Tai et al., 2018). Briefly, diluted plasma, amniotic fluid, and tissue lysates were transferred onto Vero E6 cells (ATCC) and incubated at 37°C for 1 h. The cells were further overlaid with DMEM (Thermo Fisher Scientific) containing 1% carboxymethyl cellulose (MilliporeSigma) and 2% FBS (Atlanta Biologicals), cultured at 37°C for four to five days, and stained with 0.5% crystal violet (MilliporeSigma). Viral titers are presented as PFU/ml or PFU/g of test samples.

Immunofluorescence Staining

Harvested maternal brain and placentas were fixed in 4% paraformaldehyde (MilliporeSigma), embedded in paraffin, and sectioned (Sapparapu et al., 2016; Yuan et al., 2017). The tissue slides were deparaffinized, blocked with 2% BSA (MilliporeSigma) for 30 min, and then incubated with human anti-ZIKV (EDIII-specific monoclonal antibody (mAb) ZV-64, 1:200, Absolute Antibody) and rabbit anti-vimentin antibody (1:300, Abcam), respectively, at 4°C overnight. For caspase-3 staining, the deparaffinized tissue slides were fixed and permed with FIX and PERM Cell Permeabilization Kit (Thermo Fisher Scientific), then blocked and incubated with rabbit anti-active caspase-3 antibody (1:200, Abcam) as described above. After washing with PBS, the slides were incubated with anti-human FITC (for ZIKV) or anti-rabbit Alexa Fluor® 647 (1:300, Abcam; for vimentin and caspase-3)-conjugated antibodies for 1 h. The slides were counter-stained with DAPI (4′,6-diamidino-2-phenylindole, 300 nM, Thermo Fisher Scientific) for nuclei for 5 min, and mounted in a VectaMount Permanent Mounting Medium (Vector Laboratories). All slides were imaged on confocal microscope (Zeiss LSM 880). Images were prepared using Adobe Photoshop and ZEN software, and the fluorescent signals were quantified by ImageJ software for the relative intensity (particle analysis) using a standard protocol (Wu et al., 2003). Specifically, the pictures were set up image type at 8-bit, and those from the experiment and control groups were adjusted to the same thresholds. The fluorescence intensity of the particles was then analyzed to get positive counts of each picture.

Transmission Electron Microscope (TEM)

Tissues were dissected in PBS, and 3 mm^3 fragments were fixed with 2.5% glutaraldehyde (MilliporeSigma) and 2% paraformaldehyde in sodium cacodylate buffer (0.1 M, MilliporeSigma) for at least 2 h. After fixation, samples were washed three times in sodium cacodylate buffer (0.1 M) and post-fixed by 2% OsO4 (MilliporeSigma) for 1 h. Samples were then washed and dehydrated using a series of ethanol concentrations (50–100%) with a final wash of propylene oxide (MilliporeSigma). Samples were embedded in plastic (Epon 812, EMS) and prepared for sectioning. Ultrathin sections were contrasted with UranyLess (EMS) and lead citrate (MilliporeSigma), and analyzed under the Tecnai G2 Spirit TEM.

Detection of Inflammatory Cytokines and Chemokines

Inflammatory cytokines and chemokines were measured in collected placentas and amniotic fluid using Mouse Inflammatory Cytokines Multi-Analyte ELISArray Kit and Mouse Common Chemokines Multi-Analyte ELISArray Kit according to the manufacturer's instructions (Qiagen). Briefly, mouse placental lysates and amniotic fluid were incubated with respective cytokine and chemokine capture antibodies pre-coated in the ELISA plates for 2 h at room temperature. After three washes, the plates were sequentially incubated with detection antibody for 1 h, and Avidin-HRP conjugate for 30 min at room temperature. The plates were then, respectively, incubated with development solution and stop solution to stop the reaction. Absorbance at 450 nm was measured by ELISA plate reader (Tecan).

Statistical Analysis

The values are presented as means with standard error (s.e.m). Statistical significance among different groups was calculated using GraphPad Prism Statistical Software. For data comparing viral RNA copies, viral titers, and fluorescence staining (shown on a log-based scale), Welch's *t*-test was used, and for other data, two-tailed Student's *t*-test was applied. *, ** and *** represent $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

RESULTS

Preparation of Plasma Samples at Different Stages of ZIKV Infection

To prepare plasma at early, middle, and late infection stages with low-dose ZIKV, plasma containing ZIKV at a non-lethal dose (∼90 PFU), which was previously identified via intraperitoneal (i.p.) route (Tai et al., 2018), was transfused into A129 mice, and blood was collected and pooled for plasma from defined time points after transfusion (**Figure 1A**) to detect for ZIKV viral titers. To prepare plasma at early stage (high dose) of ZIKV infection, mice were inoculated with a high, lethal-dose of ZIKV [$\sim 10^4$ PFU: previously identified through i.p. route (Tai et al., 2018)] and plasma collected at early stage post-ZIKV infection (day 3 and 5 after transfusion) (**Figure 1A**). This plasma contains significantly higher ZIKV titers than the plasma collected from mice receiving non-lethal, low-dose ZIKV at early, middle, and late stages of infection. In addition, ZIKV titers in the plasma of mice from early and middle infection stages (low dose) were also

significantly higher than those from later infection stages (low dose) (**Figure 1B**).

Transfusion of High-Dose Early-Stage ZIKV$^+$ Plasma to Pregnant A129 Mice Caused Significant Viremia and Broad Tissue Tropism, Including Brain

To investigate tissue distribution of ZIKV in pregnant A129 mice following transfusion with ZIKV$^+$ plasma, we performed the following analyses.

First, we measured viral load on day 6 post-transfusion, an optimal time point for detecting viral RNA levels and viral titers in tissues and blood (Dowall et al., 2016). We found that pregnant mice (E10–12) receiving plasma at the early-stage ZIKV infection containing a high titer (~2.6 × 10^5 ± 5 × 10^4 PFU) of ZIKV (early stage: high dose) exhibited severe ZIKV viremia, with significantly higher levels of ZIKV RNA (**Figure 2A**) and ZIKV titers (**Figure 2B**) than those receiving plasma containing a lower titer, early-stage (low dose) ZIKV$^+$ (~690 ± 285 PFU). In contrast, pregnant mice receiving middle- (~32 ± 13 PFU) and late-stage (~3 ± 2 PFU) (low dose) ZIKV$^+$ plasma had undetectable levels of viremia similar to normal plasma-transfused mice (**Figures 2A,B**). These data reveal that establishment of transfusion-transmitted ZIKV infection in pregnant mice might occur only with early-stage infected plasma containing high-dose ZIKV.

Second, analysis of tissues, including brain, on day 6 post-transfusion demonstrated that pregnant (E10–12) mice transfused with early-stage high-dose ZIKV$^+$ plasma had significantly higher ZIKV RNA (**Figure 2C**) and ZIKV titers (**Figure 2D**) in lung, spleen, kidney, heart, liver, muscle, and brain than mice receiving plasma from early stage (low dose) of ZIKV infection. In contrast, ZIKV RNA and ZIKV titers were undetectable in the above tissues of mice transfused with middle- and late-stage (low dose) ZIKV$^+$ plasma, or normal plasma (**Figures 2C,D**). The presence of ZIKV in brain tissues of pregnant mice transfused with early-stage high-dose ZIKV$^+$ plasma was confirmed by immunofluorescence staining of ZIKV E protein using a ZIKV EDIII-specific mAb (**Figure 3A**), which

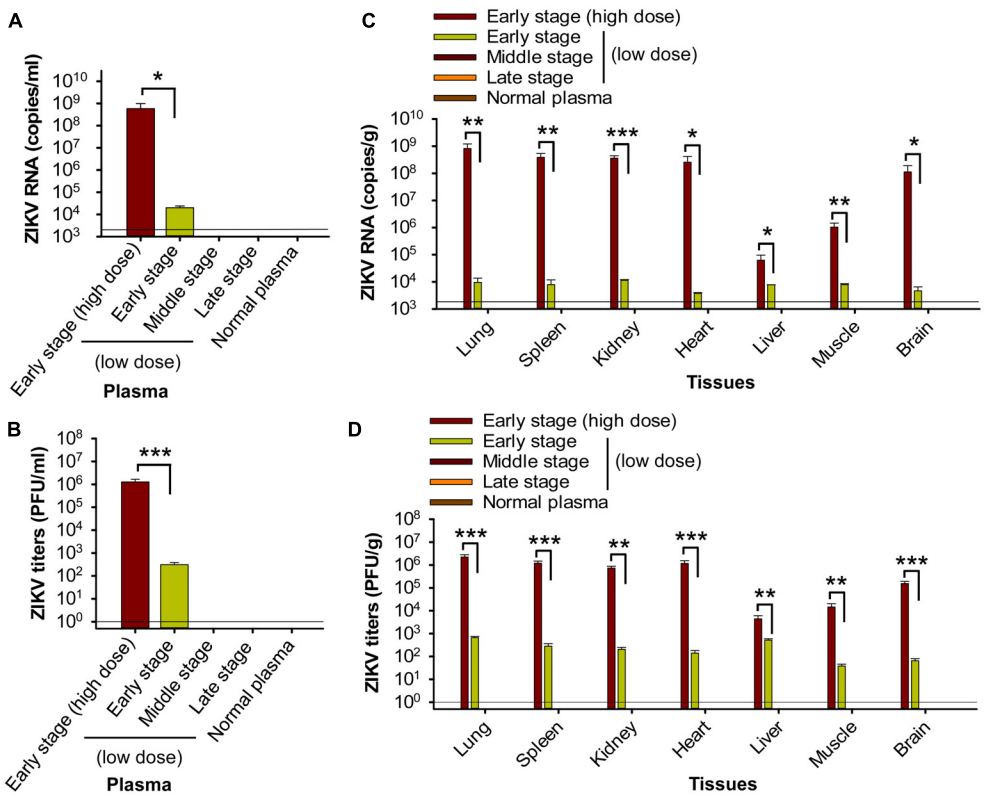

FIGURE 2 | Transfusion of pregnant A129 mice with early-stage (high dose) ZIKV$^+$ plasma resulted in significant viremia and broad tissue tropism. Pregnant (E10–12) A129 mice were transfused with ZIKV$^+$ plasma from early, middle, and late stages, respectively, and plasma and tissues were collected 6 days post-transfusion for below tests. Plasma ZIKV RNA **(A)** and ZIKV titers **(B)** were detected by qRT-PCR and plaque assay, respectively. Tissue ZIKV RNA **(C)** and viral titers **(D)** were detected by qRT-PCR and plaque assay, respectively. *, **, and *** indicate significant differences between early stage (high dose) and early stage (low dose) post-transfusion with ZIKV$^+$ plasma. The data are presented as means ± s.e.m (n = 6 mice/group). In **(A)** and **(C)**, the limit of detection, shown as the horizontal lines, was about 2.5 × 10^3 copies/ml (for **A**) or 2.5 × 10^3 copies/g (for **C**) of ZIKV RNA. This was determined based on a detection limit of 10^2 copies in a linear standard curve (at serial dilutions of 10^2 to 10^{10} copies) of ZIKV RNA, as described in Materials and Methods. In **(B)** and **(D)**, the detection limit was about 1 PFU/ml (for **B**) or 1 PFU/g (for **D**). The experiments were repeated twice, and similar results were obtained. Normal plasma: control plasma from A129 mice transfused with normal plasma without ZIKV.

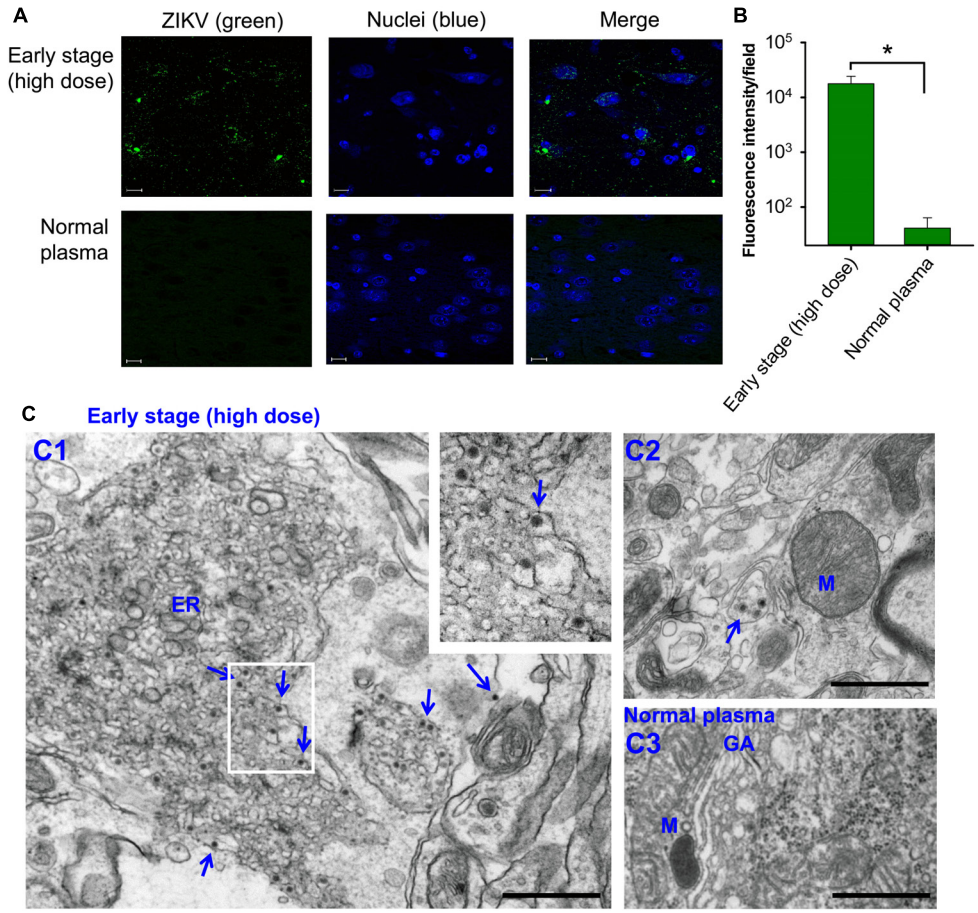

FIGURE 3 | Identification of ZIKV particles in brain tissues of pregnant A129 (E10–12) mice transfused with early-stage (high dose) ZIKV$^+$ plasma. **(A)** Immunofluorescence staining of brain tissues collected 6 days post-transfusion. Brain sections were stained for ZIKV (green) using ZIKV EDIII-specific mAb ZV-64, and representative images are listed. Nuclei were stained with DAPI, and are shown in blue. Magnification: 63X; Scale bar: 10 μm. **(B)** Quantification of ZIKV staining in **(A)** was calculated by ImageJ software for the relative intensity (particle analysis) of ZIKV staining with fluorescent signals. The data are presented as mean fluorescence intensity (e.g., ZIKV$^+$ staining) in each field ± s.e.m (n = 6, "n" indicates numbers of image from different brain tissues), and * indicates significant differences between early stage (high dose) and normal plasma groups. **(C)** TEM analysis of above brain tissues. Microphotographs of ZIKV particles formed in brain tissues of mice transfused with early-stage (high dose) ZIKV$^+$ plasma (C1–C2) or normal plasma (C3). Short arrows indicate ZIKV particles. ER, endoplasmic reticulum; M, mitochondria; GA, golgi apparatus. Scale bar: 500 nm. Normal plasma groups (A–C) indicate control mice transfused with normal plasma without ZIKV.

showed significantly more ZIKV staining than in those of mice transfused with normal plasma (**Figure 3B**), as well as by TEM analysis, which revealed multiple particles (∼50–80 nm) in highly vacuolated cells, indicative of ZIKV infection (**Figure 3C**).

The above data indicate that transfusion of ZIKV$^+$ plasma from high-dose early-stage, but not later stages, of ZIKV infection leads to severe complications in pregnant A129 recipient mice, causing high viremia and broad tissue tropism, particularly brain.

Transfusion of High-Dose Early-Stage ZIKV$^+$ Plasma to Pregnant A129 Mice Resulted in Severe Placental Infection, as Well as Vascular Damage and Apoptosis in Placentas

To further investigate whether transfusion-transmitted ZIKV causes placental infection and damage, pregnant (E10–12) A129 mice were transfused with ZIKV$^+$ plasma from different infection stages, and placentas were analyzed on day 6 post-transfusion.

Significantly higher levels of ZIKV RNA (**Figure 4A**, top) and viral titers (**Figure 4A**, bottom) were detected in the placentas of mice transfused with early-stage (high dose) ZIKV$^+$ plasma than those receiving ZIKV$^+$ plasma at early and middle stages (low dose) of ZIKV infection, whereas both ZIKV RNA and viral titers were undetectable in the placentas of mice transfused with late-stage (low dose) ZIKV$^+$ plasma or normal plasma. Immunofluorescence staining also demonstrated strong signals with ZIKV-specific mAb in the placentas of early-stage (high dose) ZIKV$^+$ plasma-transfused mice (**Figure 4B**), and greater numbers of ZIKV$^+$ signals than those of control mice (**Figure 4C**). TEM analysis of infected placental tissues revealed high numbers of particles (∼50–80 nm) in the early-stage (high dose) ZIKV$^+$ plasma-transfused placental tissues. In addition,

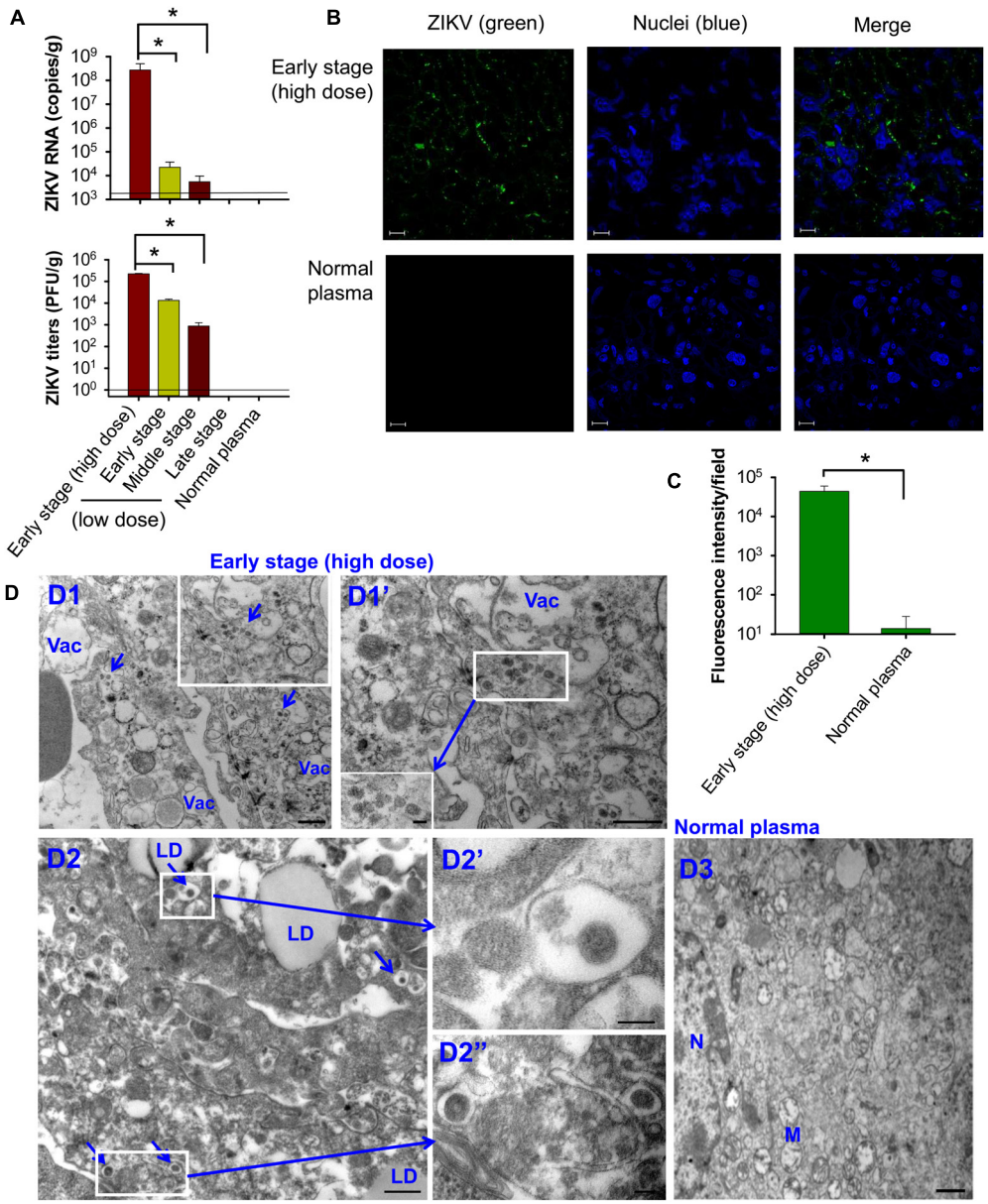

FIGURE 4 | Transfusion of pregnant A129 mice with early-stage (high dose) ZIKV$^+$ plasma led to significant placental infection. Pregnant (E10–12) A129 mice were transfused with ZIKV$^+$ plasma from early, middle, and late stages, respectively, and placentas were collected 6 days after transfusion for below tests. Detection of ZIKV RNA by qRT-PCR (**A**, top) and ZIKV titers by plaque assay (**A**, bottom). The data are presented as means ± s.e.m (n = 6 mice/group). * indicates significant differences between early stage (high dose) and early or middle stages (low dose). The detection limit, shown as the horizontal lines, for qRT-PCR and plaque assay was about 2.5 × 10^3 RNA copies/g and 1 PFU/g, respectively. **(B)** Immunofluorescence staining of placental tissues of pregnant mice transfused with early-stage (high dose) ZIKV$^+$ plasma. ZIKV (green) was stained using ZIKV EDIII-specific mAb ZV-64. Nuclei were stained with DAPI, and are shown in blue. Representative images are listed. Magnification: 63X; Scale bar: 10 μm. **(C)** Quantification of ZIKV staining in (B) was calculated by ImageJ software for the relative intensity (particle analysis) of ZIKV staining with fluorescent signals. The data are presented as mean fluorescence intensity (e.g., ZIKV$^+$ staining) in each field ± s.e.m (n = 6, "n" indicates numbers of image from different placentas). * indicates significant differences between early stage (high dose) and normal plasma groups. **(D)** TEM analysis of placental tissues collected from the mice transfused with early-stage (high dose) ZIKV$^+$ plasma. D1–D2, microphotographs show ZIKV particles in the placentas of ZIKV$^+$ plasma-transfused mice. Infected cells were highly vacuolated and abundant with lipid droplets. Short arrows indicate ZIKV particles. D3, placental tissues collected from normal plasma-transfused mice. Vac, vacuole; LD, lipid droplet; M, mitochondria; N, nuclei. Scale bar: 500 nm for D1, D1", D2, and D3, and 100 nm for D1' insertion, D2', and D2". Normal plasma: control plasma from A129 mice transfused with normal plasma without ZIKV.

the cytoplasm of placental cells of mice transfused with ZIKV$^+$ plasma, but not normal plasma control, had an abundance of vacuoles and large lipid droplets (**Figure 4D**).

To identify potential mechanisms of placental damage, placentas from pregnant mice transfused with high-dose early-stage ZIKV$^+$ plasma were also investigated for

vascular damage and apoptosis. By immunofluorescence microscopy, diminished staining for vimentin, a marker for fetal capillary endothelium and fetal blood vessels in mouse placentas (Sapparapu et al., 2016; Miner et al., 2016), was detected in placentas from mice transfused with ZIKV$^+$ plasma, with significantly reduced numbers of vimentin$^+$ signals than those of the mice transfused with normal plasma (**Figures 5A,C**), suggesting damaged vasculature associated with early-stage ZIKV$^+$ plasma transfusion. The placentas of mice transfused with high-dose early-stage ZIKV$^+$ plasma also stained positive for activated form of caspase-3, an apoptotic marker (Yuan et al., 2017), with significantly higher numbers than those from normal plasma-transfused mice (**Figures 5B,C**), demonstrating considerable cell death, consistent with reduced vimentin expression.

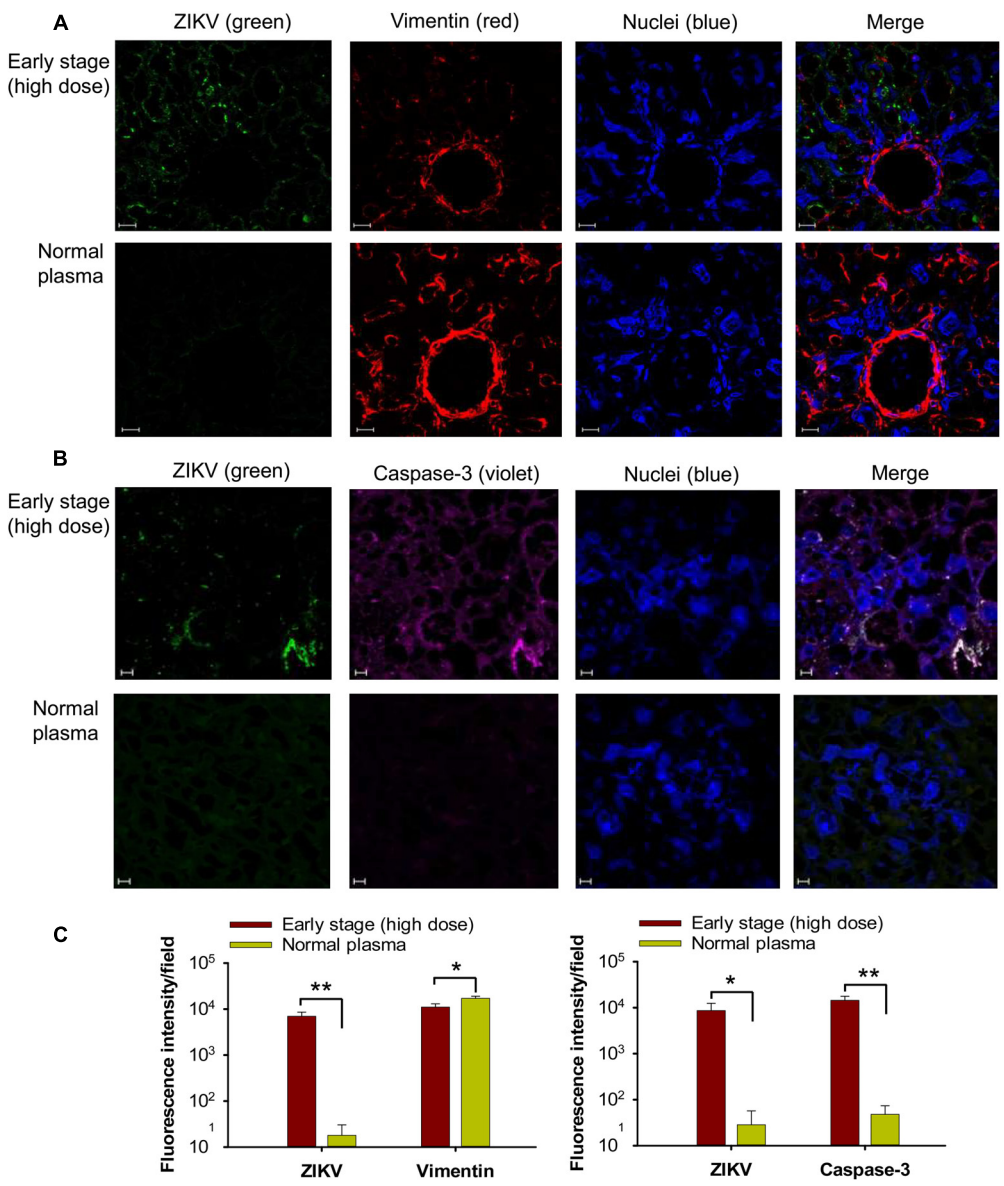

FIGURE 5 | Transfusion of pregnant A129 mice with early-stage (high dose) ZIKV$^+$ plasma caused vascular damage and apoptosis in placentas. Placentas were collected from pregnant (E10–12) A129 mice transfused with early-stage (high dose) ZIKV$^+$ plasma 6 days after transfusion, and used for below tests. Representative images of immunofluorescence staining of vimentin **(A)** and activated form of caspase-3 **(B)** in placentas are shown. ZIKV (green), vimentin (red), and activated caspase-3 (violet) were stained with anti-ZIKV, anti-vimentin, and anti-active caspase-3 antibodies, respectively. Nuclei were stained with DAPI, and are shown in blue. Magnification: 63X. Scale bar: 10 μm. **(C)** Quantification of vimentin and caspase-3 staining in **(A)** and **(B)** by ImageJ software as described above. The data are presented as mean fluorescence intensity for ZIKV$^+$, vimentin$^+$, or caspase-3$^+$ staining in each field ± s.e.m (n = 6, "n" indicates numbers of image from different placentas). * and ** indicate significant differences between early stage (high dose) and normal plasma groups. Normal plasma: control plasma from A129 mice transfused with normal plasma without ZIKV.

Altogether, these data indicate severe placental infection, vascular damage, and apoptosis following transfusion of pregnant A129 mice with early-stage ZIKV$^+$ plasma containing high titer ($\sim 2.6 \times 10^5 \pm 5 \times 10^4$ PFU) of infectious particles.

Transfusion of High-Dose Early-Stage ZIKV$^+$ Plasma to Pregnant A129 Mice Resulted in Severe Fetal Infection and Damage, Leading to Fetal and Pup Death

To test the impact of transfusion-transmitted ZIKV infection during pregnancy on fetuses and newborn pups, we first measured ZIKV in fetal amniotic fluid and embryonic brain tissue from pregnant (E10–12) A129 mice 6 days after transfusion with ZIKV$^+$ plasma. We found significantly higher ZIKV RNA (**Figure 6A**) and viral titers (**Figure 6B**), in amniotic fluid and embryonic brain of the pregnant mice transfused with early-stage (high dose) ZIKV$^+$ plasma than with plasma of early and/or middle stages (low dose) of ZIKV infection. There were also significantly higher ZIKV RNA and viral titers in amniotic fluid of the mice transfused with early-stage (low dose) than middle-stage (low dose) ZIKV$^+$ plasma. In contrast, ZIKV RNA and viral titers were undetectable in aforementioned samples of the mice transfused with other stages (low dose) of ZIKV$^+$ plasma, or normal plasma (**Figures 6A,B**).

To investigate effects of transfusion-transmitted ZIKV infection during gestation, pregnant A129 mice at E7–9 and E10–12 were transfused with early-stage (high dose) ZIKV$^+$ plasma, and then evaluated for uterus and placental morphology and fetus conditions 6 days after transfusion. All fetuses from ZIKV$^+$ plasma-transfused pregnant (E7–9) mice died in uteri and had experienced severe resorption (**Figure 6Ca**). In addition, these fetuses displayed significant growth restriction compared to those from the pregnant mice transfused with normal plasma (**Figure 6Cb**). Also, placentas from pregnant (E10–12) mice transfused with ZIKV$^+$ plasma presented obvious congestion, with significantly smaller diameter than those of the mice transfused with normal plasma (**Figure 6Cc**). Particularly, the size of the fetuses from ZIKV$^+$ plasma-transfused pregnant (E10–12) mice was significantly smaller than that of the fetuses from normal plasma-transfused mice (**Figure 6Cd**), suggesting significant fetal growth restriction during pregnancy in the ZIKV$^+$ plasma-transfused mice.

We further monitored the survival of newborn pups from pregnant (E10–12) A129 mice transfused with early-stage (high dose) ZIKV$^+$ plasma. Fewer pups were born to ZIKV$^+$ plasma-transfused pregnant mice than to normal plasma-transfused mice, and all pups from the former group died by 24 h after birth (**Figure 6D**).

The above data demonstrate that while transfusion of pregnant A129 mice with low-dose ZIKV$^+$ plasma at early ($\sim 690 \pm 285$ PFU), middle ($\sim 32 \pm 13$ PFU), and late ($\sim 3 \pm 2$ PFU) stages of infection led to low to no ZIKV replication in fetuses, transfusion with early-stage (high dose) ZIKV$^+$ ($\sim 2.6 \times 10^5 \pm 5 \times 10^4$ PFU) plasma caused severe congenital fetal infection, fetal damage, and reduced fetal growth, leading to fetal and pup death.

Transfusion of High-Dose Early-Stage ZIKV$^+$ Plasma to Pregnant A129 Mice Resulted in Inflammation With Increased Cytokines and Chemokines

To evaluate whether transfusion-transmitted ZIKV causes inflammation to pregnant mice and their fetuses, placentas and fetal amniotic fluid from pregnant (E10–12) A129 mice 6 days after transfusion were tested for inflammatory cytokines and chemokines using Multi-Analyte ELISArray Kits. Significantly higher expression of IL-1α, G-CSF, MCP-1 (CCL-2), SDF-1 (CXCL-12), and KC (CXCL-1) was identified in the ZIKV$^+$ plasma (early-stage, high dose)-transfused mouse placentas than those of the mice transfused with normal plasma (**Figures 7A,B**). In addition to the cytokines and chemokines described above, fetal amniotic fluid of the mice transfused with ZIKV$^+$ plasma also demonstrated significantly increased production of inflammatory cytokine IL-6 and chemokines MIG (CXCL-9) and Eotaxin (CCL-11), as compared with that of the normal plasma-transfused mice (**Figures 7C,D**). These data suggest that early-stage (high dose) ZIKV$^+$ plasma transfusion may lead to significant inflammation in the pregnant mice, and that fetuses might experience a more severe inflammatory response than maternal placentas.

DISCUSSION

ZIKV may be transmitted through blood transfusion. ZIKV RNA was detected in asymptomatic blood donors during the ZIKV outbreak, posing a serious threat to blood safety (Musso et al., 2016; Bierlaire et al., 2017; Slavov et al., 2017; Willyard, 2017). Here, we used ZIKV-susceptible pregnant A129 mice to study transmissibility of ZIKV through transfusion and evaluate viremia, tissue tropism, placental infection and damage, and associated pathogenic mechanisms, as well as fetal damage and fetal and pup death. Overall, the data suggest that transfusion-transmitted ZIKV from early-stage infection can cause significant viremia, broad tissue tropism, and inflammation, with devastating effects during pregnancy, particularly on the fetuses.

To establish transfusion process with ZIKV$^+$ plasma, minimize potential impact of preexisting antibodies or other factors in the transfused plasma, and maintain the recipient mice to survive during the observation period, we manually inoculated normal plasma with a contemporary ZIKV strain (R103451) at non-lethal, low-dose (e.g., ∼90 PFU) (Tai et al., 2018), transfused it to A129 mice, and collected plasma at early, middle, and late ZIKV infection stages after transfusion to test ZIKV titers. Surprisingly, viremia was detectable in the transfused mouse plasma after 45 days, with early-stage infection having the highest ZIKV titer. It has been shown that viral load in human maternal sera can be detectable for 14 weeks, and that ZIKV RNAs are detected in maternal sera 8 weeks after the onset of clinical symptoms, which potentially result from viral replication in the fetuses or placentas where the latter might serve as a reservoir (Driggers et al., 2016; Suy et al., 2016). Here

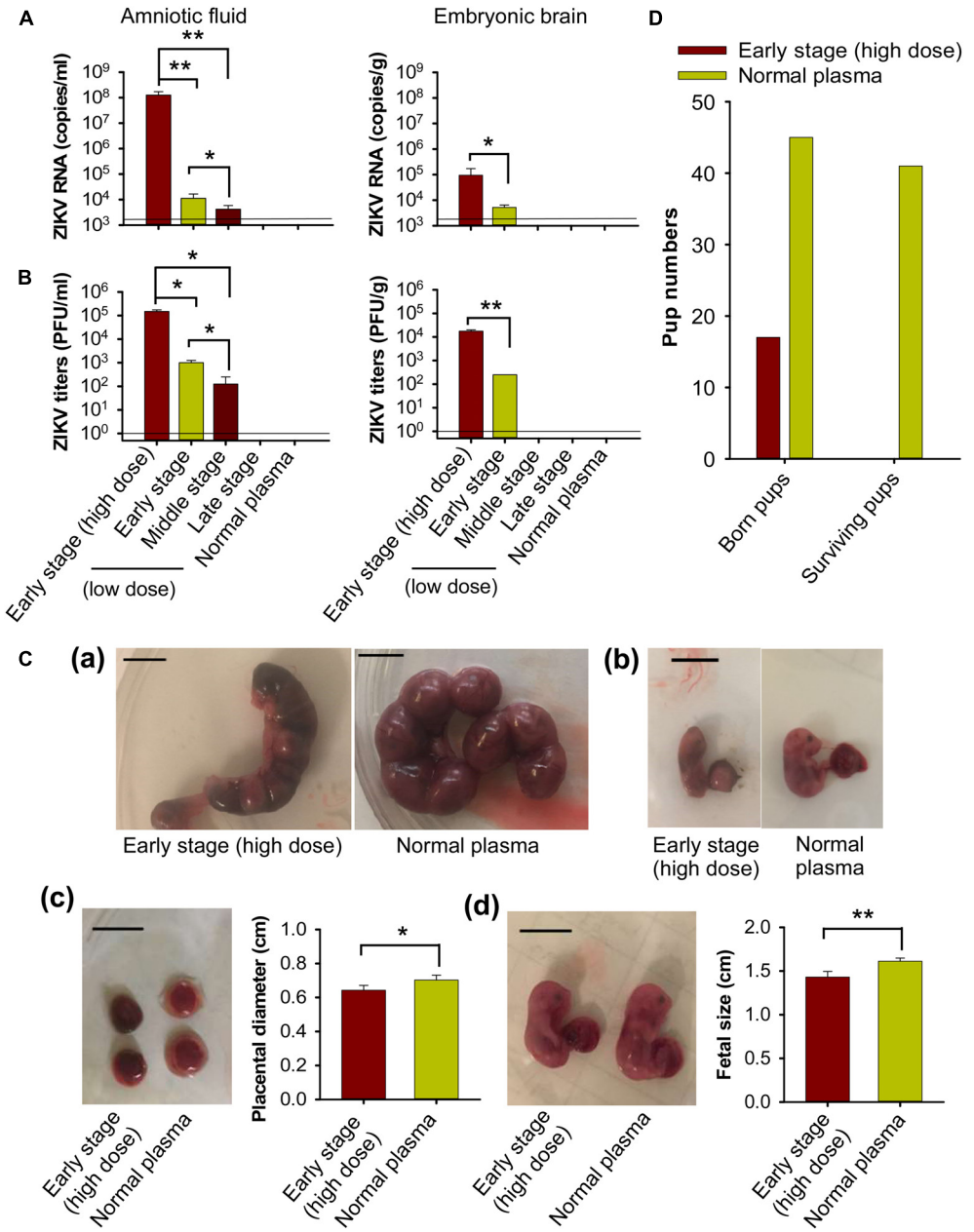

FIGURE 6 | Transfusion of pregnant A129 mice with early-stage (high dose) ZIKV$^+$ plasma led to significant fetal infection and fetal and pup death. Pregnant (E10–12) A129 mice were transfused with ZIKV$^+$ plasma from early, middle, and late stages, respectively. ZIKV RNA and ZIKV titers were detected by qRT-PCR **(A)** and plaque assay **(B)** in amniotic fluid and embryonic brain collected 6 days post-transfusion. The detection limit, shown as the horizontal lines, was about 2.5×10^3 RNA copies/ml and 1 PFU/ml (for amniotic fluid), or 2.5×10^3 RNA copies/g and 1 PFU/g (for embryonic brain), respectively, for qRT-PCR and plaque assay. * and ** indicate significant differences between early stage (high dose) and early or middle stages (low dose), or between early and middle stages (low dose). The data are presented as means ± s.e.m (n = 6 mice/group). **(C)** Morphology of mouse uteri and placentas and conditions of fetuses 6 days after transfusion of early-stage (high dose) ZIKV$^+$ plasma. **(a)** E13–15 uteri from pregnant mice transfused at E7–9. **(b)** Representative images of fetuses in **(a)**. **(c)** E16–18 placentas from pregnant mice transfused at E10–12. **(d)** Representative images of E16–18 fetuses in **(c)**. Scale bar: 1 cm. Placental diameter in **(c)** and fetal size in **(d)** are shown. * and ** indicate significant differences between early stage (high dose) and normal plasma groups. The data are presented as means ± s.e.m (n = 6 mice/group). **(D)** Total born and surviving pups 24 h after birth from pregnant (E10–12) mice transfused with early-stage (high dose) ZIKV$^+$ plasma. Normal plasma: control plasma from A129 mice transfused with normal plasma without ZIKV.

we speculate that persistent ZIKV viremia post non-lethal, low-dose ZIKV$^+$ plasma transfusion might be partially due to viral replication in organs that could act as potential reservoirs.

To determine which stages of ZIKV infection with the identified viral titers that will not cause problems to the transfused pregnant recipients, plasma collected above at early,

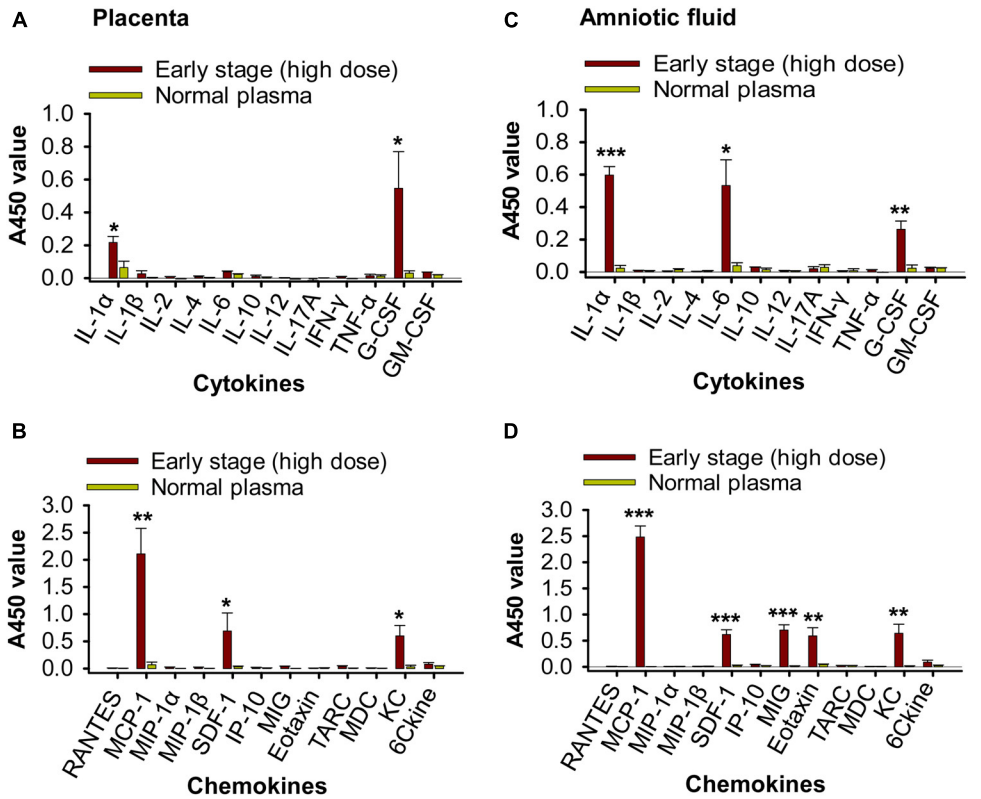

FIGURE 7 | Transfusion of pregnant A129 mice with early-stage (high dose) ZIKV$^+$ plasma led to significant inflammation. Placentas **(A,B)** and amniotic fluid **(C,D)** collected from pregnant (E10–12) A129 mice 6 days post-transfusion of early-stage (high dose) ZIKV$^+$ plasma were detected by Multi-Analyte ELISArray Kits for inflammatory cytokines **(A,C)** and chemokines **(B,D)**. *, **, and *** indicate significant differences between early stage (high dose) and normal plasma groups, and the data are presented as means ± s.e.m (n = 6 mice/group). Normal plasma: control plasma from A129 mice transfused with normal plasma without ZIKV.

middle, and late stages of ZIKV infection was transfused into pregnant A129 mice. We found that significantly higher viremia was identified in the pregnant mice receiving early-stage (high-dose) ZIKV$^+$ plasma than other stages of ZIKV$^+$ plasma, suggesting that transfusion-transmitted ZIKV infection at early stage containing high-titer ZIKV will lead to rapid ZIKV replication. Although ZIKV titers were significantly lower in the pregnant mice transfused with plasma at early-stage (low dose) ZIKV infection, it remains possible that such transfusion might have long-term effects.

ZIKV infection may trigger GBS (severe neuropathies) (Savino et al., 2017; Zhou et al., 2017; Hygino da Cruz et al., 2018; Wu et al., 2018) and it is closely associated with congenital Zika diseases (microcephaly and brain abnormalities) in humans (Franca et al., 2016; Shapiro-Mendoza et al., 2017; Zorrilla et al., 2017). ZIKV RNA replication has been identified in human brain and placental tissues (Bhatnagar et al., 2017). Our data show that while pregnant mice had low to no brain and congenital ZIKV infection upon transfusion with early-, middle-, and late-stage plasma containing the low-titer ZIKV, they had significant brain infection with identified ZIKV particles in the infected brain tissue after receiving the high-titer ZIKV$^+$ plasma. Notably, such transfusion also caused severe ZIKV replication in the placentas, amniotic fluid, and fetal brain, as well as inflammation in the placentas and fetal amniotic fluid, and had detrimental effects on the placentas, fetuses and newborn babies, causing fetal damage, and fetal and pup death. Overall, since elevated viral titers were associated with increased cell death and damaged vasculature in the placentas, loss of placental integrity in pregnant mice may have been a contributor of increased fetal and newborn mortality.

Clearly, identification of transfusion-transmitted ZIKV infection in brain and placental tissues of pregnant mice, as well as associated fetal damage and pup death by vertical transmission during pregnancy, further reinforce the need for surveillance measures to detect ZIKV in blood products. Our data from mouse studies also suggest that low ZIKV plasma titers might not be associated with establishment of transfusion-transmitted ZIKV infection in brain, placentas, and fetuses. It is thus reassuring that only high-titer ZIKV infection drives complications in pregnant mice and/or their fetuses and pups, suggesting that high risk of transfusion-transmitted ZIKV infection may be restricted to high viral titers at the early stage. Since murine and human placentas present different developmental anatomy and distinct co-expression patterns of certain genes (Georgiades et al., 2002; Soncin et al., 2018), infectious doses required for efficient ZIKV transfusion transmission in mouse and human placentas may differ. Thus, the transfusion studies performed in mice might not be completely consistent in human settings. It is possible that

humans receiving ZIKV-containing plasma or blood at different doses, particularly low dose, might have distinct consequences from mice. Nevertheless, considering that murine placentas have paralleling development to the first half of human placental development (through gestational week 16), and that there are still some similarities between human and mouse placentas in terms of structures and cell types (Georgiades et al., 2002; Soncin et al., 2018), the current data provide important information to infection window period and evaluate risk of transfusion-associated transmission by ZIKV through blood products in humans.

Our study has identified potential differences in transfusion-induced ZIKV tissue tropism and/or associated pathology compared to transmission by other routes. For example, ZIKV subcutaneous (S.C.) inoculation leads to detectable virus in peripheral nervous tissues, lymphoid tissues, joints, and uterus at day 7 post-infection (Hirsch et al., 2017). Other studies have shown that ZIKV infection via mosquito bite (e.g., route for human ZIKV infection) alters ZIKV tissue tropism, but the distribution is limited to hemolymphatic tissues, reproductive tract tissues, kidney, and liver (Dudley et al., 2017). More recently, ZIKV infection and congenital Zika syndrome were found to be also associated with critical human congenital heart disease (Angelidou et al., 2018). It is noted that pregnant mice infected with ZIKV through different routes also present variant symptoms, viral replication, and pathology in placentas, fetuses, and pups, and the doses required for efficient viral infection may vary. For instance, pregnant immunocompetent mice, such as BALB/c and C57BL/6, do not generally show significant clinical symptoms, viral replication, and tissue tropism via S.C., I.P., or intravenous (I.V.) routes after high-dose ZIKV infection ($\geq 10^5$ PFU), except for some fetal infection or fetal abnormalities (fetal resorption or growth restriction) (Yu et al., 2017; Caine et al., 2018; Szaba et al., 2018). However, pregnant C57BL/6 or BALB/c mice injected with anti-IFNAR antibodies may have significant viral replication in placentas and fetal heads after I.V. or S.C. infection of ZIKV (10^5–10^6 PFU) (Turner et al., 2017; Caine et al., 2018; Li et al., 2018). Particularly, A129 and AG129 mice infected with ZIKV through S.C. or I.P. routes demonstrate severe placental injury, fetal brain injury, shorter skull length, reduced size in fetuses and pups, or fetal and pup demise, with viral doses ranging from 10^2–10^4 PFU, focus forming unit (FFU), 50% tissue culture infectious doses ($TCID_{50}$), or 50% cell culture infectious doses ($CCID_{50}$) (Miner et al., 2016; Caine et al., 2018; Julander et al., 2018; Tai et al., 2018). Here we found that transfusion of A129 mice with early-stage $ZIKV^+$ (2.6×10^5 PFU) plasma induced broad tissue tropism 6 days post-transfusion, resulting in high viral titers in lung, spleen, kidney, heart, liver, muscle, brain, and placentas, as well as severe fetal infection and damage, accompanying with fetal and pup death. This consequence could be partially contributed to the high-dose ZIKV contained in the transfused plasma. Although other infection stages of plasma with the low-dose ZIKV did not cause significant tissue tropism at short-term (6 days) post-transfusion, ZIKV might replicate slowly but continuously, leading to long-term effects. It should be clarified that the ZIKV tissue tropisms and associated pathology resulting from various infection routes, as described above, did not count for different virus strains used for infection, hosts, age of animals, or time points post-infection. Therefore, comparative studies of viral titers by various routes of ZIKV transmission are needed to determine whether more severe outcome is linked to transfusion-transmitted ZIKV infection, and to develop a more comprehensive profile of ZIKV tropism in all tissues and related outcome to help better understand ZIKV pathogenesis.

To summarize, we report on the transmissibility and pathogenesis of transfusion-transmitted ZIKV infection in ZIKV-susceptible pregnant A129 mice. The data indicate that transfusion of plasma containing ZIKV at early-stage infection harboring high-titer ZIKV has serious consequences for pregnant mice, their fetuses and newborn pups. In contrast, transfusion-associated transmission with plasma from later stages of ZIKV infection, which contained low-titer ZIKV, might not be associated with ZIKV replication and have no serious outcome during pregnancy. Overall, the consequences from transfusion-transmitted ZIKV infection in the pregnant mice are positively correlated with ZIKV titers in the transfused plasma. Taken together, our data may help future design of studies to perform risk assessment of ZIKV transmissibility by blood transfusion in humans.

AUTHOR CONTRIBUTIONS

WT, SJ, KY, and LD designed the experiments. WT, DV, JC, and WB performed the experiments. WT, DV, JC, WB, DK, and BS analyzed the data. WT, DV, BS, SJ, KY, and LD wrote and revised the manuscript.

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Fetal-Derived MyD88 Signaling Contributes to Poor Pregnancy Outcomes During Gestational Malaria

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Placental malaria (PM) remains a severe public health problem in areas of high malaria transmission. Despite the efforts to prevent infection poor outcomes in *Plasmodium* endemic areas, there is still a considerable number of preterm births and newborns with low birth weight resulting from PM. Although local inflammation triggered in response to malaria is considered crucial in inducing placental damage, little is known about the differential influence of maternal and fetal immune responses to the disease progression. Therefore, using a PM mouse model, we sought to determine the contribution of maternal and fetal innate immune responses to PM development. For this, we conducted a series of cross-breeding experiments between mice that had differential expression of the MyD88 adaptor protein to obtain mother and correspondent fetuses with distinct genetic backgrounds. By evaluating fetal weight and placental vascular spaces, we have shown that the expression of MyD88 in fetal tissue has a significant impact on PM outcomes. Our results highlighted the existence of a distinct contribution of maternal and fetal immune responses to PM onset. Thus, contributing to the understanding of how inflammatory processes lead to the dysregulation of placental homeostasis ultimately impairing fetal development.

Keywords: mouse model, pregnancy, placenta, malaria, MyD88, *Plasmodium berghei*

INTRODUCTION

Placental malaria (PM) constitutes a serious public health issue in areas of high malaria transmission as pregnant women are particularly susceptible to *Plasmodium* infection (Fried and Duffy, 2017). Despite the significant efforts that have been done to prevent and treat malaria, every year a considerable number of newborns with low birth weight are still reported as a result of PM (Rogerson et al., 2018). In addition, malaria during pregnancy can affect the transplacental transfer of nutrients, which contributes to the impaired development of the fetus (Rogerson et al., 2007).

The negative consequences of PM are related to the accumulation of infected red blood cells (iRBCs) in the intervillous space of the placenta. During *P. falciparum* infections, this accumulation is mediated by the protein VAR2CSA (Doritchamou et al., 2012), a member of the *P. falciparum*

erythrocyte membrane protein 1 (PfEMP1) that promotes the binding of iRBCs to the chondroitin sulfate A (CSA) (Fried and Duffy, 1996; Beeson et al., 2000). The adhesion of iRBCs to the placenta is sufficient to induce an inflammatory process mainly through the recruitment of inflammatory cells and the production of pro-inflammatory mediators. Also, the immune response can disrupt a normal angiogenic process that can progress and culminate in low birth weight and preterm birth (Brabin et al., 2004; Desai et al., 2007; Umbers et al., 2011).

The trigger of pro-inflammatory mediators could be induced by the activation of the innate immune system via Pattern Recognition Receptors (PRRs) that recognize the Pathogen-associated molecular pattern (PAMPs). One of the most relevant PRRs family are the Toll-like receptors (TLRs). These receptors act as immune sensors for microorganisms and internal danger signals (Kawai and Akira, 2010). It is known that *Plasmodium* components can activate the TLR pathway (Franklin et al., 2009). As an example, *Plasmodium* glycosylphosphatidylinositol (GPI) is recognized by TLR2 and TLR4 (Krishnegowda et al., 2005), cytosolic RNA by TLR7 (Baccarella et al., 2013; Wu et al., 2014), and hemozoin and DNA by TLR9 (Parroche et al., 2007; Pichyangkul et al., 2004; Coban et al., 2010). Additionally, several TLRs polymorphisms have been associated with increased susceptibility to PM (Mockenhaupt et al., 2006; Leoratti et al., 2008; Hamann et al., 2010). All TLRs, except the TLR3, use as adaptor molecule the Myeloid differentiation primary response 88 (MyD88) protein. Upon activation of a TLR, the MyD88 allows the recruitment of several other proteins, which lead to a signaling pathway downstream that culminate in the production of pro-inflammatory proteins via MAPK and the NF-κB pathway (Kawai and Akira, 2010). The MyD88 protein was discovered in 1990 (Lord et al., 1990) and since then has been widely studied. MyD88, together with TLR7 or TLR9, has been associated as a protective factor for immunity to malaria (Gowda et al., 2012; Spaulding et al., 2016). Gowda et al. showed that MyD88$^{-/-}$ mice lacked cell-mediated immunity to malaria due to a reduction of pro-inflammatory proteins production. Additionally, the study shows that the MyD88 and TLR9 deficiency impaired NK and CD8^{+} T cell cytotoxic activity. Furthermore, other report showed that NK cell production of IFN-γ in response to *P. falciparum* infection is dependent of MyD88, which its expression in macrophages is crucial for an efficient response (Baratin et al., 2005). Accordingly, we have described that MyD88 and TLR4 signaling are fundamental to the placental inflammatory process induced by *Plasmodium* infection (Barboza et al., 2014, 2017).

Albeit the studies which agree that local inflammation in response to *Plasmodium* infection is crucial for the development of PM, little is known about the differential influence of maternal and fetal tissue to local immune response. Recently, Rodrigues-Duarte et al. (2018) presented results showing that fetal-derived TLR4 contributes to the iRBCs uptake by trophoblasts and to placental innate immune responses triggered in response to *P. berghei* infection.

Herein, we used an experimental mouse model that manifests several clinical features of the human disease in order to determine the contribution of maternal and fetal innate immune activation on PM pathogenesis. Therefore, we conducted a series of cross-breeding between mice with different MyD88 genotypes to generate several fetal genotypes born from mothers with distinct MyD88 backgrounds. Our findings have shown that the expression of fetal-derived MyD88 is detrimental for illness-induced complications of PM. These results extend and complete our previous observations showing the crucial role of MyD88 for local inflammation and consequent disease onset (Barboza et al., 2014).

MATERIALS AND METHODS

Animals

C57Bl/6 WT, MyD88$^{+/-}$, and MyD88$^{-/-}$ mice with 8- to 10-weeks were bred and maintained in conventional housing with constant light-dark cycle (12 h:12 h) at the Animal Facility of the Department of Parasitology from the Institute of Biomedical Sciences at the University of São Paulo (ICB/USP). Mice received water and were fed *ad libitum* with commercial NUVILAB CR-1 ration (Nuvital®, Brazil). All experiments were performed in accordance with the ethical guidelines for experiments with mice, and the protocols were approved by the Animal Health Committee of the Institute of Biomedical Sciences of the University of São Paulo (CEUA No. 015fls82livro2). The guidelines for animal use and care were based on the standards established by the National Council for Control of Animal Experimentation (CONCEA).

Cross-Breeding Experiments

To study the contribution of maternal and fetal MyD88 expression to PM development, heterogenic litters from females with heterozygous (**Figure 1A**) or null (**Figure 1B**) MyD88 expression were generated and then compared to WT and MyD88 full knockout fetuses/mothers. The generation of knockout (MyD88$^{-/-}$) and heterozygous (MyD88$^{+/-}$) fetuses from heterozygous mothers started by mating MyD88$^{-/-}$ males with C57Bl/6 WT females (I from **Figure 1A**) to obtain a heterozygous progeny (II from **Figure 1A**). From these offspring, MyD88$^{+/-}$ females were backcrossed with MyD88$^{-/-}$ males (II from **Figure 1A**) and the fetuses and placentas (diamonds, sex not defined) from generation III were evaluated. Additionally, to obtain MyD88$^{+/-}$ fetuses from MyD88$^{-/-}$ mothers, we crossed C57Bl/6 WT males with MyD88$^{-/-}$ females (I from **Figure 1B**), and the fetuses and placentas (diamonds, sex not defined) from generation II were evaluated.

MyD88 Genotyping

To determine the genetic background of the generated offspring, tail genomic DNA was isolated, and genotyping was performed through conventional PCR using the following set of primers: Forward – 5′ TGG CAT GCC TCC ATC ATA GTT AAC C 3′; Reverse – 5′ GTC AGA AAC AAC CAC CAC CAT GC 3′; and, MyD88 neo – 5′ ATC GCC TTC TAT CGC CTT CTT GAC G 3′ (Leadbetter et al., 2002). The forward primer is common for both PCR reactions, while the reverse is specific for the wild-type gene and the neo is specific for the deficient gene. The PCR products

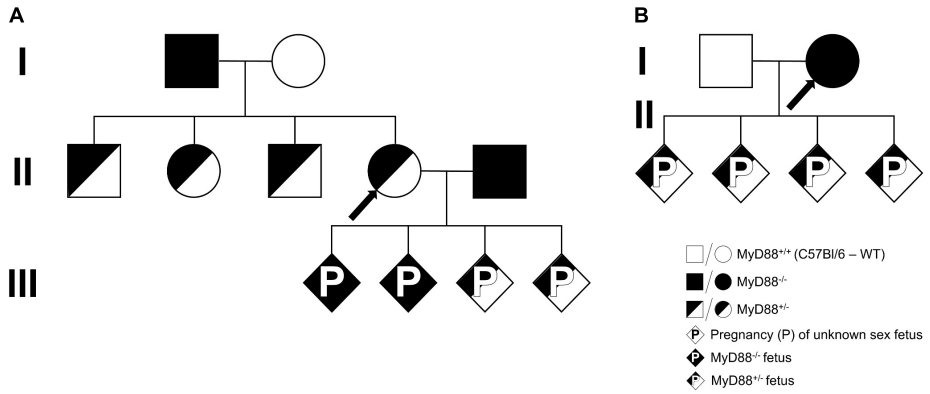

FIGURE 1 | Breeding scheme. A schematic of the breeding strategy used to generate mice containing combinations of the maternal and fetal MyD88 genotypes. **(A)** MyD88 deficient (MyD88$^{-/-}$) males (black square) mated with C57Bl/6 (MyD88$^{+/+}$) females (white circle), generation **I**, to produce a heterozygous progeny (MyD88$^{+/-}$, half-shaded symbols). MyD88$^{+/-}$ females (half-shaded circle) from generation **II** were backcrossed with MyD88$^{-/-}$ males (black square) to obtain fetus with MyD88$^{+/-}$ (half-shaded diamond) and MyD88$^{-/-}$ (black diamond) genotypes, generation **III**. **(B)** C57Bl/6 (MyD88$^{+/+}$) males (white square) mated with MyD88 deficient (MyD88$^{-/-}$) females (black circle), generation **I**, to obtain fetus with MyD88$^{+/-}$ (half-shaded diamond) genetic background, generation **II**. Diamond represents pregnancy (P) of a fetus with unknown sex. The arrows depict the MyD88 maternal genotype for each MyD88 fetal genotype (**IIIA, IIB**).

were subjected to 1% agarose gel electrophoresis to verify the presence of a MyD88 allele (**Supplementary Figure 1**).

Pregnancy Monitoring and Experimental Infection

Pregnancy was monitored as described elsewhere (Freyre et al., 2006). Briefly, we combine the detection of a vaginal plug and body weight measurement to determine the gestation time. The day in which a vaginal plug was detected was considered gestational day 1 (G1) and, from there on, pregnancy progression was monitored every day by regularly controlling weight variation. Successful fecundation was confirmed between G10 and G13 when females exhibited an average increase of 3–4 g in their body weight. Therefore, weight gain was taken as an indication of pregnancy, whereas unexpected weight loss was interpreted as an indicator of pregnancy complications or interruption. As such, pregnant mice were intravenously inoculated at G13 with 10^5 *P. berghei* NK65^{GFP} iRBCs stored in frozen vials, and parasitemia was assessed. G13 was determined as the optimal time point for the infection, allowing to analyze the pathological features of malaria along the course of pregnancy and in the developing fetus. The infection at an earlier stage would not allow reaching the pregnancy term, which is consistent with previous reports (Neres et al., 2008; Marinho et al., 2009). Parasitemia was assessed by Giemsa-stained thin blood smears until G19, gestational day that cesarean section was performed. Fetal weight was measured, and placentas were used for histopathological analysis and RNA extraction. Non-pregnant infected and non-infected pregnant mice were used as controls whenever appropriate.

Placenta Collection and Morphometric Analysis

Placentas from infected and non-infected pregnant mice were processed similarly. Briefly, placentas were divided into halves: one-half was fixed in 1.6% paraformaldehyde supplemented with 20% sucrose for further processing, and the other half was collected into an RNA stabilizer (RNAlater^{TM}, Invitrogen^{TM}, CA) for subsequent RNA extraction. Non-consecutive paraffin-embedded placentas were sectioned and stained with hematoxylin-eosin (H&E) for further microscope examination.

The placental morphometric analysis was performed as previously described (Marinho et al., 2009; Rodrigues-Duarte et al., 2012). In short, vascular spaces were quantified by analyzing the hematoxylin-eosin (H&E) stained sections. For each section, three areas of intervillous space were randomly selected for image acquisition (200× magnification) using a Zeiss color camera (Axio Cam HRc) connected to a Zeiss light microscope (Axio Imager M2). Images were analyzed using an Image J software[1]. Briefly, images were subjected to an automated light analysis procedure in which noise removal was applied to ensure color and image quality standardization across sections and specimens. Images were given a color threshold to cover the area corresponding to blood space lumen. Percentage of coverage was calculated as the ratio between the number of pixels covered by the threshold-defined area and the total amount of pixels in the image. Blood vascular area in each placenta was assessed from the analysis of three non-consecutive sections. Reported results correspond to individual pregnant mice, representing the average result from 3–9 placentas.

Gene Expression Analysis by qPCR

Total RNA was extracted from each placenta obtained at G19 using the RNeasy Minikit (Qiagen) in accordance with manufacturer's protocol for animal tissue (Animal Cell 1). One microgram of mRNA was converted into cDNA using First Strand cDNA Synthesis Transcriptor kit (Roche, Penzberg, Germany). The expression of *Il6*, *Il10*, *Tnf*, *Cxcl1* (KC),

[1]http://rsbweb.nih.gov/ij/

Ccl3 (MIP-1α) and *Ccl4* (MIP-1β) was quantified by using the following TaqMan® probes: *Il6* (Mm00446190_m1), *Il10* (Mm01288386_m1), *Tnf* (Mm00443258_m1), *Cxcl1* (Mm04207460_m1), *Ccl3* (Mm00441259_g1) and *Ccl4* (Mm00443111_m1). Gene expression quantifications were performed according to the manufacturer's instructions on Applied Biosystems 7500 Fast Real-Time PCR System. All results were obtained through the comparative ΔΔCT method after normalization to the constitutive expression of the GAPDH gene (*gpdh*: Mm99999915_g1).

Statistical Analysis

Statistical analysis was performed using the GraphPad Prism version 7.0 software (GraphPad Software, San Diego, CA, United States). To evaluate survival curves, the Long-rank test was used, the differences between groups were evaluated using the One or Two-way analysis of variance (ANOVA) as indicated at the figure's legends. *P*-values < 0.05 were considered statistically significant.

RESULTS

MyD88 Expression Influences the Progression of Murine Malaria Infection

In a previous study, we have shown that the MyD88 pathway is essential for the development of PM (Barboza et al., 2014). However, the results did not allow for individualizing the fetal-

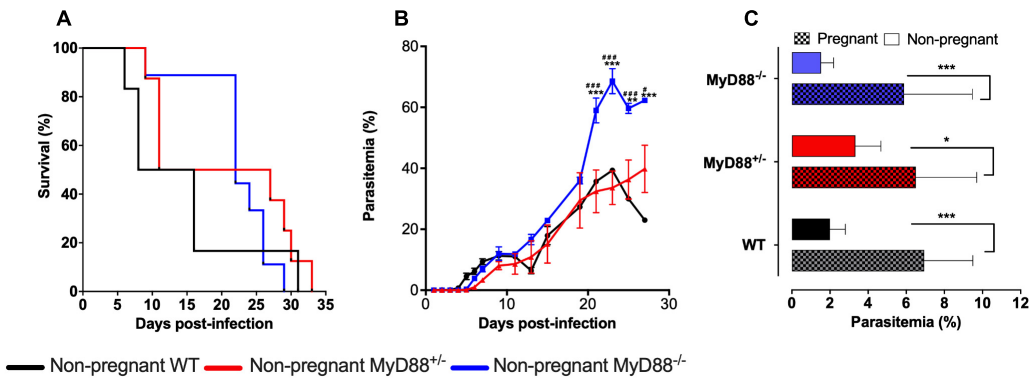

FIGURE 2 | MyD88 expression influences disease progression in mice infected with *P. berghei* NK65. **(A,B)** Non-pregnant C57Bl/6 (WT), MyD88 deficient (MyD88$^{-/-}$) and heterozygous (MyD88$^{-/-}$) mice were infected intravenously with 10^5 *P. berghei* NK65^{GFP} iRBCs. Evaluation of Kaplan-Meier survival curves **(A)** and parasitemia levels **(B)**. **(C)** Pregnant WT, MyD88$^{+/-}$ and MyD88$^{-/-}$ mice were intravenously infected at gestational day 13 (G13) with 10^5 *P. berghei* NK65^{GFP} iRBCs. Parasitemia was measured at G19 before C-section and compared with non-pregnant mice, also with 6 days of infection. Plain bars represent non-pregnant mice, and square-patterned bars represent pregnant mice. In B and C data are presented as mean ± SD. The statistical differences were achieved by a Long-rank (Mantel-Cox) test **(A)** and Two-way analysis of variance (ANOVA) with the Bonferroni's *post hoc* test **(B,C)**. $^{**}P$-value <0.01 and $^{***}P$-value < 0.001 when compared with WT **(B)** or non-pregnant mice **(C)**; $^{\#}P$-value < 0.05 and $^{\#\#\#}P$-value < 0.001 when compared with MyD88$^{+/-}$ mice **(B)**.

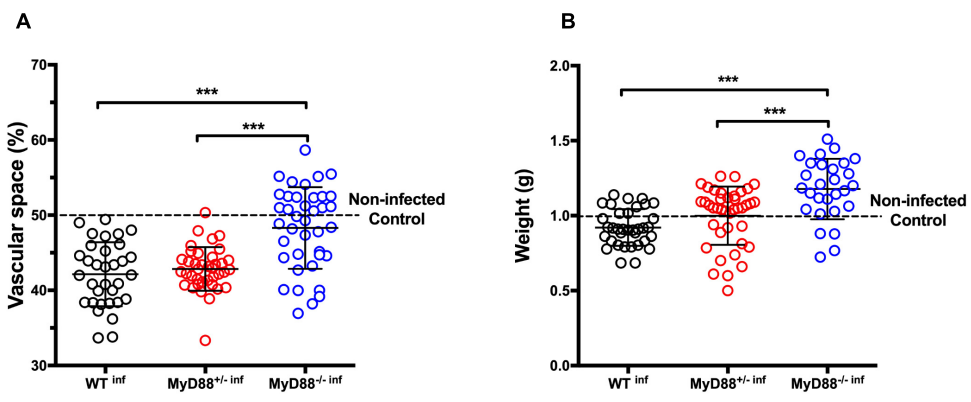

FIGURE 3 | MyD88 expression is associated with reduced placental vascular space and fetal weight in mice infected with *P. berghei* NK65. Pregnant C57Bl/6 (WT), and MyD88 deficient (MyD88$^{-/-}$) and heterozygous (MyD88$^{-/-}$) mice were intravenously infected (^{inf}) with 10^5 *P. berghei* NK65^{GFP} iRBCs at gestational day 13 (G13) and C-section performed at G19. The placenta/fetus share the same maternal MyD88 genotype. **(A)** Placental vascular spaces (n: WT^{inf} – 33; MyD88$^{+/-inf}$ – 38; MyD88$^{-/-inf}$ – 39) and **(B)** fetal weight (n: WT^{inf} – 35; MyD88$^{+/-inf}$ – 40; MyD88$^{-/-inf}$ – 28) measures. Data are presented as scatter plot with indication of the median ± SD; each dot represents an independent measurement. Dotted line represents mean values of the control group (non-infected pregnant WT mice; n: 34, vascular space, and 32, fetal weight). The statistical differences were achieved by a One-way analysis of variance (ANOVA) with *post hoc* Tukey's test. $^{***}P$-value < 0.001.

and maternal-derived MyD88 specific role in PM pathogenesis. To ascertain this, a series of experiments were conducted using C57Bl/6 (MyD88$^{+/+}$ – WT), MyD88$^{+/-}$ and MyD88$^{-/-}$ mice, evaluating their survival and parasitemia progression upon infection with *P. berghei* NK65-iRBCs. The distinct groups of mice did not present significant differences regarding the survival rates when compared among each other (**Figure 2A**). However, it was possible to note that the MyD88$^{-/-}$ group started to die later, probability due to the augment of parasitemia levels observed from the twentieth day post-infection onward (**Figure 2B**).

The evaluation of parasitemia did not show significant differences, with MyD88$^{+/-}$ mice behaving similarly to the WT group (**Figure 2B**). Moreover, our data corroborate previous studies showing that pregnancy increases mice susceptibility to infection, irrespective of the maternal genotype (**Figure 2C**).

Although there were no differences between peripheral parasitemia of pregnant mice with different genotypes, we hypothesized that heterozygous and null MyD88 expression might be involved in placental damage and poor pregnancy outcomes. Therefore, we evaluated the placental vascular spaces as an indicator for placental abnormalities. The expression of only one MyD88 allele is sufficient to induce placental structural changes as similarly to WT infected mice (WT^{inf}), MyD88$^{+/-}$ infected mice (MyD88$^{+/-inf}$) presented reduced vascular spaces (**Figure 3A**). On the other hand, the absence of the two MyD88 alleles (MyD88$^{-/-inf}$) showed no infection deleterious impact in the placental vasculature, identical to the non-infected controls (dashed line). Moreover, fetal weight exhibited a similar pattern. Like fetuses born from WT^{inf}, the progeny from heterozygous MyD88 pregnant mice (MyD88$^{+/-inf}$) also presented reduced

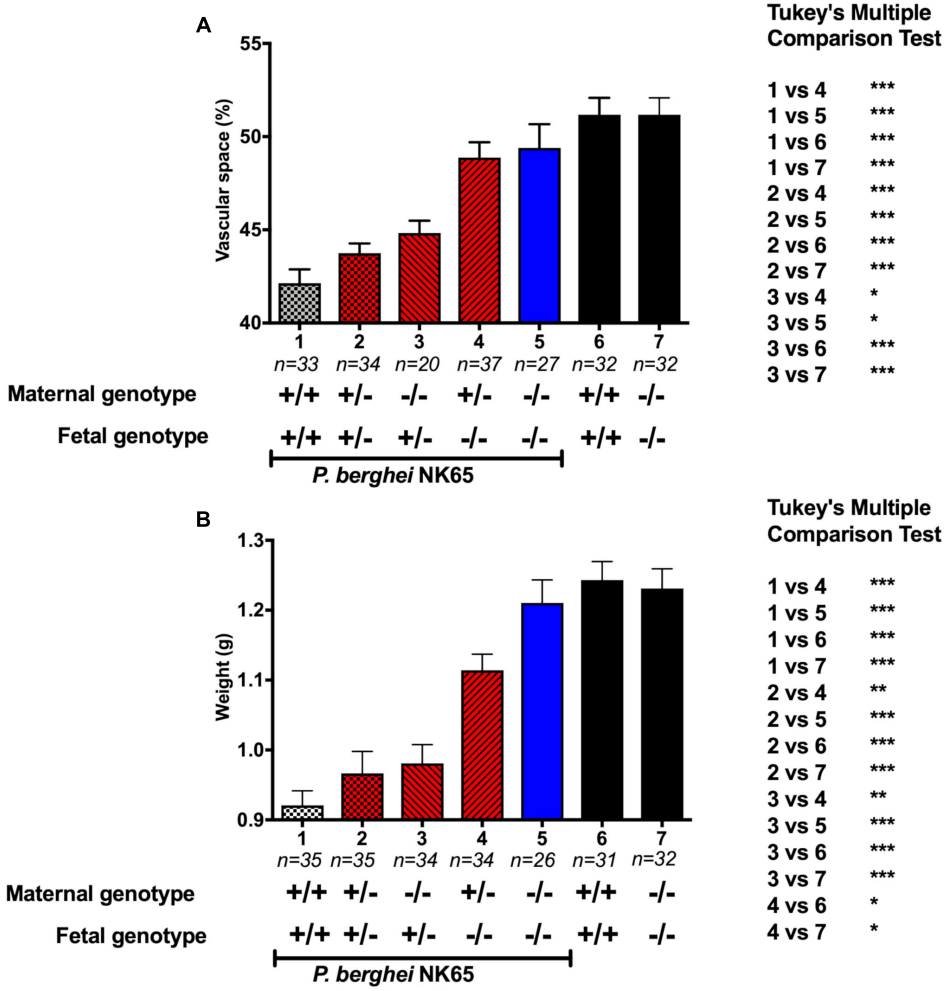

FIGURE 4 | Fetal MyD88 expression is detrimental to disease development in an experimental mouse model of PM. To ascertain the differential contribution of the maternal- and fetal-derived MyD88 expression different combinations of maternal/fetal genotypes were evaluated. Pregnant mice were intravenously infected with 10^5 *P. berghei* NK65^{GFP} iRBCs at gestational day (G13), with the following maternal-fetus pairs: WT/WT (1), MyD88$^{+/-}$/MyD88$^{+/-}$ (2), MyD88$^{-/-}$/MyD88$^{+/-}$ (3), MyD88$^{+/-}$/MyD88$^{-/-}$ (4), and MyD88$^{-/-}$/MyD88$^{-/-}$ (5). C-section was performed at G19 and fetus were genotyped and evaluated. **(A)** Placental vascular spaces and **(B)** fetal weight measures. Infected WT maternal-fetus pair is a positive control (1) and non-infected WT and MyD88$^{-/-}$ maternal-fetus pairs are negative controls (6 and 7, respectively). Data are presented as mean ± sem. The statistical differences were achieved by a One-way analysis of variance (ANOVA) with Tukey's *post hoc* test. **P*-value < 0.05; ***P*-value < 0.01; ****P*-value < 0.001.

fetal weight upon infection. Notably, in the fetuses born from MyD88$^{-/-}$ infected mice malaria did not impair their weight when compared to WT^{inf} and MyD88$^{+/-inf}$ mice (**Figure 3B**). The fetal weight in these fetuses reached values resembling those from healthy WT mice.

MyD88 Expression on Fetal-Derived Tissue Is Detrimental for Pregnancy Outcomes

To investigate the role of maternal- and fetal-derived MyD88 in PM-associated pregnancy outcomes. We performed a comparative analysis between placental vascular spaces (**Figure 4A**) and fetal weight (**Figure 4B**) associated with different MyD88 genotypes, pairing maternal and fetal genetic backgrounds. Including an infected WT maternal-fetus pair as a positive control (group 1) and as negative controls a non-infected WT and MyD88$^{-/-}$ maternal-fetus pairs (group 6 and 7, respectively). The results demonstrate that the presence of only one fetal-derived MyD88 allele is sufficient to contribute to a prominent reduction of placental vascular spaces (**Figure 4A**, groups 2 and 3) and fetal weight (**Figure 4B**, groups 2 and 3) upon *P. berghei* NK65 infection when comparing to full knockout fetuses (**Figure 4**, group 4 and 5). Also, these results show that there are no influence of maternal MyD88 genotype since MyD88$^{-/-}$ fetuses from infected MyD88$^{+/-}$ did not have a negative impact on either vascular spaces or fetal weight (**Figure 4**, group 4).

To validate our results, we performed a second multivariable statistical analysis, excluding the control groups (1, 6, and 7) from the analysis. The new analysis reinforced the idea that the fetuses genotype has a significant effect on both fetuses' weight and the placental vascular space while the maternal genotype does not (**Supplementary Figure 2** and **Supplementary Table 1**). Together, our results support the idea that fetal-derived MyD88 is associated with local parasite sensing, triggering inflammation and, consequently leading to poor pregnancy outcomes and PM development. Interestingly, the deletion of one maternal MyD88 allele was sufficient to negatively modulate the expression of placental inflammatory cytokines mRNA, except for the *Il10* (**Supplementary Figure 3**), indicating the possible involvement of other mechanisms.

DISCUSSION

Innate immune activation during pregnancy represents a substantial problem for the mother and the growing fetus. Regarding *Plasmodium* infection, it has been shown that innate immune activation in response to parasite accumulation results in placental injuries, which ultimately leads to impaired fetal development. Previously, we have demonstrated that MyD88 signaling is associated with poor pregnancy outcomes (Barboza et al., 2014, 2017). Other studies also have unveiled that the MyD88 protein plays an important role in inducing immune protection to *Plasmodium* infection (Baratin et al., 2005; Gowda et al., 2012; Spaulding et al., 2016). Herein, using mice cross-breeding strategies, we managed to obtain distinct MyD88 genetic backgrounds and determine the maternal- and fetal-derived MyD88 contribution to PM. Our results extend previous observations, showing that fetal-derived MyD88 is markedly associated with murine PM development.

Since the placenta is constituted by cells from at least two different individuals, one can argue that those cellular components can differentially influence placental physiology. The results presented here evidence the contribution of fetal-derived MyD88 expression in the disease onset, highlighting the influence of fetal components in detriment of the maternal components. Such impact is perceived when we evaluate placental vascular spaces of infected pregnant mice. Our work revealed that placental vascular spaces reduction upon infection is mediated by fetal-derived MyD88, even when only one allele is carried (**Figure 4A** group 2 and 3 and **Supplementary Figure 2**). Of note, the importance of this observation is supported by the minor impact of maternal MyD88 expression (**Figure 4A** group 4 and **Supplementary Figure 2**). Previously, it was described that in murine PM the reduction of placental vascular spaces is correlated with low birth weight (Neres et al., 2008; Marinho et al., 2009; Medeiros et al., 2013; Barboza et al., 2014). In fact, we also observed a parallel reduction of fetal weight, which was equally determined by fetal-derived MyD88 expression (**Figure 4B** group 2 and 3). We should emphasize that the deletion of the MyD88 gene does not impact the mice growth nor produces abnormalities (Adachi et al., 1998), as well as have no influence in pregnancy in general, as non-infected MyD88$^{-/-}$ and MyD88$^{+/-}$ have no differences in the pregnancy outcome.

In line with our observation, Rodrigues-Duarte et al. (2018) have recently reported that maternal and fetal counterparts act in opposite directions during murine PM. This study showed that TLR4 and IFNAR1 expression in the maternal tissue has a deleterious effect on fetal development, which is counteracted by fetal tissue. The role of these family of innate immune receptors on PM has been extensively studied in humans and murine malaria models (Adegnika et al., 2008; McDonald et al., 2015; Odorizzi and Feeney, 2016; Barboza et al., 2017; Rodrigues-Duarte et al., 2018). These studies have shown that local immune activation via innate immune receptors is correlated with placental damage, which results in impaired fetal development.

The negative impact of innate immune activation observed in the fetuses is correlated with a rupture of maternal-fetal interactions during pregnancy. For instance, to achieve a successful pregnancy it is necessary that trophoblasts (fetal-derived cells which populate most of the placenta) and maternal decidual immune cells interact, allowing the development of the embryo in the uterus (Prabhudas et al., 2015). One way of promoting this rupture is through the activation of MyD88-dependent pathways induced by TLR activation in trophoblasts, which are known to broadly express TLRs (Abrahams et al., 2004; Mitsunari et al., 2006; Lucchi and Moore, 2007; Aldo et al., 2010; Tangerås et al., 2014). Moreover, TLR activation has been associated with pregnancy complications (Mockenhaupt et al., 2006; Rindsjö et al., 2007; Leoratti et al., 2008; Hamann et al., 2010; Pineda et al., 2011; Koga et al., 2014). Thus, TLR activation by the parasite components can lead to NF-κB activation and, as a consequence, to the production of pro-inflammatory mediators

such as chemokines and cytokines, contributing to tissue damage and PM development.

Nevertheless, our work may present some limitations. The PM murine models recapitulate many features observed in the human's disease, which represent numerous advantages in the research and acquisition of knowledge of this disease (Hviid et al., 2010). Though, human and mouse pregnancy differences do not allow us to make direct assumptions, such as the placental architecture or the immunological responses (Moffett and Loke, 2006; Carter, 2007). Hence, it would be of crucial relevance the validation of murine results with human samples, whenever is possible. Following our results in murine models, it would be interesting to ascertain the correlation of the maternal and fetal genetic interplay with pregnancy outcomes upon *Plasmodium* infection.

Our results on the placental vasculature and fetal weight obtained using heterogenic mice progeny from different maternal MyD88 genetic backgrounds have evidenced a marked impact of the fetal immune system in the PM onset. In summary, this study highlights the importance of maternal and fetal immune response in the context of pregnancy-associated malaria, which ultimately contributes to the placental pathology and poor pregnancy outcomes.

AUTHOR CONTRIBUTIONS

RB and CM designed the study. LH, RB, OM, AB, EP, FL, AR, LG, SE, and CM were involved in data acquisition and scientific input. RB, LH, LG, SE, and CM contributed to the analysis and/or interpretation of the data. RB, LG, and CM wrote the manuscript. SE revised the manuscript. CM, SE, and RB were the main funders of this work. CM has full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. All authors reviewed and approved the definitive version of this manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00068/full#supplementary-material

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Maternal-Fetal Conflict During Infection: Lessons From a Mouse Model of Placental Malaria

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Infections that reach the placenta via maternal blood can target the fetal-placental barrier and are associated with reduced birth weight, increased stillbirth, miscarriage and perinatal mortality. Malaria during pregnancy can lead to infection of the placental tissue and to adverse effects on the unborn child even if the parasite is successfully cleared, indicating that placental sufficiency is significantly compromised. Human samples and animal models of placental malaria have been used to unravel mechanisms contributing to this insufficiency and have implicated molecular pathways related to inflammation, innate immunity and nutrient transport. Remarkably, fetal TLR4 was found to take part in placental responses that protect the fetus, in contrast to maternal TLR4 responses that presumably preserve the mother's health but result in reduced fetal viability. We propose that this conflict of fetal and maternal responses is a determinant of the clinical outcomes of placental malaria and that fetally derived trophoblasts are on the front lines of this conflict.

Keywords: placenta, malaria, maternal-fetal conflict, toll-like receptors, pregnancy

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1. INTRODUCTION

Pregnant women are at a higher risk of malaria infection (Espinosa et al., 2000; Lindsay et al., 2000). Infection by *Plasmodium falciparum* contributes to adverse outcomes including premature delivery, intra-uterine growth restriction, stillbirth and perinatal death alongside worsened maternal anemia and increased maternal parasite loads (Menendez et al., 2000; Crocker et al., 2004). These outcomes have been found to occur in pregnancies several months after clearance of the parasite, illustrating that treatment of the infection alone may be of little benefit (Schmiegelow et al., 2017).

Placental infection is a key determinant of these outcomes and the molecular basis of placental malaria pathology has been intensively studied (recently reviewed by Fried and Duffy, 2017). Parasite sequestration in the placenta is the primary pathological event, and, in the case of *Plasmodium falciparum*, is primarily mediated by infected erythrocytes binding to chondroitin sulfate A on the surface of syncytiotrophoblasts (Fried and Duffy, 1996; Abrams et al., 2003; Miller et al., 2013; Moya-Alvarez et al., 2014). Interactions between infected erythrocytes and placental tissue trigger significant infiltration of maternal inflammatory cells (Fried and Duffy, 2017) and alterations in the profile of cytokines secreted in the placenta, namely increases in TNFα and IFN-γ which are linked to adverse pregnancy outcomes (Moormann et al., 1999; Muehlenbachs et al., 2007).

Placentas from infected women show functional alterations including reduction in the activity of system A, a group of sodium dependent amino acid transporters which actively uptake small

amino acids into the trophoblast layer (Boeuf et al., 2013). Placental glucose transporter activity is reduced when infection is accompanied by intervillositis (Chandrasiri et al., 2013). Malaria also reduces placental megalin, a transporter for a vast array of proteins (Lybbert et al., 2016). These nutrient transport pathways depend on an adequate placental blood supply to function effectively and placentas from women infected with malaria exhibit reduced placental perfusion (Dorman et al., 2002; Brabin and Johnson, 2005), impaired trophoblast invasion (Umbers et al., 2013), and alterations in various angiogenic factors within the placenta (Ataíde et al., 2015) which corroborates suboptimal placental perfusion.

More recently, a prospective study revealed that blood levels of L-arginine, a precursor to the potent vasodilator nitric oxide, were reduced in women with placental malaria while levels of dimethylarginine, an inhibitor of nitric oxide biosynthesis, were increased. These changes were strongly correlated with worse birth outcomes (McDonald et al., 2018). Earlier studies have highlighted a potential role for vascular endothelial growth factor (VEGF) and its receptors in the response to placental malaria in primigravid mothers, with soluble receptors for VEGF being more abundantly expressed in the placenta. This further implicates circulatory impairments in the disease pathology and provides the first evidence that placental responses to infection may not be in harmony with maternal responses, as maternal cells in the placenta showed elevated VEGF levels whereas fetal syncytiotrophoblasts produced more sVEGFR1, reducing VEGF bioavailability (Muehlenbachs et al., 2006).

These findings suggest that the intertwining of inflammatory signals, vasoregulatory systems, and nutrient transport pathways in the placenta are critical components of human placental malaria pathophysiology. However, experimental demonstration of the pathogenic mechanisms operating in the placenta relies on available mouse models of disease. In this perspective article we explore evidence generated from a mouse model of acute placental malaria that highlights the role of toll-like receptor 4 (TLR4) in controlling the outcomes of pregnancy ergo providing an interesting example of infection provoking conflict between the mother and the unborn child.

2. PATHOGENESIS OF MURINE ACUTE PLACENTAL MALARIA

Several murine experimental systems have been used to model specific aspects of malaria in pregnancy, but extrapolations to human disease should be considered with caution (Hviid et al., 2010). These experimental systems model different aspects of malaria in pregnancy, including: use of recrudescent *Plasmodium berghei* ANKA to study maternal susceptibility to infection (Marinho et al., 2009); a system using *P. berghei* K173 strains to infect mice both prior to and during gestation as would occur in high transmission settings (Van Zon and Eling, 1980); and a system making use of *Plasmodium chabaudi* which allows for the study of infections in early stages of pregnancy (Poovassery et al., 2009). Here, we will focus on a model which makes use of *Plasmodium berghei* infection during gestation and which models acute malaria during pregnancy in women. Briefly, infecting naïve, primigravid BALB/c females with 10^6 *P. berghei ANKA* infected erythrocytes intra-venously on the 13th day of gestation results in severe disease outcomes, such as intra-uterine growth restriction, decreased fetal viability, post-natal growth impairment and increased maternal parasitemia and anemia (Neres et al., 2008). Similar results are obtained with the use of the NK65, K173 and ANKApm4 lines of *P. berghei* in primigravid C57BL/6 mice, following the same mating and dosage protocols (Rodrigues-Duarte et al., 2012). Examination of the placentas with acute infection revealed an accumulation of infected erythrocytes and hemozoin in the blood sinusoids (Sharma et al., 2012b), thickening of the labyrinthine zone, deposits of hemozoin, fibrinoid necrosis, hyperplasia of the syncytiotrophoblasts, reduced blood sinusoid area, and a significant infiltration of maternal macrophages and monocytes (Neres et al., 2008).

This model has allowed investigation of the underpinnings of placental dysfunction, particularly by linking inflammatory responses to alterations in angiogenic and vasoregulatory pathways. This is illustrated by descriptions of increases in the amounts of angiopoietin 1 and in the ratio of angiopoietin 1 to angiopoietin 2 in infected placentas belonging to viable, low birth weight offspring (Silver et al., 2010) as well as by the reduced expression of bradykinin receptor B2 and NOS3 genes, both known to be involved in vasodilatory responses (de Moraes et al., 2018). Human studies have also revealed that infection with *P. falciparum* during pregnancy increases levels of these angiopoietins and complement C5a while reducing nitric oxide bioavailability (Conroy et al., 2013; McDonald et al., 2018). Genetically ablating C5a receptor in mice infected with *P. berghei* during pregnancy increased placental vascular branching and ameliorated the increase in resistance to flow caused by infection (Conroy et al., 2013). Similar results were obtained by dietary supplementation with L-arginine, a nitric oxide precursor (McDonald et al., 2018). Furthermore, intra-vital imaging (Lima et al., 2014) has revealed how infected erythrocytes accumulate in areas of slower flow in the placental labyrinth, possibly adhering to, or being phagocytosed by, the syncytiotrophoblasts and suggesting that infection impairs local circulatory regulation (de Moraes et al., 2013). Additionally, oxidative stress has been implicated in malaria during pregnancy, with a combination of increased lipid perodixation (Sharma et al., 2012a), decreased catalase activity and increases in apoptosis markers being observed in the placentas of infected mice while the absence of alterations in Fas expression and Caspase 8 indicate that the damage caused is primarily via the mitochondrial pathway of apoptosis (Sharma et al., 2012b). Treating the infected mice with chloroquine or with sulfadoxine pyrimethamine abrogated oxidative stress, apoptosis, and placental damage, consequently improving birth weight. Interestingly, anti-malarial treatment of mice with placental malaria did not improve fetal survival, indicating that placental insufficiency is not recoverable by parasite clearance alone (Sharma and Shukla, 2014). This raises the possibility that innate immune stimulation during pregnancy results in enduring placental dysfunction. Here, we argue that responses mediated by Toll-like receptors (TLRs), particularly

TLR4, have a decisive impact on the development of placental pathologies during infection.

3. TLR4, MALARIA, AND PREGNANCY

TLRs are a class of pattern recognition receptors involved in the detection of, and in the response to, pathogen and damage associated molecular patterns (PAMPs and DAMPs) by activation of downstream signaling pathways which induce immunity mediators including pro-inflammatory cytokines and interferons. TLR signaling makes use of either of two adaptor proteins, MyD88 or TRIF, with TLR4 being unique in its ability to use both of these pathways (**Figure 1D**) (Lu et al., 2008; Kawasaki and Kawai, 2014).

TLRs play a significant role in the response to malaria infection, participating in the recognition of glycosylphosphatidylinositol anchors, peroxiredoxin and fibrinogen/hemozoin complexes as well as host derived microvesicles and heme (Eriksson et al., 2014; Gazzinelli et al., 2014) (**Figure 1C**). TLR4 polymorphisms have also been associated with disease severity, particularly the hyporesponsive polymorphisms *Tlr4* Asp299Gly and *Tlr4* Thr399Ile which predispose children to severe malaria (Schmitt et al., 2002; Mockenhaupt et al., 2006a). However, contrasting results suggested that these polymorphisms may be beneficial in adults (Esposito et al., 2012; Basu et al., 2014) and a recent meta-analysis found no association between *Tlr4* Asp299Gly and the outcomes of malaria (Dhangadamajhi et al., 2017). In mice, TLR4 has been implicated in dendritic and mast cell activation during malaria (Furuta et al., 2008; Seixas et al., 2009), potentially contributing to the resistance of DBA/2 mice to infection with *P. yoelii*, although TLR4 has not been linked to the pathology of experimental cerebral malaria (Togbe et al., 2007).

3.1. TLR4 in Pregnancy

Several TLRs are expressed in fetally derived placental tissues and surrounding maternal tissue Koga and Mor (2010) but cumulative evidence suggests a specific role for TLR4 in the outcomes of pregnancy. It has been observed that fetoplacental TLR4 expression is decreased in miscarriages (Kolben et al., 2019) and in preeclampsia patients (Kulikova et al., 2016), while the *Tlr4* Asp299Gly polymorphism in the fetus is associated with severe prematurity (Rey et al., 2008). In contrast, increases in TLR4 expression on maternal monocytes, which may be responding to fibrinogen, are correlated with spontaneous preterm labor (Pawelczyk et al., 2010; Al-ofi et al., 2014), and increased expression in maternal decidua has been linked to recurrent miscarriages (Li et al., 2016). These data suggest that the role of maternal and fetal TLR4 in pregnancy may be in opposition, with reductions in fetal activity and/or increases in maternal activity being detrimental to the outcomes of the pregnancy.

Various mouse models have illustrated the role of TLR4 in pregnancy associated infections and disorders, including malaria (Barboza et al., 2017; Rodrigues-Duarte et al., 2018), bacterial infections (Liu et al., 2007; Arce et al., 2012; Chin et al., 2016), lipopolysaccharide exposure (Breen et al., 2012; Wahid et al., 2015) and uterine ischemia (Thaete et al., 2013). It should be noted that these studies (barring that by Rodrigues-Duarte et al., 2018) have focused on completely eliminating TLR4 signaling and, consequently, do not differentiate between fetal and maternal TLR4 responses. Furthermore, increased fetal TLR4 activity has been found in models of maternal ethanol-induced inflammation (Zheng et al., 2014) as well as in maternal cigarette smoke exposure (Chan et al., 2016). These studies support that maternal factors may be contributing to alterations in fetal innate immune responses as well as having direct impacts on the outcomes of pregnancy. Still, in all of these cases, the downstream actions of TLR4 are yet to be fully understood.

3.2. TLR4 in Placental Malaria

The role of TLR4 in malaria during pregnancy has also been examined in genetic association studies. The *Tlr4* Asp299Gly and *Tlr4* Thr399Ile maternal polymorphisms appeared more frequently in women who had a higher parasitemia and severe anemia, and translated to a significantly increased risk of low birth weight. They had no impact on prematurity, viability or the incidence of placental malaria (Mockenhaupt et al., 2006b), suggesting that maternal TLR4 takes part in responding to infection, but may not be linked to severe placental dysfunction during malaria. In mice, examination of TLR4 was preceded by work on MyD88, which was shown to contribute to reductions in placental vascular space and fetal weight (Barboza et al., 2014). Genetic ablation of several TLRs which use this adaptor protein demonstrated that alterations in vascular space, TNFα production and detrimental outcomes are directly linked to TLR4 (Barboza et al., 2017).

Genetic ablation of TLR4 confers striking protection from fetal death induced in murine placental malaria. The roles played by fetally derived placental cells in protecting fetal viability were discerned by comparing pregnancy outcomes when the fetal placenta either expressed TLR4 or did not. As expected, we observed improvements in fetal viability in TLR4KO females which were carrying TLR4KO offspring, suggesting that the TLR4 response to infection was deleterious to the fetuses, as observed in the other mouse models of disease during pregnancy mentioned above. Unexpectedly, TLR4KO females carrying placentas expressing fetally derived TLR4 showed further improvements in the outcomes of pregnancy with stillbirth rates similar to those of uninfected mothers (Rodrigues-Duarte et al., 2018). This showed that TLR4 in the fetal compartment was protective for the litter, whereas having it in the maternal compartment was harmful, a conflict which is yet to be investigated in other infections. Although the mechanism behind this protection has not been fully elucidated, alterations in glucose (Chandrasiri et al., 2013) and amino acid (Boeuf et al., 2013) transport, observed using human samples from malaria infected individuals, suggest that a conflict may arise over the allocation of metabolic resources. On the other hand, altered nitric oxide bioavailability (McDonald et al., 2018) and VEGF levels further point toward a role for fetal TLR4 responses in regulating placental perfusion.

While other models of infection during pregnancy have not yet been interrogated in a manner which allows for the

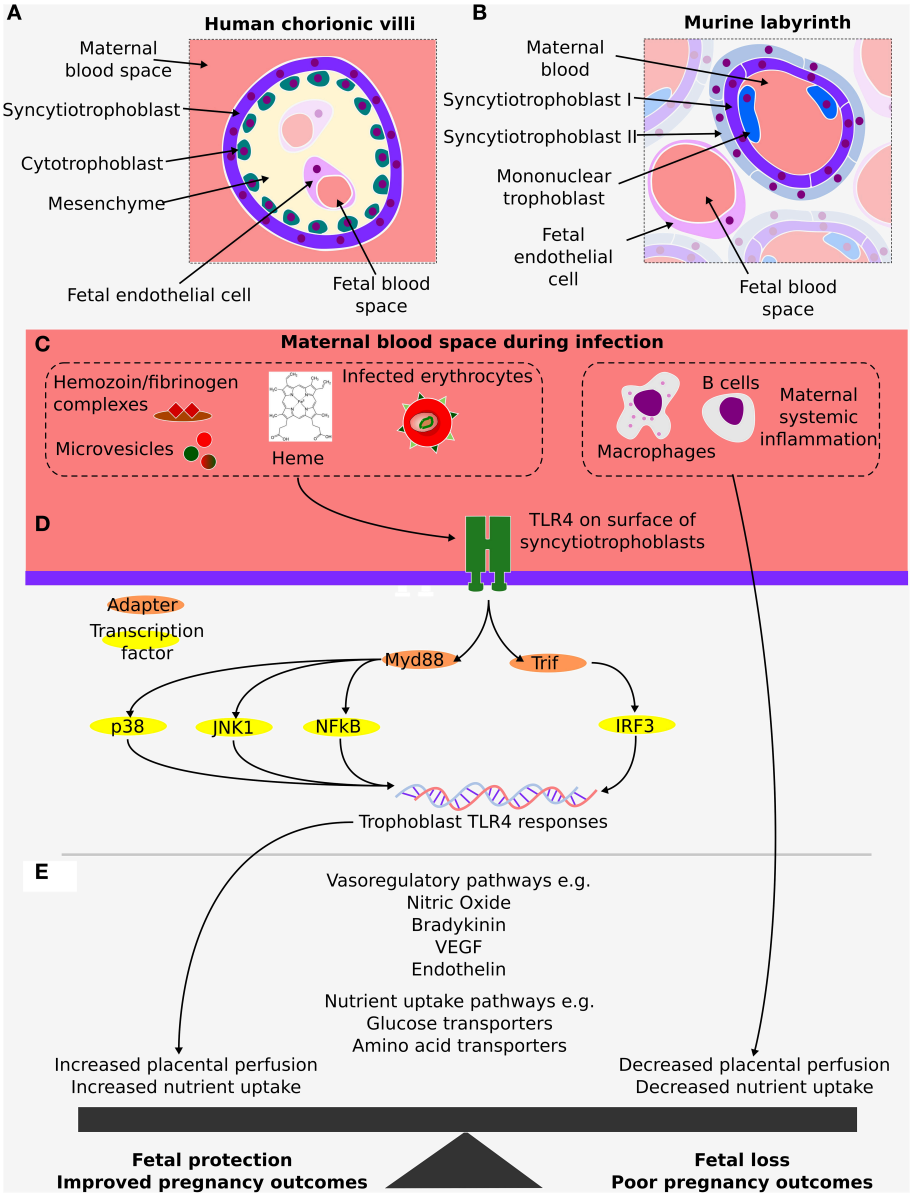

FIGURE 1 | (A) Cross section of a human placental terminal chorionic villus, which is part of highly branched tree of fetally derived tissue anchored in the chorionic plate and surrounded by maternal blood. At the terminal chorionic villi, the placental barrier is composed by the endothelial cells enclosed in mesenchyme followed by a layer of cytotrophoblasts and an outermost layer of syncytiotrophoblasts in contact with maternal blood. **(B)** Cross section of the analogous murine tissue, the placental labyrinth where the maternal fetal barrier is made up of a layer of fetal capillary endothelial cells, two layers of syncytiotrophoblasts and a discontinuous layer of mononuclear trophoblasts that is in direct contact with maternal blood. **(C)** In both the human and murine placentas, fetally derived syncytiotrophoblasts come into direct contact with maternal blood, an important similarity, as during a malaria infection, these are the fetal cells exposed to maternal inflammatory mediators and to components of parasite origin such as infected erythrocytes and microvesicles. **(D)** TLR4 detects and responds to several of these stimuli via either MyD88 or TRIF, activating several transcriptional factors including p38 and IRF3. **(E)** Transcriptional changes in the syncytiotrophoblast contribute to alterations in local production of vasoactivators such as nitric oxide, bradykinin and endothelin as well as altering nutrient transport pathways in a manner which result in fetal protection. In contrast, maternal responses impair these pathways and worsen the outcomes of pregnancy.

disentanglement of fetal and maternal responses, there are strong similarities between the impact of malaria infection on placental TLR4 and the impact of a variety of other pathogens. In wild type mice infected with *P. berghei*, the amount of TLR4 protein detected in the placenta is significantly increased (Barboza et al., 2017), a pattern which is replicated with *Campylobacter rectus*, *Porphyromonas gingivalis* (Arce et al., 2009) and murine cytomegalovirus infections (Liao et al., 2018). It would be particularly important to determine if the maternal component is responsible for the outcomes of these infections and assess the

impact of maintaining the fetal response. Bearing in mind that the first point of contact for fetally derived TLR4 with infectious agents in the maternal blood is at the placental barrier, examining fetally derived placental cells may be key to better understand the conflict with maternal responses.

4. PRIMARY TROPHOBLAST RESPONSES TO INFECTION

The fetal cell type directly in contact with maternal blood in both humans and mice are the syncytiotrophoblasts. They are responsible for the exchange of nutrients and waste, as well as forming the barrier between maternal and fetal circulation (Zeldovich et al., 2013; Blackburn, 2015; Maltepe and Fisher, 2015) (**Figures 1A,B**). While TLRs are found in fetally derived cells in the placenta (Koga and Mor, 2010), their expression and activity is greatly altered in placental cell lines, showing a lack of suitability for examining certain aspects of trophoblast behavior (Amirchaghmaghi et al., 2013; Gierman et al., 2015), and highlighting a requirement for the employment of primary cultures.

Although they are rarely used for studies with malaria (Lucchi et al., 2006, 2008), primary human trophoblasts have been successfully isolated and used to examine responses to a variety of infectious agents such as *Brucella spp.*, zika virus and *Toxoplasma gondii* (Salcedo et al., 2013; Aagaard et al., 2017; Ander et al., 2018), the latter being further examined in villous explants (Ander et al., 2018). This experimental system has also been used to study *Trypanosoma cruzi* infection (Díaz-Luján et al., 2016; Medina et al., 2018; Triquell et al., 2018), which is known to provoke changes in several immune related genes during pregnancy (Juiz et al., 2018). Trophoblasts are reported to show changes in amino acid and glucose uptake in response to lipopolysaccharide (a TLR4 ligand) (Liong and Lappas, 2017), pathways which are also altered in human placental malaria samples (Boeuf et al., 2013; Chandrasiri et al., 2013). Therefore, the use of primary human trophoblasts to study responses to malaria infection may provide insights into the pathology of placental malaria.

As an alternative, murine trophoblasts, isolated from term placentas or of stem cell origin, represent a powerful tool for studying infection during pregnancy. TLR4 responses have been examined in cell culture studies using *P. berghei*. These studies have revealed a reduction in the amount of trophoblast-associated parasite in absence of TLR4 as well as marked reduction in the expression of *Ifnar1* in these cells (Rodrigues-Duarte et al., 2018). Along the same lines, *Listeria monocytogenes* has been shown to be taken up by trophoblast giant cells in a MAPK dependent manner, using innate sensing systems heavily influenced by TLR2, subsequently downregulating HO-1 and resulting in cell death (Hashino et al., 2015). In a study not linked to TLRs, trophoblasts exposed to *Toxoplasma gondii* have been demonstrated to undergo apoptosis as well as alter production of various cytokines, with increased oxidative stress and subsequent mitochondrial damage (Liu et al., 2013; Xu et al., 2015). Thus, it is clear that trophoblasts respond to pathogens infecting maternal blood, raising the interesting possibility that they are the initiators of fetal protective responses in placental infections, particularly when the pathogens do not cross the placental barrier.

5. CONCLUDING REMARKS

It is expected that a variety of pathways are impacted upon during placental infection, affecting inflammatory responses, nutrient transport and vasoregulatory responses, and contributing to placental insufficiency that leads to poor pregnancy outcomes. A key finding from the acute murine placental malaria model has been the importance of TLR4 in the determination of the outcomes of pregnancy. Interestingly, this trait is shared with several other models of disease in pregnancy that also show signs of placental dysfunction. The strong pathological similarities between these models supports the proposal that innate immune recognition by the placental tissue may improve fetal survival in other infections.

Taken together, the work highlighted here leads us to propose that maternally driven TLR4 responses to malaria infections, and other illnesses during pregnancy, are deleterious for the fetus, impairing nutrient/waste exchange, hampering placental perfusion and worsening the outcomes of pregnancy. In contrast, feto-placental TLR4 responses are protective, as demonstrated by Rodrigues-Duarte et al. (2018) and may compensate for the maternal actions by activating mechanisms to increase nutrient uptake and placental perfusion (**Figure 1E**). This re-inforces the notion that malaria infection induces maternal-fetal conflict, as proposed by Muehlenbachs et al. (2006), in their examination of soluble VEGF receptor 1 in human placental malaria, but further hints that fetal responses which preserve placental function are initiated by innate immune recognition and downstream signaling to effector mediators in trophoblasts.

The syncytiotrophoblasts are at the front line of this conflict between mother and fetus and their examination, using *in vitro* and *in vivo* experimental systems, as well as in human tissue samples, will be required for the identification of critical components of the fetal protective responses. Making use of the vast range of existing genetic mouse models in conjunction with the gamut of infection systems which have been developed for the study of pathological pregnancies, will provide a clearer understanding of the mechanisms that protect from adverse outcomes of pregnancy.

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YP contributed to writing the paper. CP-G contributed to writing the paper.

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Blood Microbial Communities During Pregnancy Are Associated With Preterm Birth

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Microbial infection of the placenta, amniotic fluid, vaginal canal, and oral cavity is known to significantly contribute to preterm birth (PTB). Although microbes can be translocated into the blood, little is known regarding the blood microbiota during pregnancy. To assess changes in the microbiome during pregnancy, blood samples were obtained 2 or 3 times during pregnancy from a cohort of 45 pregnant women enrolled between 2008 and 2010. To analyze the association with PTB, we conducted a case-control study involving 41 pregnant women upon admission for preterm labor and rupture of membrane (20 with term delivery; 21 with PTB). Bacterial diversity was assessed in number and composition between the first, second, and third trimesters in term delivered women according to 16S rRNA gene amplicon sequencing, and data were analyzed using Quantitative Insight Into Microbial Ecology (QIIME). Taxonomy was assigned using the GreenGenes 8.15.13 database. Dominant microorganisms at the phylum level in all pregnant women were identified as *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria*. However, the number and composition of bacteria in women with PTB differed from that in women with term delivery. *Firmicutes* and *Bacteroidetes* were more abundant in women with PTB than in women with term delivery, while *Proteobacteria* was less prevalent in women with PTB. At the genus level, *Bacteroides*, *Lactobacillus*, *Sphingomonas*, *Fastidiosipila*, *Weissella*, and *Butyricicoccus* were enriched in PTB samples. These observational results suggest that several taxa in the maternal blood microbiome are associated with PTB. Further studies are needed to confirm the composition of the blood microbiota in women with PTB. Additionally, the mechanism by which pathogenic microbes in maternal blood cause infection and PTB requires further analysis.

Keywords: blood microbiota, pregnancy, preterm birth, 16S rRNA gene sequencing, microbiome

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INTRODUCTION

Approximately 15 million babies are born prematurely each year (Lawn et al., 2013), defined as parturition before 37 weeks of gestation. In Korea, the rate of preterm birth (PTB) has continuously increased by 1.4-fold, from 4.7% in 2005 to 6.9% in 2015 (Hwang et al., 2015; Korean Statistical Information Service, 2016). Prematurely born babies account for a significant proportion of infant

morbidity and mortality (Simmons et al., 2010; Yoo et al., 2018). Approximately 40% of PTBs spontaneously occur because of infection and inflammation, including associations with subclinical intrauterine, intra-amniotic, and extrauterine maternal infections, such as periodontal disease (Goldenberg and Culhane, 2006; Mysorekar and Cao, 2014).

Over the course of pregnancy, the microbiome in every organ of the body undergoes profound changes associated with metabolic alterations and immunological adaptations (Koren et al., 2012). However, bacterial pathogens and associated products can induce local inflammatory responses in gestational tissues (acute chorioamnionitis), leading to preterm labor (Romero et al., 1988, 1989; Yoon et al., 1998). Most intra-amniotic infections are thought to occur when the microbiome in the lower genital tract (vagina and/or cervix) gains access to the amniotic fluid (Romero et al., 2006). Changes in the microbial ecosystem of the vagina have been implicated in the genesis of ascending intrauterine infection (Hillier et al., 1995; Hitti et al., 2001; Racicot et al., 2013). Similarly, microbial infection of the amniotic fluid, vaginal canal, and oral cavity is known to significantly contribute to PTB (Ryan and Ray, 2004; Menon, 2008; Yoo et al., 2016).

High-throughput, culture-independent technologies can be used to assess the microbiome of each organ in the body at high taxonomic resolution (Hillier et al., 1995; Hitti et al., 2001; Menon, 2008; Racicot et al., 2013). We previously reported analyses of the microbial communities of amniotic fluid and urine by sequencing of the bacterial 16S ribosomal RNA gene in women who delivered preterm (Yoo et al., 2016; You et al., 2016). We identified *Sneathia sanguinegens* and *Fusobacterium nucleatum* in amniotic fluid samples from two preterm delivered women (You et al., 2016). In urine samples, *Ureaplasma* spp. and *Veillonellaceae* family members, including *Megasphaera* spp., were more abundant in preterm delivered women than in women who delivered at term (Yoo et al., 2016). While antibiotic use can alter the composition and structure of the microbiota in specific cases, such as in chorioamnionitis and cervical infection (You et al., 2016), microbes such as *F. nucleatum*, *Leptotrichia* (*Sneathia*), *Ureaplasma urealyticum*, *Mycoplasma hominis*, *Streptococcus agalactiae*, *Escherichia coli*, and a species of the order *Clostridiales* can induce local inflammatory responses in gestational tissues, causing PTB (Peltier, 2003; Han et al., 2006; Wang et al., 2013). However, the microbial composition of the blood of women who undergo preterm delivery has not been established.

The aim of this study was to characterize the composition of the blood microbiome during healthy pregnancy and to compare the blood microbiome of pregnant women with those of term and preterm delivery using 16S rRNA gene sequencing-based methods.

MATERIALS AND METHODS

Study Population

To analyze changes in the microbiome during pregnancy, blood samples were obtained 2 or 3 times from 45 pregnant women enrolled in a cohort study between 2008 and 2010 when they visited Ewha Womans University MokDong Hospital for regular pregnancy check-ups in the first, second, and third trimesters. Only pregnant women who underwent term delivery with healthy singletons were included in this cohort. We also conducted a case-control study involving 41 pregnant women (20 with term delivery; 21 with PTB) between 2014 and 2015 to compare the blood microbiome of pregnant women with term and preterm delivery. The normal term delivery group (who underwent term delivery at $\geq$37 weeks of gestation) was selected from among women who had undergone prenatal examinations and were followed up until delivery in our hospital. When pregnant women in the case-control study were admitted for the first time with symptoms of labor and/or rupture of membrane, maternal blood was collected in EDTA-containing tubes, and blood cells and plasma were separated within 24 h and stored at –70°C. The inclusion criteria were a singleton birth and gestational age of 25–42 weeks at delivery. We excluded women who had multiple births, stillbirths, infants with congenital anomalies, chronic hypertension, pregnancy complication, placenta previa, and abruption placenta.

Ethics Statement

The present study was approved by the Institutional Review Board of Ewha Womans University Hospital (ECT 127-07 and EUMC 2014-06-010). The methods were conducted in accordance with the approved guidelines. All participants were fully informed regarding the study and provided written informed consent.

DNA Extraction and 16S rRNA Gene Sequencing

Bacterial DNA was extracted from batches of blood cells using a PowerMax Soil DNA Isolation Kit (MOBIO, Carlsbad, CA, United States) following the manufacturer's protocol. The V3–V4 hypervariable region of bacterial genomic DNA was amplified according to Illumina 16S metagenomic sequencing library protocols (Illumina, San Diego, CA, United States). The barcoded fusion primer sequences used for amplification were 16S_V3_F (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACG GGNGGCWGCAG-3′) and 16S_V4_R (5′-GTCTCGTGGGCT CGGAGATGTGTATAAGAGACA GGACTACHVGGGTATCT AATCC-3′). The libraries were prepared using PCR products according to the MiSeq System guide (Illumina) and quantified using a QIAxpert (QIAGEN, Hilden, Germany). After PCR products were extracted and quantified, equimolar ratios from each mixture were pooled and sequenced on the MiSeq (Illumina) platform according to the manufacturer's recommendations.

Analysis of Blood Microbiome

Raw pyrosequencing reads obtained from the sequencer were filtered according to the barcode and primer sequences using MiSeq (Illumina). Taxonomic assignment was performed with the profiling program MDx-Pro ver.1 (MD Healthcare, Seoul, Korea). High-quality sequencing reads were selected after

filtering based on read length (≥300 bp) and quality score (average Phred score ≥20). Operational taxonomic units (OTUs) were clustered using the sequence clustering algorithm CD-HIT (Fu et al., 2012). Subsequently, taxonomy assignment was performed using UCLUST (Edgar, 2010) and QIIME (Caporaso et al., 2010) against the 16S rRNA gene sequence database in GreenGenes 8.15.13[1]. All 16S rRNA gene sequences were assigned to taxonomic levels based on sequence similarity. The bacterial composition at each level was plotted as a stack bar. When case clusters could not be assigned at the genus level because of a lack of sequences or redundant sequences in the database, taxa were assigned at higher levels, which are indicated in parentheses. Data were normalized to have a mean of 0 and standard deviation of 1 by linear normalization. Principal coordinate analysis and two-dimensional scatter plots with axes of the first and second principal components were calculated and drawn using Matlab 2011a (Lee et al., 2017).

Statistical Analysis

Results are presented as the mean ± standard deviation. Basic patient characteristics, including age, maternal features, and birth outcomes, were compared between term and preterm delivered women using a Student *t*-test. Based on significant differences in the Shannon index, clustering characteristics were compared using the Kruskal–Wallis test (PERMANOVA). Statistical analyses were performed using SAS software (Version 9.3; SAS Institute, Cary, NC, United States). Results were considered statistically significant when the probability value (p) was <0.05.

[1]http://qiime.org/home_static/dataFiles.html

RESULTS

Blood Microbial Diversity and Composition of Pregnant Women in Cohort

To characterize the blood microbial composition during healthy pregnancy, we obtained blood samples from 45 subjects in their first, second, and third trimesters. Mean patient ages and gestational ages at delivery were 32.0 years (range: 26–40 years) and 39 weeks 3 days (range: 37 weeks 1 day–41 weeks 2 days), respectively. The data set comprised 1,609,012 high-quality gene sequences from blood samples, with an average of 16,243 reads per sample. After filtering out low-quality reads and trimming extra-long tails, the remaining representative reads were clustered into OTUs based on a 97% sequence similarity cut-off at the genus level.

We analyzed the taxonomic diversity and profiles of bacterial DNA sequences in the blood during healthy pregnancy by assessing the number and abundance of distinct types of organisms. **Figure 1** presents the Shannon index, principal component analysis, and relative abundances of OTUs at the phylum and genus levels in each sample. Analysis of the Shannon index and principal component analysis did not

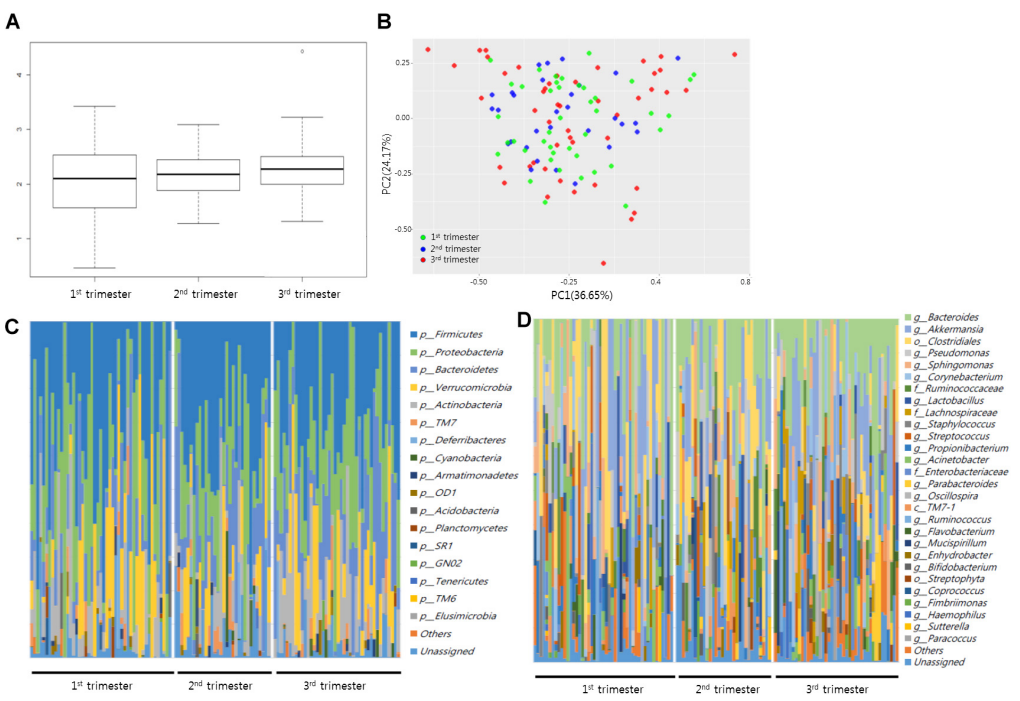

FIGURE 1 | Microbial diversity and profiling during healthy pregnancy by 16S rRNA gene sequencing. **(A)** Comparison of Shannon index (community richness) in first, second, and third trimesters in maternal blood. **(B)** Plot of principal component analysis. Relative abundances of operational taxonomic units (OTUs) accounting for >0.1% of the total bacterial community are shown. **(C)** Bacterial profiling plot of relative abundances of OTUs at the phylum level and **(D)** genus level.

identify significant differences among the first, second, and third trimesters. Microorganisms with a relative abundance of >0.1% at the phylum level predominantly included *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria*, and those at the genus level included *Bacteroides*, *Pseudomonas*, *Sphingomonas*, *Ruminococcaceae*, *Staphylococcus*, *Propionibacterium*, and *Streptococcus*.

Blood Microbial Diversity and Composition of Pregnant Women in Case-Control Study

To compare the bacterial compositions of term and preterm groups, we analyzed the bacterial diversity and relative abundances of OTUs in those in the case-control study. The general characteristics of the pregnant women in this study are shown in **Table 1**. The mean ages of the women in the term and preterm groups were 31.6 and 30.9 years, respectively. Gestational age at delivery, weight of the neonate, and Apgar score were significantly lower in the preterm group ($p < 0.05$).

The data set comprised 1,679,505 high-quality gene sequences, with an average of 16,243 reads per sample. After filtering out low-quality reads and trimming extra-long tails, the remaining representative reads were clustered into OTUs with a 97% sequence similarity cut-off at the genus level. The Shannon index was significantly higher in the preterm group than the term group ($p < 0.05$). The principal component analysis revealed differences between the term and preterm groups (**Figure 2**).

TABLE 1 | General characteristics of study subjects.

	Women with term delivery (*N* = 20)	Women with preterm delivery (*N* = 21)	*p*-value
Mothers			
Age (years)	31.60 ± 2.91	30.91 ± 4.37	0.55
BMI (kg/m^2)	26.81 ± 3.13	26.12 ± 4.67	0.59
Parity, *n* (%)			
Nulliparous	10 (50.0)	9 (42.9)	0.71
Multiparous	10 (50.0)	12 (57.1)	
Mode of delivery, *n* (%)			
Vaginal	13 (65.0)	16 (76.2)	0.43
C-section	7 (35.0)	5 (23.8)	
Education (years)			
≤12	5 (26.3)	10 (52.6)	0.10
>12	14 (73.7)	9 (47.4)	
Infants			
Gestational age (weeks)	39.65 ± 1.04[a]	29.67 ± 3.58	<0.0001
Sex, *n* (%)			
Male	11 (55.0)	12 (57.1)	0.89
Female	9 (45.0)	9 (42.9)	
Weight (kg)	3.36 ± 0.36[a]	1.48 ± 0.60	<0.0001
AS at 1 min	9.65 ± 0.59[a]	6.33 ± 3.06	<0.0001
AS at 5 min	10.00 ± 0.00[a]	7.48 ± 3.12	0.001

Data are expressed as means ± SD or number (%). [a]p-values were calculated by Student t-test. BMI, body mass index of pregnant women at delivery; C-section, cesarean section; and AS, Apgar score.

The blood microbiota of the two groups were enriched primarily in *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria*. While *Firmicutes* and *Bacteroidetes* were more abundant in women with PTB than in women with term delivery ($p < 0.05$), *Proteobacteria* was less abundant in women with PTB (**Supplementary Table S1**, $p < 0.001$). Notably, at the class level, *Betaproteobacteria* and *Gammaproteobacteria* were significantly less abundant in women with PTB than in those with term delivery (**Supplementary Table S2**, $p < 0.001$). Among genera with abundances of >0.1%, *Bacteroides*, *Lactobacillus*, *Sphingomonas*, *Fastidiosipila*, and *Butyricicoccus* were enriched in preterm samples, after adjusting for maternal age, pregnant BMI, delivery mode, and sex of the newborn (**Table 2**). At the genus level, the archaeon *Methanobrevibacter* and uncultured bacteria belonging to *Ruminococcaceae*, *Saccharibacteria*, and *Lachnospiraceae* were enriched in the preterm samples. In contrast, *Delftia*, *Pseudomonas*, *Massilia*, and *Stenotrophomonas* belonging to the *Proteobacteria* phylum were enriched in term samples ($p < 0.05$).

DISCUSSION

Our study investigated the characteristics of the blood microbiota during healthy pregnancy and its association with PTB by sequencing the V3–V4 region of the 16S rRNA gene. During healthy pregnancy, bacterial diversity was similar in number and composition between the first, second, and third trimesters. However, the microbial diversity in women with PTB differed from that in women with term delivery. The blood microbiome of all pregnant women enrolled in the study was largely composed of nonpathogenic commensal bacteria from the *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria* phyla. While bacterial enrichment during healthy pregnancy did not differ from this overall picture, several taxa, such as *Bacteroides*, *Lactobacillus*, *Delftia*, and *Pseudomonas* exhibited differential enrichment between blood samples of women who delivered preterm and term in a case-control study. Our results suggest that several taxa in the maternal blood microbiome are associated with PTB.

In healthy pregnancy, the microbiota in all organs of the body undergoes profound changes associated with metabolic alterations and immunological adaptations (Goldenberg and Culhane, 2006). Moreover, the similarity between the oral and placental microbiome suggests that the placental microbiome becomes colonized primarily as the result of hematogenous bacterial spread via the circulation (Ramos et al., 2015). Bacterial infections threaten pregnant women and the fetus by gaining access to gestational tissues, such as the decidua, placenta, and fetal membranes (Vinturache et al., 2016). Notably, the virulence properties assigned to specific oral pathogenic bacteria, for example, *F. nucleatum*, *Porphyromonas gingivalis*, *Filifactor alocis*, *Campylobacter rectus*, and others, render them potential collaborators in adverse outcomes of pregnancy (Cobb et al., 2017), and these pathogenic bacteria can be transmitted from the oral cavity to gestational tissues via hematogenous spread (Han et al., 2006; Aagaard et al., 2014).

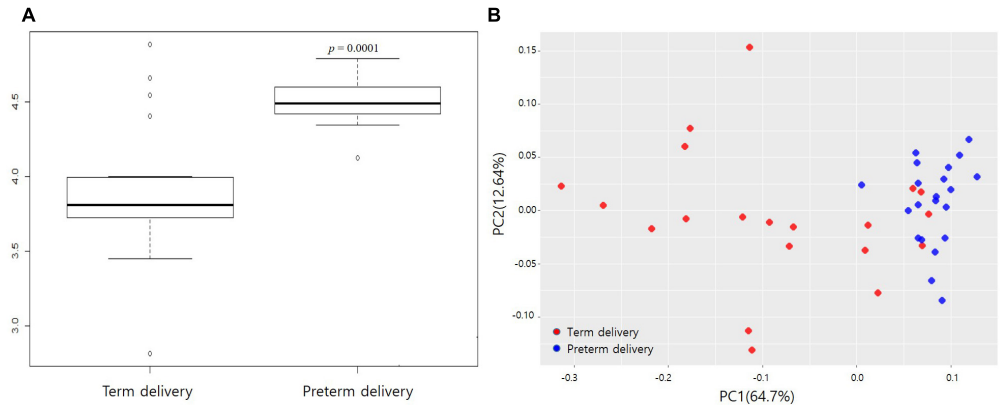

FIGURE 2 | Differences in microbial diversity between term and preterm delivery. **(A)** Comparison of Shannon index (community richness) between term and preterm delivery (p = 2.02E-10). **(B)** Plot of principal component analysis. Relative abundances of operational taxonomic units (OTUs) accounting for >0.1% of the total bacterial community are shown.

TABLE 2 | Abundances of genera differed in peripheral blood of women who experienced preterm and term delivery.

Taxon	Term delivery		Preterm delivery		*p*-value*	Fold
	Mean	SD	Mean	SD		change
Bacteroides	3.425	2.614	8.722	1.891	9.01E-09	2.55
Lactobacillus	1.665	1.175	3.391	1.353	1.30E-04	2.04
Sphingomonas	0.944	0.938	2.051	0.911	5.91E-04	2.17
Rhizobium	1.192	0.661	1.898	1.046	1.65E-02	1.59
Clostridiales vadinBB60 group	0.758	0.767	1.353	0.693	1.52E-02	1.78
Ruminococcaceae	0.207	0.407	0.852	0.440	2.86E-05	4.11
Delftia	3.050	2.263	0.828	0.359	4.14E-04	0.27
Eisenbergiella	0.474	0.554	0.814	0.428	3.78E-02	1.72
Pseudomonas	3.680	2.913	0.800	0.502	3.89E-04	0.22
Ruminiclostridium 5	0.388	0.596	0.754	0.398	2.87E-02	1.95
Fastidiosipila	0.130	0.268	0.603	0.414	1.42E-04	4.65
Saccharibacteria	0.134	0.264	0.466	0.344	1.71E-03	3.47
Lachnospiraceae	0.163	0.315	0.449	0.414	2.01E-02	2.76
Syntrophaceticus	0.120	0.230	0.373	0.307	6.25E-03	3.10
Butyricicoccus	0.045	0.135	0.277	0.234	5.84E-04	6.14
Massilia	0.743	0.701	0.272	0.219	1.03E-02	0.37
DA101 soil group	0.096	0.172	0.256	0.198	1.09E-02	2.65
Stenotrophomonas	1.395	1.313	0.230	0.232	1.09E-03	0.17
Anaerolineaceae	0.058	0.145	0.221	0.207	6.91E-03	3.83
Methanobrevibacter	0.015	0.066	0.186	0.336	3.59E-02	12.40
Methanocella	0.023	0.039	0.171	0.280	2.91E-02	7.56
Flavonifractor	0.024	0.072	0.170	0.166	1.23E-03	7.10
Woesearchaeota (DHVEG-6)	0.026	0.113	0.150	0.205	2.51E-02	5.69
Prochlorococcus	0.017	0.053	0.137	0.198	1.54E-02	7.90
Gemmata	0.024	0.062	0.122	0.185	3.30E-02	5.19
Morus notabilis	0.027	0.090	0.122	0.149	2.08E-02	4.53
planctomycete WY108	0.017	0.072	0.117	0.167	2.02E-02	6.94
[Eubacterium] hallii group	0.010	0.038	0.108	0.143	7.06E-03	11.17

**p-values were calculated using ANCOVA adjusting for maternal age, pregnant BMI, type of delivery mode, and newborn's gender.*

Based on our results, the richness of the microbial community (Shannon index) did not differ among trimesters during healthy pregnancy, but it was increased in the blood samples of women with preterm delivery compared to that of term delivery. This indicates that the community structure of the blood microbiota in women with PTB differs from that

in women with term delivery, even though the microbial community structure does not change during healthy pregnancy. However, a previous study reported that PTB is associated with distinct microbial DNA changes detected in midtrimester maternal serum (Subramaniam et al., 2018). Another study reported the increased richness and diversity of the vaginal microbiome in spontaneous PTB (Freitas et al., 2018). These findings suggest that a more diverse microbiome may be important in the pathogenesis of some bacteria.

The microbial communities of all pregnant women in the study were primarily composed of *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria*. This composition of the blood microbiota is similar to that of the human gut microbiota (Khanna and Tosh, 2014). In our study, while the composition did not change during healthy pregnancy, *Firmicutes* and *Bacteroidetes* were enriched in the samples of women delivered preterm, while *Proteobacteria* was reduced. A previous study reported that the gut microbiota consisted of mostly *Firmicutes* and *Bacteroidetes* in the first trimester but shifted substantially in phylogenetic composition and structure over the course of pregnancy (Goldenberg and Culhane, 2006). The enrichment of *Proteobacteria* and *Actinobacteria* was observed during the third trimester of pregnancy in most cases (Goldenberg and Culhane, 2006). Notably, enrichment of the *Proteobacteria* in the third trimester has been observed repeatedly under inflammation-associated dysbiosis (Païssé et al., 2016). Although pregnancy is not a disease, this suggests that a shift in the gut microbiota during pregnancy causes dysbiosis of the blood microbiota.

The class *Bacteroidia* of the phylum *Bacteroidetes* is composed of a single order of environmental bacteria. *Bacteroides* spp. provide some benefits to their host by excluding potential pathogens (Stevens et al., 2012). However, *Bacteroides* such as *B. fragilis* and *B. thetaiotaomicron* can quickly become opportunistic pathogens if they are translocated outside the gastrointestinal tract, and they have been associated with abscess formation across multiple body sites, such as the abdomen, brain, liver, pelvis, and lungs, as well as serious bloodstream infections (McGregor et al., 1991; Wexler, 2007). Phospholipase C production by *B. fragilis*, *B. bivius*, and *B. thetaiotaomicron* has been implicated in various reproductive tract infections as well as PTB (McGregor et al., 1991). In addition, among women in preterm labor, a study reported an increased rate of preterm delivery ($\leq$34 weeks) in pregnant women with high concentrations of *B. bivius* and *B. fragilis* in their vaginal fluid (Krohn et al., 1991).

Abundant *Lactobacillus* spp. in the vaginal microbiome are important for maintaining pregnancy (Romero et al., 2014). However, our results showed that *Lactobacillus* was more abundant in the blood of women with PTB compared to that in women with term delivery. A study reported that enrichment of *Lactobacillales* was frequently observed in the intestinal microbiota of women in a PTB group compared to that in a term delivered group (Sato et al., 2014; Shiozaki et al., 2014). Although *Pseudomonas* commonly causes conjunctivitis in hospitalized preterm infants (Shah et al., 1999), *Pseudomonas*, belonging to the *Proteobacteria*, was enriched in term samples in this study. In addition, *Delftia*, which in amniotic fluid has been linked to PTB (DiGiulio et al., 2008), was also enriched in term samples. Further study is needed to explain the relatively high abundance of *Lactobacillus* and the deficiency of *Pseudomonas* and *Delftia* in preterm blood samples compared with that in term samples.

Although the *Firmicutes* to *Bacteroidetes* ratio in stool samples is associated with obesity (Ley et al., 2005; Schwiertz et al., 2010), *Bacteroides* and *Lactobacillus* were still enriched in preterm samples in this study. In addition, maternal age, delivery mode, and newborn sex did not affect the enrichment of *Bacteroides* and *Lactobacillus* in preterm samples. A previous study reported that infant sex contributes to the dynamic development of the gut microbiome in preterm infants (Cong et al., 2016). The study reported that the abundances of *Enterobacteriales*, *Lactobacillus*, and *Clostridiales* were influenced by sex in preterm infants. However, in our analysis, *Lactobacillus* and *Clostridiales* were enriched in blood samples collected from women with preterm deliveries after adjusting for infant prenatal sex. Thus, our results suggest that the increased prevalence of *Bacteroides* and *Lactobacillus* in the maternal blood microbiota is associated with PTB.

We acknowledge several limitations of our study. The primary limitation is the small number of patients in the case cohort and the ethnic homogeneity of the participants. Another limitation is the lack of a direct comparison between our blood microbiome data and the oral, gut, and placental microbiome derived from the subjects enrolled in the study. Lastly, although in 2015, obesity rate (body mass index, BMI $>$30) was 5.3% in Korea (OECD, 2017), our study did not include information on pre-pregnancy BMI. Thus, further studies by other investigators are needed to confirm our results.

In summary, we found that the number and abundances of distinct types of organisms did not change in peripheral blood during healthy pregnancy. However, the microbial diversity in women who experienced PTB differed from that in women delivered at term. While bacterial enrichment did not change during healthy pregnancy, a case-control study demonstrated that several taxa, such as *Bacteroides*, *Lactobacillus*, *Delftia*, and *Pseudomonas* exhibited differential enrichment between women delivered preterm and term. This suggests that changes in the microbiota in various locations in the body during pregnancy can be detected in the blood. Further studies are needed to confirm the composition of the blood microbiota in pregnant women and women with PTB. Moreover, the mechanism by which pathogenic microbes cause infection requires further analysis.

AUTHOR CONTRIBUTIONS

Y-AY and YK conceived and designed the experiments. Y-AY, JY, and EK performed the experiments. Y-AY, JY, and EK analyzed the data. YK contributed reagents, materials, and analysis tools. Y-AY and YK wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.01122/full#supplementary-material

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Trypanosoma cruzi Infection at the Maternal-Fetal Interface: Implications of Parasite Load in the Congenital Transmission and Challenges in the Diagnosis of Infected Newborns

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Trypanosoma cruzi is the protozoan unicellular parasite that causes Chagas disease. It can be transmitted from infected mothers to their babies *via* the connatal route, thus being able to perpetuate even in the absence of Triatomine insect vectors. Chagas disease was originally endemic in Central and South America, but migration of infected women of childbearing age has spread the *T. cruzi* congenital infection to non-endemic areas like North America, Europe, Japan, and Australia. Currently, 7 million people are affected by this infection worldwide. This review focuses on the relevance of the *T. cruzi* parasite levels in different aspects of the congenital *T. cruzi* infection such as the mother-to-child transmission rate, the maternal and fetal immune response, and its impact on the diagnosis of infected newborns. Improvements in detection of this parasite, with tools that can be easily adapted to be used in remote rural areas, will make the early diagnosis of infected children possible, allowing a prompt trypanocidal treatment and avoiding the current loss of opportunities for the diagnosis of 100% of *T. cruzi* congenitally infected infants.

Keywords: *Trypanosoma cruzi*, mother-to-child transmission, parasitemia, infected pregnant women, congenitally infected infants, early diagnosis

EPIDEMIOLOGY OF THE CONNATAL CHAGAS DISEASE

The American trypanosomiasis, or Chagas disease, is caused by the protozoan parasite *Trypanosoma cruzi*, which affects about 6–7 million people worldwide, with most of the cases in Latin America (WHO | Chagas disease (American trypanosomiasis), 2019).

Given the great success in the control of *Triatoma infestans*, after which Brazil, Paraguay, Uruguay, and Chile were free of *T. cruzi* vectorial transmission, and appropriate control of blood supply that interrupted parasite infection through blood transfusion in most endemic countries, interruption of mother-to-child *T. cruzi* transmission became the new challenge in research and in public health policies. Around 9,000 babies are born to *T. cruzi*-infected mothers each year, and it is estimated that 1.1 million women of childbearing age are infected with *T. cruzi* in 21 countries from Mexico to Argentina, where this neglected tropical disease is endemic (WHO | Chagas disease (American trypanosomiasis), 2019).

Currently, *T. cruzi* infection is globally distributed and has been increasingly detected in countries where vector transmission is absent, mainly due to migration of infected individuals from Latin America. Among the non-endemic countries, the United States is home to the largest number of Chagas infection cases, estimated to be more than 300,000, of which a small number of cases were reported as autochthonous vector-borne transmission in the southern US (Bern et al., 2011; Manne-Goehler et al., 2016), whereas the number of *T. cruzi*-infected people has exceeded 100,000 in Europe (Strasen et al., 2013). It has had a smaller impact in Canada, Australia, and Japan (Buekens et al., 2008; Imai et al., 2014; Jackson et al., 2014). There is a substantial proportion of *T. cruzi*-infected women of childbearing age and congenitally infected infants among the Latin American migrants (Soriano-Arandes et al., 2016). Conversely, *T. cruzi* transmission through blood transfusion or organ transplants are of less epidemiological importance, since non-endemic countries with large immigrant populations have begun to intervene in blood-borne *T. cruzi* transmission (Gascon et al., 2010).

The outcome of congenital infection with *T. cruzi* is due to the result of complex interactions among the parasite, the placenta and the immune responses of the mother and the fetus, and studies about the mechanism of congenital infection are scarce. Understanding these relationships would help in successfully preventing congenital transmission of the parasite or facilitate better access to diagnosis and treatment of the newborns, which would eventually contribute to decreasing the number of cases of this disease around the world.

T. cruzi MOTHER-TO-CHILD TRANSMISSION

It has been reported that *T. cruzi* maternal-fetal transmission occurs in about 1–12% of the pregnancies, taking into account reports with the largest number of infected pregnant women studied in endemic areas (Russomando et al., 1998; Torrico et al., 2005; De Rissio et al., 2010; Salas Clavijo et al., 2012; Bua et al., 2013). The rate of parasite transmission is variable in different countries: 6% in Argentina, 4.1% in Bolivia, and 4.3% in Paraguay (Carlier and Truyens, 2015), 1.7% in Brazil (Martins-Melo et al., 2014), and between 0.8 and 4.08 in Mexico (Cardoso et al., 2012; Montes-Rincón et al., 2016), with an average rate of around 5% (Howard et al., 2014). The wide variation in the reported rates is probably due to studies performed in different areas with and without vector transmission, with heterogeneous populations, experimental conditions and different diagnostic methods.

MATERNAL PARASITEMIA AND VECTOR EXPOSURE

The vertical transmission rate of *T. cruzi* is different in areas with or without the presence of insect vectors, as geographic regions where the disease is endemic are twice as likely to have congenital transmission, compared to the countries free of transmission vectors, 5.0 vs. 2.7% respectively, according to studies performed mainly in Spain (Howard et al., 2014). It was intuitive that parasite load would be enhanced under continuous vector exposure in endemic areas, increasing the risk of parasite congenital transmission (Dias et al., 2002; Torrico et al., 2006). However, it was also demonstrated that infected women living in houses under active vector control had significantly higher parasite loads compared to those women who lived in infested houses (Sánchez Negrette et al., 2005; Rendell et al., 2015). This is probably due to repeated parasite inoculations, which induce an enhanced immune response that helps to control the parasite levels (Rendell et al., 2015).

MATERNAL PARASITEMIA AND RISK OF CONNATAL PARASITE TRANSMISSION

A correlation between high parasitemia in pregnant women and the risk of maternal-fetal *T. cruzi* transmission was observed when the blood from mothers of infected children showed a higher frequency of positive parasite hemocultures (Hermann et al., 2004). A higher parasitemia was also observed in the blood buffy coats of women that transmitted the parasite to their offspring compared to those who did not (Salas et al., 2007; Brutus et al., 2010). A higher frequency of vertical parasite transmission was observed in *T. cruzi* acute infection, which is usually associated with an increased parasitemia (Moretti et al., 2005). Among the studies that quantified parasite load in seropositive pregnant women, mothers of infected babies had significantly higher parasitemia, compared to the mothers of non-infected babies (Virreira et al., 2007; Bern et al., 2009; Bua et al., 2012; Kaplinski et al., 2015; Rendell et al., 2015). When the parasitic load, quantified by quantitative polymerase chain reaction (qPCR), was correlated with the parasite transmission rate in 128 *T. cruzi*-infected pregnant women from Bolivia, researchers found that 31.3% of women with a high parasite load (35 Pe/mL or more) delivered infected children, compared to 15.4% in women with a moderate parasite load (between 1 and 34 Pe/mL), and 0% in women with a parasite load of less than 1 Pe/mL (Rendell et al., 2015). Similar results were obtained in another study in Spain, with migrants from Bolivia and Paraguay, where 31% of pregnant women with detectable *T. cruzi* DNA by conventional PCR delivered infected children, whereas a 0% parasite transmission rate was observed in babies born to chronic infected mothers with negative PCR findings (Murcia et al., 2013).

A high *T. cruzi* parasite load was also detected in patients co-infected with HIV (Rosemberg et al., 1992), and a 100% parasite transmission rate was observed in children born to mothers with reactivated Chagas disease, due to immunosuppression in four different studies (Freilij and Altcheh, 1995; Nisida et al., 1999; Scapellato et al., 2009; Agosti et al., 2012).

Congenital *T. cruzi* infection cannot be prevented during pregnancy as there are no studies on the possible teratogenic effects in pregnant women treated with trypanocidal drugs, benznidazole or nifurtimox. However, six different retrospective studies showed that no congenital infection was detected in

infants delivered from a total of 243 infected women that had been treated with benznidazole or nifurtimox prior to pregnancy, in childhood or even in early adulthood (Sosa-estani et al., 2009; Murcia et al., 2013, 2017; Fabbro et al., 2014; Moscatelli et al., 2015; Álvarez et al., 2017).

Altogether, research in this field has strongly supported that parasitemia during pregnancy is a key factor for *T. cruzi* connatal transmission, considering that 100% of the infants were born infected with *T. cruzi* when their mothers displayed high parasitemia during pregnancy. On the other hand, 100% of the children born to drug-treated women or women with naturally very low or no parasitemia were uninfected (Murcia et al., 2013; Rendell et al., 2015). These studies reinforce the notion that decreasing the parasite load might be beneficial in avoiding congenital infection, and supports the idea that either a specific trypanocidal treatment or a possible preconceptional therapeutic vaccine with *T. cruzi* recombinant proteins in near future (Dumonteil et al., 2019) should be offered to women of childbearing age who could potentially transmit the infection to their babies.

PARASITEMIA AND MATERNAL IMMUNE RESPONSE

Interferon-gamma (IFN-γ) and tumor necrosis factor (TNF) are key mediators that control *T. cruzi* infection. IFN-γ activates monocytes/macrophages and stimulates, in synergy with TNF-α, the generation of nitric oxide which kills the parasite (Carlier and Truyens, 2015).

Regarding the maternal immune response during pregnancy, it has been observed that mothers that gave birth to infected children have decreased plasma levels of TNF-α (Cardoni et al., 2004; García et al., 2008) and moderately decreased circulating levels of soluble TNF receptor 1 (sTNF-R1), compared to mothers of uninfected children. Soluble TNF receptors downregulate the biological activity of TNF-α by competing with its membrane receptors (García et al., 2008).

A decrease in production of IFN-γ, in response to parasite antigens, was found in the blood cells derived from the mothers of infected children before and after delivery. However, similar levels of intracellular IFN-γ, within $CD3^+$ cells derived from both groups of infected mothers, were found after polyclonal activation, indicating that they have a comparable ability to produce IFN-γ. Mothers that gave birth to infected children also showed decreased percentages of activated T lymphocytes and monocytes, compared to those who did not transmit the infection to their offspring (Hermann et al., 2004).

Another study that compared cytokine production in *T. cruzi*-infected women that gave birth to uninfected children showed that the mothers with detectable parasitemia presented increased levels of IFN-γ and TNF-α in peripheral, placental and cord blood (Cuna et al., 2009), compared to infected mothers with undetectable parasitemia. These results indicate that, when a higher parasite load is associated with a more robust but pro-inflammatory response, there is no congenital transmission.

Altogether, these findings indicate that the ability to control the infection through an appropriate innate and adaptive immune response against *T. cruzi* to maintain a low parasite load in mothers is associated with lower rates of vertical transmission. Alterations in the control of the inflammatory response may have direct consequences on the congenital transmission and on the children's immune response. Taking into account the challenges related to congenital Chagas diagnosis, the identification of immunological mediators could be very useful for the development of new biomarkers of vertical transmission risk.

PARASITEMIA AND FETAL IMMUNE RESPONSE

T. cruzi infection in pregnant women can induce the activation of T lymphocytes in the fetus *in utero*, as supported by the production of proinflammatory cytokines like interleukin (IL) 1β, IL-6 and TNF-α, in response to *T. cruzi* antigens in uninfected infants born to *T. cruzi*-infected mothers (Vekemans et al., 2000; Hermann et al., 2002; García et al., 2008).

The study of serum cytokines showed a distinct immune profile in congenitally infected infants, with a vigorous innate immune response skewed towards a Th17 profile. Decreased levels of IFN-γ, but increased levels of IL-17A, monokine induced by gamma interferon (MIG) and monocyte chemoattractant protein-1 (MCP-1), were revealed as early predictors of *T. cruzi* infection in the presence of either high or low parasitemia, while *T. cruzi*-infected infants also displayed increased levels of IL-6 and IL-17F, but only in the presence of low parasitemia (Volta et al., 2016).

These demonstrations of a distinct and polarized profile of cytokines and chemokines in the circulation of infants born to *T. cruzi*-infected mothers, and its correlation with the newborn parasite load, reinforce the role of the immune system in restricting the severity of this parasitic infection, preventing the morbidity and mortality of a possible congenital Chagas disease.

PARASITE DIVERSITY AND THE PLACENTAL BARRIER

T. cruzi parasites display genetic differences that have been defined by molecular markers and can be differentiated into six discrete typing units or DTUs (TcI to TcVI), with a localized geographical distribution (Zingales et al., 2012). Although efforts have been made to correlate the different *T. cruzi* DTUs with parasite virulence or clinical manifestations in humans, there has not been any clear association so far (Del Puerto et al., 2010).

T. cruzi parasites from almost all DTUs, except TcIV, have been found in babies born to infected mothers. TcV is the predominant DTU reported in the congenital cases in the Southern cone countries of Latin America (Burgos et al., 2007; Virreira et al., 2007; Corrales et al., 2009). In our laboratory, 38 parasite isolates were obtained from 382 *T. cruzi*-infected pregnant women, which represents 10% positive hemocultures in this group. All of the isolated parasites were identified as

TcV, among which only six belonged to mothers who have gave birth to infected children (Bua et al., 2012); thus, we were not able to associate any parasite DTU with connatal transmission. Nevertheless, it would be interesting to study the genetic differences between parasites isolated from mothers who did not transmit the infection in two or even three different pregnancies and those parasites isolated from infected children (Bua et al., 2013), looking at other biomarkers that would probably help in discriminating the divergences in virulence and pathogenicity under the DTU classification.

Many studies have tried to mimic the human maternal-fetal *T. cruzi* transmission in experimental models with rare offspring infections (Carlier and Truyens, 2015), but some studies with TcI, TcII and TcVI strains revealed that infected pups were obtained only from acutely infected mice with Y and Tulahuen strains which were TcII and TcVI, respectively (Cencig et al., 2013). This suggests that the connatal transmission in experimental models were related more to mice parasitemia than DTU differences. It was possible to obtain a congenital transmission rate of 3.7% in chronically infected mice with the *T. cruzi* strain RA (TcVI), although maternal parasitemia in those mice was significantly higher than the mice infected with K98 clone/TcI, from which no congenitally infected offspring were obtained (Solana et al., 2002).

Another interesting study on the genetic response of the placenta in chronic experimental infections in mice compared the virulence of *T. cruzi* K98 clone with an isolated parasite from a congenitally infected child (VD/TcVI), and demonstrated that the murine placental infection with the VD isolated parasite was associated with upregulation of genes related to components of the innate immune system and IFN-γ. Even so, no congenital transmission was observed in pups born to infected mice with VD nor K98 parasites (Juiz et al., 2017). The VD/TcVI parasite proved to be more infective in the human trophoblast-derived cell line BeWo compared to the *T. cruzi* Y strain/TcII (Medina et al., 2018), probably due to a higher virulence and placental tropism, as it was isolated from a human case of congenital infection (Risso et al., 2004). Nevertheless, no significantly different infection levels could be observed on placental explants with a *T. cruzi* isolated from a congenitally-infected newborn (Lucky, TcII/VI) compared to the Tulahuen strain (TcVI), although the isolated parasite Lucky showed a greater survival rate in a deleterious placental milieu (Triquell et al., 2009). It was demonstrated that a high inoculum of these two parasites resulted in increased infection of placental explants, producing structural and physiological changes through nitric oxide synthase and oxidative-nitrosative stress of the placental barrier (Triquell et al., 2018).

The human placenta forms an anatomical barrier between the maternal blood and fetal tissue, and when infected by *T. cruzi,* a reorganization of the extracellular matrix occurs (Duaso et al., 2012), and a differential expression of pro-inflammatory and immune-modulating cytokines has been observed in infected human placental explants (Castillo et al., 2018), confirming the important role of this organ in avoiding parasite infectivity (Liempi et al., 2014; Díaz-Luján et al., 2016; Juiz et al., 2017).

PARASITEMIA AND DIAGNOSIS IN CONGENITALLY INFECTED INFANTS

Since most *T. cruzi*-infected pregnant women and children are asymptomatic, this parasite infection can go undetected. Additionally, there is a current under-diagnosis of this infection due to losses of opportunities in the prenatal care and proper child follow-up by the health system surveillance programs (Carlier et al., 2015).

The diagnosis of *T. cruzi* congenitally infected children under 8–10 months of age relies primarily on the detection of the parasite, usually live parasites in blood by microscopic methods, as specific antibodies are usually transferred by their seropositive mothers. Only when parasitological assays fail to detect the infection are infants required to be monitored over time for the detection of parasite-specific antibodies, which confirm additional cases of *T. cruzi* infection when maternal antibodies disappear (De Rissio et al., 2010).

In most of the Latin American countries, an early diagnosis of infants born to *T. cruzi*-infected pregnant women relies on the direct examination of the buffy coat from fresh blood samples collected in microhematocrit heparinized tubes or microtubes. This micromethod has limited analytical sensitivity (40–50 parasites/mL) and strongly depends on trained operators (Freilij and Altcheh, 1995; De Rissio et al., 2010), due to the fact that this method needs a minimal 30 min of microscopic observation per sample. Since the micromethod only detects 40–60% of congenitally infected newborns, it is necessary to perform additional serological tests at 8–10 months of age, a period in which around 40–60% of the children do not complete the follow-up for the final diagnosis of this infection (De Rissio et al., 2010; Bua et al., 2013).

MOLECULAR APPROACHES FOR THE PARASITOLOGICAL DIAGNOSIS OF THE CONNATAL *T. cruzi* INFECTION

T. cruzi nucleic acid amplification by PCR has been utilized since 1998 (Russomando et al., 1998) for the detection of *T. cruzi* in congenitally infected babies, offering a higher sensitivity and specificity than parasitological methods involving direct microscopic examination of blood buffy coat samples (Schijman et al., 2003; Virreira et al., 2003; Mora et al., 2005). Later, qPCR technology was developed (Piron et al., 2007; Virreira et al., 2007; Duffy et al., 2009, 2013; Ramírez et al., 2015) and was able to detect 0.85 or 0.43 parasite equivalents per mL (Pe/mL) of satellite DNA and kinetoplastid DNA, respectively, providing more sensitivity than the conventional PCR technique (Cura et al., 2017). qPCR emerged as a potential tool for an accurate and early diagnosis of congenital *T. cruzi* infection (Virreira et al., 2007; Bua et al., 2013). However, a positive amplification of parasitic DNA in newborns could be ambiguously interpreted as a result of maternal parasite DNA debris not related to the passage of live parasites (Virreira et al., 2007), and thus for a positive DNA amplification in babies close to birth it would not be confirmative of parasite infection (Carlier et al., 2015).

This represents a disadvantage over microscopic detection methods, which rely on the observation of viable and motile parasites (Freilij et al., 1983). To avoid misinterpretations, PCR diagnosis would be more reliable at 1 month after delivery (Bua et al., 2013) or for the confirmation of diagnosis with a subsequent blood sample (Murcia et al., 2017).

It is important to highlight that qPCR requires highly equipped laboratories and robust quality controls, frequently found in urban areas or reference health centers but rarely available in maternities or primary point of care units in endemic areas (Porrás et al., 2015; Messenger and Bern, 2018; Picado et al., 2018). In fact, PCR is not included as a tool for diagnosis of congenital *T. cruzi* infection in the Latin American guidelines, with the exception of Chile, although its use is sometimes recommended (Picado et al., 2018). In Argentina, this molecular technique is in the process of being transferred to different laboratories of the national public health network (Cura et al., 2017).

Other molecular methods that could be implemented for the *T. cruzi* diagnosis are the techniques based on isothermal amplification of DNA. These methods overcome the needs for specialized PCR equipment and have been proven to amplify the *T. cruzi* DNA successfully. Loop-mediated isothermal amplification (LAMP) can be performed at a constant temperature of 60–65°C with a simple heat-block (Besuschio et al., 2017; Rivero et al., 2017), and the recombinase polymerase amplification (RPA) can be run at 37–42°C (Castellanos-Gonzalez et al., 2018) with a sensitivity similar to that of the quantitative PCR amplification (Besuschio et al., 2017; Jimenez-Coello et al., 2018). These new molecular approaches await the necessary standardization and validation, but, as with all molecular techniques, the main issue is that parasite DNA amplification cannot be performed without purification of DNA from patient blood samples, which cannot be performed easily in health centers in rural areas, as it requires experienced operators, infrastructure and the necessary quality controls recommended by good practice guidelines.

PARASITEMIA IN *T. cruzi* INFECTED-CHILDREN AT A 1-YEAR FOLLOW-UP STUDY

Parasitemia levels in infants congenitally infected with *T. cruzi* are significantly higher at birth than in their infected mothers, who are usually in the chronic phase of this infection (Schijman et al., 2003; Virreira et al., 2007; Bern et al., 2009; Bua et al., 2012), and no correlation has been observed between the parasitemia of pregnant women and their babies (Bua et al., 2012).

Parasite load was quantified by qPCR in 51 infected babies born to *T. cruzi*-infected mothers in a retrospective study. These babies were grouped according to the time and method in which congenital infection was diagnosed during 1-year follow-up after delivery. A group of 19 newborns diagnosed by micromethod at 1 month showed the highest median parasitemia, around 1,700 Pe/mL. The infected infants that came back for a second parasitological diagnosis at 6 months of age showed a median parasitemia of around 20 Pe/mL in the sample obtained at 1 month of age, which was under the threshold of the micromethod sensitivity. This group of 10 infants could be diagnosed by microscopy at 6 months of age because parasitemia increased up to 500 Pe/mL. In the infants (22/51) negative for the first and second parasitological control, who required serological diagnosis at around 1 year of age, the median parasite load was 5,800 and 20 Pe/mL in the blood samples obtained at 1, 6, and 12 months after delivery, respectively. This study helped to understand the differences among diverse groups of *T. cruzi* congenitally infected children during 1 year follow-up in centers where molecular techniques are not available (Bua et al., 2013). An infected child that is not diagnosed at 8–10 months after delivery and not treated will experience a drastic decrease of parasitemia, indicating the transition from the acute phase to the chronic phase of the *T. cruzi* infection, and most importantly, will be excluded from the possibility of being treated with trypanocidal drugs.

Although DNA amplification has shown great sensitivity for the detection of cases of *T. cruzi* mother-to-child transmission, qPCR does not detect 100% of congenital cases, and in case of negative PCR findings, it is necessary to detect the congenital infection by serology at 8–10 months of age. Quantitative PCR was able to detect *T. cruzi* infection in 50/51 babies in the first control visit, and we did not observe any false positive PCR in the babies diagnosed by micromethod at 1 month after delivery (Bua et al., 2013). The only *T. cruzi*-infected infant that could not be diagnosed by qPCR at 20 days nor at 6 months of age was infected by a TcI *T. cruzi* parasite that was isolated by hemoculture at 7 months of age, a period in which the specific anti-*T. cruzi* serology was also positive (Volta et al., 2018).

SEARCH OF BIOMARKERS FOR THE EARLY SEROLOGICAL DIAGNOSIS OF THE CONNATAL *T. cruzi* INFECTION

As mentioned, the detection of anti-*T. cruzi* specific antibodies in infants born to seropositive mothers can be performed when they are 8–10 months of age, when maternal passively transferred antibodies are no longer detectable (Moya et al., 1989). A positive serological result at this time is a conclusive diagnosis for the *T. cruzi* congenital infection in infants where previous parasitological methods failed to detect the parasite. Unfortunately, only 40–60% of congenitally infected children complete the required 1-year follow-up (Sosa-Estani, 2005; De Rissio et al., 2010). Efforts are being made to find specific serological markers to diagnose this infection at an earlier stage and overcome the loss of opportunities to detect 100% of the *T. cruzi* infected children as soon as they are born.

The *T. cruzi* Shed Acute Phase Antigen (SAPA) (Affranchino et al., 1989) proved to be a reliable and highly sensitive marker for the early parasite detection of congenital *T. cruzi* infection (Reyes et al., 1990). An ELISA test available in Paraguay detects anti-SAPA IgG antibodies in children born to infected mothers at 3 months of age (Russomando et al., 2010). The anti-SAPA IgG levels in binomial blood samples from seropositive mothers

and their babies also allowed for diagnosis in 90.5% of the *T. cruzi*-infected children at around 1 month of age (Volta et al., 2015) by subtracting the anti-SAPA OD value of the mother from the one in the child (Mallimaci et al., 2010). We observed a positive correlation between parasitemia levels in mothers and infants, evaluated by qPCR, and the anti-SAPA IgG antibody titers detected by ELISA, which more likely accounts for the secretion of SAPA antigen by the trypomastigotes in the bloodstream (Volta et al., 2015).

Trypomastigote secreted/excreted (TESA) protein bands of 120–200 kDa were blotted on membranes (Umezawa et al., 1996) and recognized by anti-SAPA IgM antibodies in acute and congenital *T. cruzi*-infected children, but with lower sensitivity than qPCR (Messenger et al., 2017). Western blotting with TESA antigen helped discriminate the chronic maternal infection by detecting IgG bound to TESA (a single 150–160 kDa band) from the blood of newborns with acute infection, highlighting the presence of four to six SAPA-specific protein bands between 120 and 200 kDa on the IgM TESA-blot (Noazin et al., 2018). Although the sensitivity of the anti-SAPA IgM on TESA blots reached 80% in *T. cruzi*- infected newborns (Noazin et al., 2018), the immunodetection of membrane strips has several issues of reproducibility and standardization in care units outside urban areas (Messenger and Bern, 2018).

The SAPA antigen has been considered as a promising biomarker for the diagnosis of *T. cruzi* infection, and some new approaches for the development of diagnostic assays which include detection of SAPA along with other antigenic determinants in single multiplex assays, to confirm the *T. cruzi* infection in humans, are being developed (Granjon et al., 2016). SAPA has also been included in the design of chimeric molecules, named as CP1 and CP3, which were sensitive enough to circumvent inconclusive diagnosis in subjects with serodiscordant findings (Peverengo et al., 2018). These multi-epitope constructs are currently being tested for an improved detection of congenital infected newborns (Dr. Ivan Marcipar, personal communication).

Another recent development was to span *T. cruzi* linear B-cell epitopes and design antigenic short peptides to achieve an accurate diagnosis of this infection in chronic human samples by ELISA (Mucci et al., 2017). The next step is to extend this approach with the aim of an early accurate diagnosis for congenital infection (Dr. Fernán Agüero, personal communication).

In summary, the current search for highly sensitive serological diagnostic tests based on multiple antigenic determinants in multiplex assays could offer the possibility to detect *T. cruzi*-infected children born to seropositive women. A prompt diagnosis may prevent dropout during the 1-year serological follow-up after delivery required for the accurate diagnosis of *T. cruzi* infection. Ideally, serological diagnosis for early parasite detection in newborns could be available as a lateral flow immunochromatographic test, with an affordable cost for public health systems, easily performed by operators with minimal training, without the need of any specialized and costly equipment, no reagent preparations, with immediate results and easy adaptability for use in primary health care facilities, public hospitals or maternities in endemic area. As established by WHO, *T. cruzi* infection is curable if treatment is initiated soon after infection (Carlier et al., 2011; WHO | Chagas disease (American trypanosomiasis), 2019). Many reports have shown that benznidazole and nifurtimox treatments are well tolerated in children and resulted in undetectable parasite load (Russomando et al., 1998; Blanco et al., 2000; Schijman et al., 2003; Altcheh et al., 2005; Luquetti et al., 2005).

Infants who fail to complete the required follow-up period for parasite diagnosis will be deprived of access to immediate drug treatment and parasite clearance and will become *T. cruzi*-infected adults. It is crucial to develop improved, rapid and simple diagnostic methods for a timely detection of *T. cruzi* congenital infection, soon after birth and before the newborn leaves the care unit, especially in rural areas where access to the health system can be limited.

CONCLUSION

Mother-to-child transmission of *T. cruzi* infection represents a challenge in controlling parasite dissemination in endemic and non-endemic regions. Parasitemia in infected women plays a key role in congenital Chagas outcome, as it directly affects transmission rate and maternal and fetal protective immune response against the parasite. In fact, decreasing parasite load by trypanocidal treatment administered to women of childbearing age proved to be highly efficient in avoiding congenital infection. Parasite levels in congenitally infected newborns have a direct impact on their diagnosis, so it is crucial to develop improved diagnostic methods to facilitate access to treatment.

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Contribution of Murine Models to the Study of Malaria During Pregnancy

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Annually, many pregnancies occur in areas of *Plasmodium* spp. transmission, particularly in underdeveloped countries with widespread poverty. Estimations have suggested that several million women are at risk of developing malaria during pregnancy. In particular cases, systemic infection caused by *Plasmodium* spp. may extend to the placenta, dysregulating local homeostasis and promoting the onset of placental malaria; these processes are often associated with increased maternal and fetal mortality, intrauterine growth restriction, preterm delivery, and reduced birth weight. The endeavor to understand and characterize the mechanisms underlying disease onset and placental pathology face several ethical and logistical obstacles due to explicit difficulties in assessing human gestation and biological material. Consequently, the advent of murine experimental models for the study of malaria during pregnancy has substantially contributed to our understanding of this complex pathology. Herein, we summarize research conducted during recent decades using murine models of malaria during pregnancy and highlight the most relevant findings, as well as discuss similarities to humans and the translational capacity of achieved results.

Keywords: murine models, *Plasmodium* spp., malaria, pregnancy, placental malaria

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MALARIA IN HUMAN PREGNANCY

Malaria still represents a serious public health issue for several communities distributed worldwide. Estimations from 2017 have shown that at least 219 million cases occurred in areas of *Plasmodium* spp. transmission, in which approximately half a million victims died from the disease (World Health Organization, 2018). These estimations encompass pregnant women, who are particularly more susceptible to developing severe clinical manifestations resulting from malaria in pregnancy (MiP) induced by *Plasmodium* spp. Although outdated, estimations performed by Dellicour et al. (2010) noted 125.2 million pregnancies occurring in malaria-endemic areas. This study came as a follow-up of previous estimations suggesting that 25 million pregnancies occur in areas of *P. falciparum* transmission alone, distributed across sub-Saharan Africa (Desai et al., 2007). Nevertheless, current epidemiologic knowledge is inaccurate and imprecise and might support outdated and underestimated predictions, hiding a much more alarming reality.

This concerning epidemiological scenario highlights the importance of conducting preventive measures to control MiP, which might drastically evolve to placental malaria (PM), a pathology frequently associated with the occurrence of poor outcomes during pregnancy, such as maternal and fetal mortality, intrauterine growth restriction (IUGR), preterm birth and reduced birth weight

(Desai et al., 2007; Umbers et al., 2011; Rogerson et al., 2018). These deleterious consequences affecting both the mother and the growing fetus are reflections of histological and physiological changes occurring within the placenta [recently summarized elsewhere (Sharma and Shukla, 2017)]. One of the key features of *P. falciparum* PM is the accumulation of *P. falciparum*-infected erythrocytes (IE) in the placenta (Beeson et al., 2002; Muthusamy et al., 2004). Upregulation of the VAR2CSA protein, a variant of the highly polymorphic adhesion peptide *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) (Salanti et al., 2003), is responsible for parasite sequestration upon preferential binding to chondroitin sulfate A (CSA), which is abundantly expressed in the placenta (Fried et al., 2006; Muthusamy et al., 2007). Consequently, a severe local inflammatory process is triggered, characterized by the infiltration of monocytes and leukocytes in the placenta and eventual inflammation in response to parasite accumulation (Ismail et al., 2000; Parekh et al., 2010; Lucchi et al., 2011; Souza et al., 2013). This process has been frequently associated with placental histological alterations during MiP, such as dysregulation of placental architecture, formation of syncytial knots, fibrin deposition, necrosis, and placental barrier thickening (Walter et al., 1982; Ismail et al., 2000; Souza et al., 2013), and has been reported to occur during IUGR and preterm delivery, as well as in cases of reduced birth weight (Moormann et al., 1999; Menendez et al., 2000; Rogerson et al., 2003; Umbers et al., 2011).

Although we have obtained substantial knowledge in the field, studies on the epidemiology and pathology of MiP are frequently challenging due to related ethical and logistic difficulties. Long gestational periods, uncontrollable experimental planning, difficult access to biological and placental samples, and critical constraints associated with human experiments represent significant barriers that slow research progress and the understanding of this severe and complex disease. Therefore, alternatives have emerged with the advent of murine models to study MiP that have brought fundamental knowledge to the field. Herein, we have compiled research conducted for the past four decades using experimental rodent models, highlighting the most relevant findings, similarities to humans and, consequently, the translational capacity of achieved results.

HUMAN AND MURINE GESTATION: COMPARISON AND TRANSLATIONAL CHALLENGES FOR THE STUDY OF MiP

Comparative Gestation Development

In parallel with some other features, gestation length and development are somewhat different between human and murine mammals. Human gestation lasts for approximately 38 weeks (three trimesters) in contrast to rodents, in which gestation takes place over a 3-week period (Murray et al., 2010). In addition, there are also slight differences between species regarding the implantation period. Murine implantation takes place between the fourth and fifth days of gestation, somewhat sooner than in humans, in which this phenomenon occurs between the fifth and sixth days (Rossant and Tam, 2017). After implantation, gestation will progress toward a shift in fetal nutritional means. During early gestation in both humans and rodents, the growing fetus will survive through means of histiotrophic nutrition, in which nutrients are acquired by the uptake of substances secreted from the uterine glands present in the endometrium (Burton et al., 2002; Georgiades et al., 2002). However, the nutritional strategy changes around mid-gestation when the maternal blood supply to the placenta is completely established. Accordingly, the nutrient and gas exchanges between the mother and the fetus become functional, marking the onset of placental hemotrophic nutrition (Burton et al., 2001; Georgiades et al., 2002). In humans, this phenomenon occurs between the end of the first and the beginning of the second trimester, while in mice and rats, the same scenario occurs specifically at mid-gestation (between the twelfth and thirteenth days) (Georgiades et al., 2002). This shift in nutritional strategy occurs with the onset of organogenesis and fetal development, after which growth will continue until gestational term is reached.

Comparative Placental Function, Structure, and Histology

Human and murine placentas have a considerable degree of similarity and are nearly identical from physiological and functional perspectives (Rossant and Cross, 2001; Georgiades et al., 2002). In both mammalian species, this transient organ ensures nutrient and gas exchange between the mother and the growing fetus (Lager and Powell, 2012), maintains tolerance to the maternal immune system (Kanellopoulos-Langevin et al., 2003), and works as a physical and immunological barrier against endogenous pathogens (Robbins and Bakardjiev, 2012).

The placentas in both species are discoid organs in which maternal blood coming from the endometrium circulates and is in direct contact with trophoblasts, the fetal-derived cells responsible for regulating most placental physiological functions. In both species, the placenta can be structurally divided into three main areas: (1) an outer layer, consisting of uterine decidual cells and maternal blood vessels; (2) a middle layer, considered the implantation site where the placenta attaches to the uterus; and (3) an inner layer, in which the maternal blood interacts with trophoblasts to promote vital metabolic exchanges [reviewed elsewhere (Georgiades et al., 2002)]. Accordingly, the outer area can be mainly described as being composed by the myometrium and decidua basalis, which are extensively infiltrated by maternal arteries. This area is partially invaded by fetal-derived trophoblasts during the implantation process, which is considerably similar between both mammalian species. Moving toward the inner area of the human placenta, it is possible to distinguish a structural layer with no counterpart in the murine placenta known as the basal plate. This implantation site contains several distinct trophoblast subpopulations, such as extravillous (EVT) and cytotrophoblast (CT) cells. Nevertheless, an analogous area defined as the junctional zone occurs in murine animals, which is populated by specific types of cells such as trophoblast giant cells (TGC) and spongiotrophoblasts (SPG). Inside the placenta, more pronounced differences are observed regarding

morphology and structure. The central area, which is commonly referred to as the human fetal placenta, is constituted by villi in an extremely ramified tree-like structure, which increases the contact surface with the maternal blood freely circulating within the intervillous space (IVS) (**Figure 1A**). On the other hand, a similar structure known as the labyrinth develops in mice and rats (**Figures 2A,C**). This impacts maternal blood circulation inside the placenta, where blood stays confined to the tortuous and sinusoidal channels (Rossant and Cross, 2001). Additionally, the surface area contacting the maternal blood is somewhat distinguished between humans and rodents from both cytological and structural perspectives. In the human placenta, maternal blood is separated from fetal capillaries by a single layer of syncytiotrophoblasts (ST), beyond which CT cells are present together with the basal lamina and fetal endothelium (schematically represented in **Figure 3**). According to these characteristics, the human placenta is classified as hemomonochorial (Takata et al., 1997; Georgiades et al., 2002). In opposition, three layers compose the murine labyrinthine wall: one composed of mononuclear trophoblasts dispersed throughout the surface contacting maternal blood and two ST layers definitively separating the maternal and fetal compartments (illustrated in **Figure 3**). Accordingly, murine placentas are classified as hemotrichorial (Takata et al., 1997).

Nevertheless, the structural divergences of human and murine placentas have occurred in parallel with an extensive variety of resident cell populations that are independent of their analogs but not similar in development and characteristics and are equally responsible for the same physiological and functional processes in human, mouse and rat placentas (**Figure 3**).

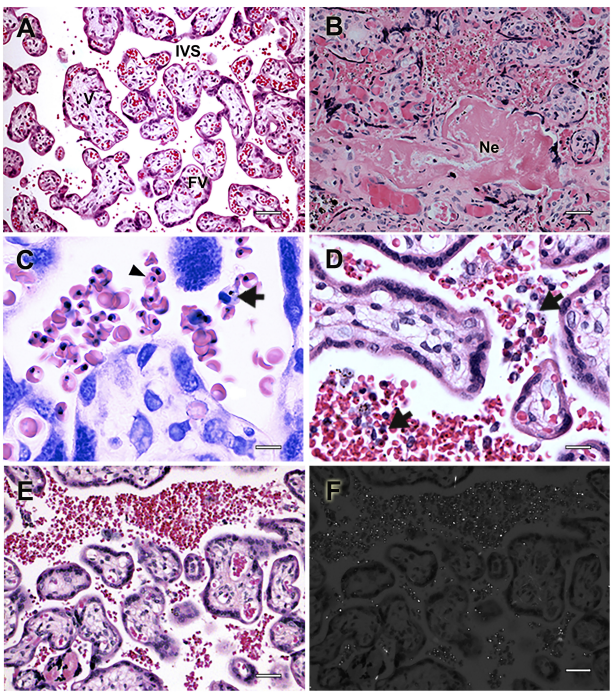

FIGURE 1 | Histologic events associated with placental pathology during human malaria in pregnancy. Placental histologic sections of non-infected **(A)** and *P. falciparum*-infected **(B–F)** women stained with Hematoxylin-Eosin. **(A)** Healthy human placenta without tissue damage and normal architecture in which intervillous spaces (IVS), usually bathed in maternal blood are in contact with placental villi (V), feeding the growing fetus through the fetal vessels (FV). **(B)** *P. falciparum*-infected placentas often characterized by extensive necrotic areas (Ne) and tissue disorganization. **(C,D)** Mononuclear cells infiltrate (arrow) is frequently patent due to the accumulation of parasitized erythrocytes (arrowhead). **(E)** Massive sequestration of *P. falciparum*-infected erythrocytes in the placenta is accompanied by **(F)** the accumulation of hemozoin in the IVS, which can be detected by polarized light microscopy. Scale bars represent **(A,B,E,F)** 100 μm, **(C)** 5 μm, and **(D)** 15 μm. Microscopy pictures were taken by Rodrigo Medeiros de Souza.

Using Murine Models to Study Malaria in Human Pregnancy

It is required that a suitable experimental model includes a group of features that lead to valid and translatable findings. As such, it is pivotal that rodent physiology and genetics, as well as pathologic manifestations during disease, resemble those in humans to a certain extent, ultimately validating them as models for human research (Justice and Dhillon, 2016). Accordingly, after several models were reviewed, mice and rats were validated by Desowitz as suitable to study MiP (Duffy and Fried, 2001) under the assumption that physiological and pathological similarities observed between rodents and humans were sufficient to consider them appropriate to study this disease. Shared placental characteristics, such as hemochorial and discoid structure (Georgiades et al., 2002; Wildman et al., 2006), hemotrophic nutrition (Burton et al., 2001; Georgiades et al., 2002) and analogous placental cell populations (Rossant and Cross, 2001; Georgiades et al., 2002), encourage their usage as models of MiP. These similarities are supported by molecular phylogenetic analysis, which clusters both rodents and humans into the same evolutionary clade (Wildman et al., 2006). The molecular similarities can be extended to immunity, an important aspect to consider when studying a disease in which poor outcomes are predominantly dependent on the host's immune response to malaria (Moormann et al., 1999; Rogerson et al., 2003). Hence, despite controversial discussion, it was shown that patterns of gene expression and biological pathways altered in mouse models of inflammatory diseases were significantly correlated with those from corresponding human conditions (e.g., sepsis) (Takao and Miyakawa, 2015), further supporting the usage of these animals for studying inflammatory diseases such as MiP. Together with the features mentioned above, the short gestational period, capacity for frequent and successive pregnancies, as well as large litter sizes, allow the rapid gathering of a considerable number of biological samples, which makes the rodents a powerful model for studying any pregnancy-related disease.

In addition to host characteristics, parasite intrinsic features are essential to validate experimental models of MiP. Above all, the most relevant features to consider should be the similarities between human and murine parasites cytoadhesion mechanisms. It is well known that the pathogenesis mechanisms involved in severe malaria induced by *P. falciparum* are associated with

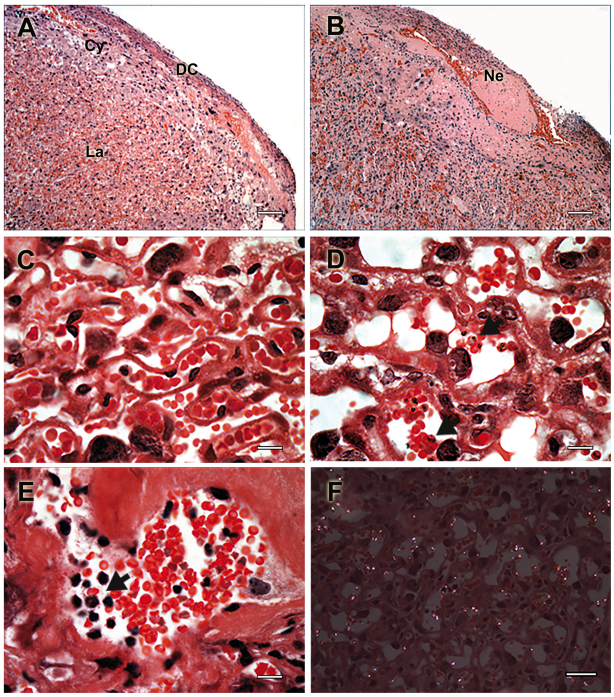

FIGURE 2 | Histologic events associated with placental pathology during murine malaria in pregnancy. Placental histology of non-infected **(A,C)** and *P. berghei*-infected **(B,C,E,F)** mice stained with Hematoxylin-Eosin. **(A)** Healthy placenta with normal histologic structure characterized by normal distribution of layer-specific cell such decidual cells (DC), trophoblastic cells (Cy), and labyrinthic cells (La), having **(C)** a normal labyrinth organization. **(B)** Fibrinoid necrosis (Ne) is depicted in section of *P. berghei*-infected placentas with **(D)** massive tissue disorganization and trophoblast membrane thickening. **(E)** Mononuclear cell infiltrate is visible (arrow), which occurs in response to **(D)** parasite accumulation (arrow) and **(F)** hemozoin deposition observed using polarized light microscopy. Scale bars represent **(A,B)** 100 μm, **(C–E)** 15 μm, and **(F)** 30 μm. Figure was modified from Neres et al. (2008) and Marinho et al. (2009) upon permission granted on behalf of journal's editorial board.

tissue- and organ-specific sequestration of IE (Schofield and Grau, 2005). More specifically, *P. falciparum* MiP may lead to severe PM as a result of IE sequestration in the placenta, which is mediated by the PfEMP1 variant VAR2CSA (which binds to placental CSA). The wide range of PfEMP1 variants known to be encoded by 50–60 *var* genes (Kraemer and Smith, 2006) have no known homologs in murine parasites (Hall et al., 2005). In fact, only some genetic signatures are similar between the murine and human *Plasmodium* species that encode alternate variable surface antigens (VSA), which were grouped in the multigenic *pir* superfamily (*Plasmodium* interspersed repeats) (Janssen et al., 2004; Hall et al., 2005). Nevertheless, murine parasite strains such as *P. berghei* have been shown to accumulate in specific tissues such as brain, fat, lung and spleen in a CD36-dependent and independent manner, which is also a well conserved pathogenesis mechanisms of severe malaria induced by *P. falciparum* (Franke-Fayard et al., 2010). In the same extent, the expression of alternate VSA and the binding capacity to placental CSA observed in murine parasites support the conclusions regarding the similarities between the pathogenesis mechanisms involved in murine and human disease (Hall et al., 2005; Neres et al., 2008; Marinho et al., 2009; Hviid et al., 2010). Distinct mechanisms of pathogenesis might also be directly linked with the biology of the parasites. As such, it is advisable to consider that the usage of murine parasites needs to be adjusted with caution since specific strains might better mimic the distinct diseases associated with the unrelated biology of human parasites (e. g. *P. falciparum* or *P. vivax*). Accordingly, disease severity in mice tends to be higher in *P. berghei* ANKA causing lethal anemia and cerebral malaria in C57BL/6 for instance (resembling *P. falciparum* in humans); however, it has a tropism for reticulocytes (similar to *P. vivax*). Differently, *P. chabaudi* invades mature erythrocytes and is responsible for a less severe pathology shown by the resistance to infection observed in BALB/c and C57BL/6 mice. In the same extent, different parasites might also have different tendencies to accumulation in specific tissues, being better used to study specific diseases and host-pathogen interactions (reviewed in Lamb et al., 2006).

Despite the aforementioned host and parasite characteristics, one must carefully translate findings obtained in murine models to humans, especially regarding preclinical observations, due to the observed differences between both mammals. Nevertheless, the following sections will depict some of the current knowledge acquired using murine models of MiP (research details for each model are presented in **Table 1**) during the past decades while describing the parallel characteristics existing between humans and rodents in regard to poor pregnancy outcomes and MiP placental pathology (summarized list in **Table 2**).

CONTRIBUTION OF MURINE MODELS TO THE STUDY OF MiP

Understanding Recrudescence Using Murine Models of MiP

Currently, we have been facing the emerging problem of drug resistance acquisition observed in *P. falciparum* infections, the outcome of which might be the occurrence of recrudescence (without a new infection episode) as a consequence of subcurative therapy (Cattamanchi et al., 2003; Berrevoets et al., 2013). In addition to treatment incapacity to clear circulating parasites, the immune depression observed in specific situations, such as during pregnancy, might elicit the emergence of recrudescent parasites that are either dormant or at submicroscopic levels (Giobbia et al., 2005; Mayor et al., 2009; Laochan et al., 2015; Al Hammadi et al., 2017; Malvy et al., 2018). In this context, some of the first available studies using murine models of MiP appeared during the early 1980s, when van Zon and Eling first described recrudescence in pregnant mice infected with *P. berghei* (van Zon and Eling, 1980; van Zon et al., 1982). Females of different mouse strains (Swiss, C3H/StZ, BALB/c, and B10LP) challenged with *P. berghei* K173 were treated with chloroquine and sulfadiazine to decrease parasitemia and

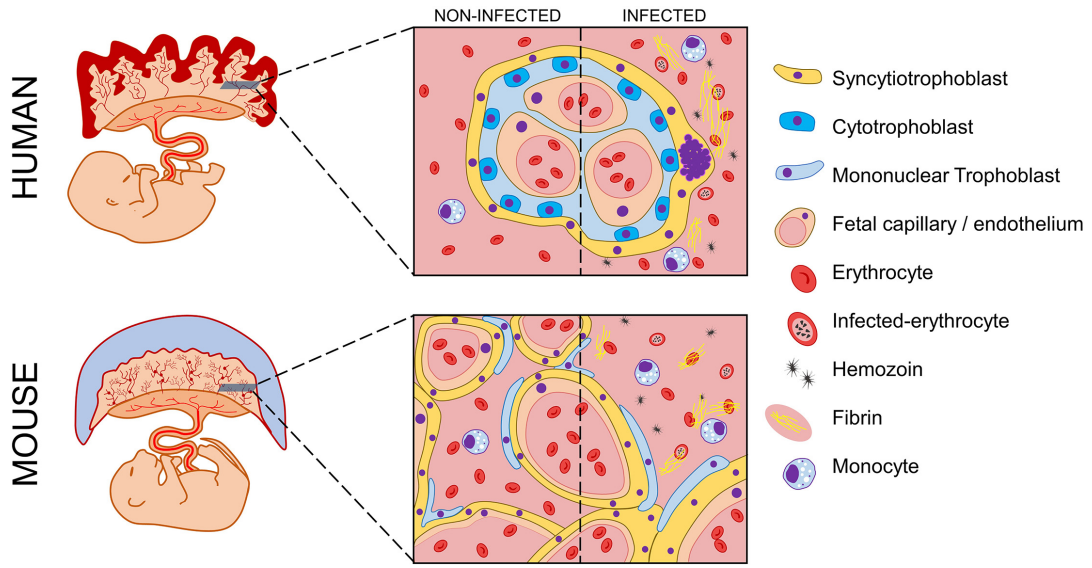

FIGURE 3 | Schematic representation of possible histologic events in human and murine placentas during malaria in pregnancy. Human and murine fetuses are represented with corresponding placentas. Magnification of transversal slices of each placental core illustrate histologic architecture, which widely differs between human placental villi and murine labyrinthine structure. During malaria in pregnancy, both human and murine placentas experience histopathologic manifestations that impair local homeostasis and lead to poor pregnancy outcomes. A comparison between both infected placentas and their non-infected counterparts is depicted. The presence of monocyte infiltrate is visible, which occurs in response to parasite and hemozoin accumulation. Histologic modifications such as fibrin deposition and syncytial knots occur due to the onset of severe local inflammation. Legend of picture components is vertically displayed beside the scheme.

promote the acquisition of immunity before mating. Afterward, pregnancy was shown to induce recrudescence, which was clearly shown to be strain- and gravidity-dependent (van Zon and Eling, 1980) and linked to preterm delivery and maternal mortality (van Zon et al., 1982). Consequently, the pioneering results enabled the authors to draw brief conclusions on similarities observed between human and murine recrudescence by determining that the phenomenon could be associated with (1) the drugs' incapacity to completely clear the parasite in a previous malaria episode and (2) the particular immune status associated with pregnancy, recapitulating the features of recrudescence in human MiP (Giobbia et al., 2005; Al Hammadi et al., 2017; Malvy et al., 2018). Pregnancy-associated immune modulation was then shown to be linked to increased production of corticoids during murine gestation, thereby facilitating the onset of recrudescence (van Zon et al., 1982, 1985). Notwithstanding the relevance of van Zon and coworkers' findings, parasite reappearance in maternal circulation was often ensured by experimental reinfection, raising questions about the translational reliability of murine recrudescence results obtained in these models (van Zon and Eling, 1980; van Zon et al., 1982).

Despite the promising advances made during that period, recrudescence in murine models was poorly addressed until the last decade. Only in 2009, two studies have addressed questions about the topic (Marinho et al., 2009; Megnekou et al., 2009). By reevaluating the pioneering experimental model implemented by van Zon and coworkers, the team of Megnekou addressed the production of specific anti-VSA antibodies in pregnant BALB/c mice immunized against *P. berghei* K173. Although previously immunized, protection was lost during pregnancy due to the recrudescence of parasites expressing specific VSA. Consequently, protection was shown to be gravidity-dependent since the less susceptible multiparous mice have raised antibodies against parasite-specific VSA during their previous pregnancies (Megnekou et al., 2009). However, some parasite reappearances were once again ensured by experimental infections, raising the same translational limitations discussed above. During that same year, a study from Marinho et al. (2009) established a model to study recrudescence in BALB/c mice, however, without recurring to mid-gestation reinfection. Accordingly, non-pregnant mice were infected with *P. berghei* ANKA and administered a subcurative treatment to clear apparent parasitemia. Mice were then mated approximately 1 month later and analyzed for recrudescence, which occurred in up to 58% of pregnant mice from gestational day (G)12 onward (Marinho et al., 2009). Maternal mortality and parasitemia were shown to be increased in primigravidae, which diminished with increased gravidity, supporting the observations of Megnekou et al. (2009) regarding acquired immunity in subsequent pregnancies.

Nevertheless, both recrudescent and newly inoculated parasites constitute a risk for highly susceptible pregnant women (Laochan et al., 2015). Accordingly, a recent study has shown that *P. chabaudi* CB sporozoites are capable of inducing recrudescence in pregnant C57BL/6 mice, being more closely related to the human parasite life cycle. However, poor pregnancy outcomes, such as maternal mortality and reduced birth weight, were likely to occur upon heterologous reinfection with *P. chabaudi* AS rather than in the cases of recrudescence. Preacquired immunity against the recrudescent

TABLE 1 | Summary of the principal findings and observations done using murine models of malaria in pregnancy.

Author(s)	*Plasmodium* strain	Murine strain	Recrudescence	Anemia	Maternal mortality	Abortion Resorption Stillbirth Preterm delivery	Litter size Newborn/fetal weight/health	Placental abnormalities/alterations
van Zon and Eling, 1980	*P. berghei* K173	Swiss Albino C3H/StZ BALB/c B10LP	↑ Primigravidae ↓ multigravidae ↑ due to ↓ immunity	X	↑ In recrudescence ↑ primigravidae	X	X	X
van Zon et al., 1982	*P. berghei* K173	Swiss Albino	→ To pregnancy	X	↑ In recrudescence	Preterm delivery ↑ in recrudescence	X	X
Oduola et al., 1982	*P. berghei* NK65	A/J ICR	→ To pregnancy	↑ In pregnancy	↑ In early infection ↓ in late infection ↑ parturition in late infection	Stillbirth → to early infection	Normal litter size ↓ Weight → to late infection ↑ spleen weight No congenital malaria	Impaired labyrinth structure Trophoblast barrier thickening IEs/Hz MΦ
van Zon et al., 1985	*P. berghei* K173	Swiss Albino B10LP	→ To pregnancy	X	X	X	X	X
Vinayak et al., 1986	*P. berghei* NICD	Swiss Albino	X	X	↑ In early infection Death before parturition	Stillbirth → to mid gestation infection ↑ resorption	↓ Litter size due to resorption ↓ weight ↑ Spleen weight No congenital malaria	Hyperplasia ↓ placental sinusoids IEs/Hz
Oduola et al., 1986	*P. berghei* NK65	Sprague-Dawley ICR	X	X	X	X	X	Hyperplasia Necrosis/fibrin Impaired labyrinth structure Trophoblast barrier thickening IEs/Hz MΦ/Leu
Desowitz et al., 1989	*P. berghei* NYU-2	Wistar	No recrudescence	↑ In pregnancy	↑ In early infection ↓ in mid gestation infection with ↑ parasitemia at term	No preterm delivery	Normal litter size	↑ Placental parasitemia
Hioki et al., 1990	*P. berghei* NK65	BALB/c	X	↑ In pregnancy ↑ in mid gestation infection	↑ In early infection (death before term) ↓ in late infection (live until term)	X	X	X
Pathak et al., 1990	*P. berghei* NICD	Swiss Albino VRC	No recrudescence	X	↑ Maternal mortality ↓ in treated pregnant mice	Stillbirth → to mid gestation infection	↓ Weight ↓ Litter size	Hyperplasia IEs
Pavia and Niederbuhl, 1991	*P. yoelii* YM *P. yoelii* 17X	ICR	No recrudescence	X	↑ Maternal mortality in non-immunized pregnancies ↓ with ↓ parasite virulence	X	Delivery of dead pups in non-immunized pregnancies No congenital malaria	X

(Continued)

TABLE 1 | Continued

Author(s)	*Plasmodium* strain	Murine strain	Recrudescence	Anemia	Maternal mortality	Abortion Resorption Stillbirth Preterm delivery	Litter size Newborn/fetal weight/health	Placental abnormalities/alterations
Tegoshi et al., 1992	*P. berghei* NYU-2	Wistar	X	X	X	X	X	Hyperplasia Necrosis/fibrin Impaired labyrinth structure Trophoblast barrier thickening ↑ IEs after late infection MΦ/Leu
Adachi et al., 2000	*P. berghei* ANKA	BALB/c	X	X	X	X	Congenital malaria	X
Poovassery and Moore, 2006	*P. chabaudi* AS	C57BL/6	X	↑ In pregnancy → to ↑ parasitemia	↑ Mortality → pregnancy	↑ Abortion/resorption at mid gestation → to early infection	↓ Fetal viability	↑ IEs at mid gestation ↓ monocyte accumulation
Neres et al., 2008	*P. berghei* ANKA	BALB/c	X	X	↑ Mortality → pregnancy	↑ Abortion/resorption during infection ↑ preterm delivery during infection	↓ Fetal/birth weight ↓ fetal blood flow/content ↓ viability	Hyperplasia Necrosis/fibrin Impaired labyrinth structure Trophoblast barrier thickening IEs/Hz MΦ ↓ Placental vasculature
Poovassery and Moore, 2009	*P. chabaudi* AS	C57BL/6	X	X	X	↑ Abortion at mid gestation → to early infection	↓ Fetal viability	IEs/Hz
Poovassery et al., 2009	*P. chabaudi* AS	C57BL/6	X	↑ In pregnancy → to ↑ parasitemia	↑ Mortality → pregnancy	↑ Abortion/resorption at mid gestation → to early infection	↓ Fetal viability → to IFN-γ/TNF-α	Fibrin thrombi Placental hemorrhage Impaired labyrinth structure Monocytes/macrophages
Megnekou et al., 2009	*P. berghei* K173	BALB/c	→ To pregnancy ↑ primigravidae	→ To recrudescence	→ To recrudescence ↑ due to ↓ immunity	X	→ To recrudescence ↓ Litter size ↓ Weight	iRBCs/Hz
Marinho et al., 2009	*P. berghei* ANKA	BALB/c	→ To pregnancy	X	→ To recrudescence ↑ primigravidae ↓ multigravidae	X	→ To recrudescence ↓ Litter size ↓ Weight ↓ Primigravidae ↑ Multigravidae	Trophoblast barrier thickening IEs MΦ/Tc/NKc ↓ Placental vascular spaces
Silver et al., 2010	*P. berghei* ANKA	BALB/c	X	X	X	↑ Abortion/resorption at late-gestation → to mid gestation infection	↓ Fetal weight at late gestation ↓ fetal viability	X
Sarr et al., 2012	*P. chabaudi* AS	A/J C57BL/6	X	↑ In pregnant A/J mice when compared to C57BL/6	↑ In pregnant A/J mice when compared to C57BL/6	↑ Abortion/resorption in A/J and C57BL/6	X	↑ Placental IEs in A/J when comparing to C57BL/6

(Continued)

TABLE 1 | Continued

Author(s)	*Plasmodium* strain	Murine strain	Recrudescence	Anemia	Maternal mortality	Abortion Resorption Stillbirth Preterm delivery	Litter size Newborn/fetal weight/health	Placental abnormalities/alterations
Rodrigues-Duarte et al., 2012	*P. berghei* K173 NK65 ANKAΔpm4	C57BL/6	X	X	↑ In pregnant mice infected with *P. berghei* NK65	↑ Stillbirth → mid gestation infection	↓ Fetal weight at late gestation ↓ newborn viability	Necrosis/fibrin Impaired labyrinth structure Trophoblast barrier thickening IEs ↓ Placental vascular spaces
Avery et al., 2012	*P. chabaudi* AS	C57BL/6	X	↑ In pregnancy → to ↑ parasitemia	X	↑ Abortion at mid gestation → to early infection → to coagulation and impaired fibrinolysis	↓ Fetal viability	Necrosis/fibrin Impaired labyrinth structure
Conroy et al., 2013	*P. berghei* ANKA	BALB/c Wildtype C5aR$^{-/-}$	X	X	X	X	↑ Fetal weight and ↑ viability in infected C5aR$^{-/-}$ mice	↑ Placental vascular remodeling in infected C5aR$^{-/-}$ mice
de Moraes et al., 2013	*P. berghei* ANKA	BALB/c ♀ C57BL/6 ♂	X	X	X	X	X	Impaired labyrinth structure Trophoblast barrier thickening Placental vasculature remodeling IEs in ↓ blood flow areas
Barboza et al., 2014	*P. berghei* NK65	C57BL/6 Wildtype MyD88$^{-/-}$	X	X	X	X	↑ Fetal weight in infected MyD88$^{-/-}$ mice ↑ survival in newborn from infected MyD88$^{-/-}$ mice	↑ Placental vascular spaces in infected MyD88$^{-/-}$ mice
Sharma and Shukla, 2014	*P. berghei* NK65	BALB/c	X	X	↓ In pregnant-infected mice treated with CQ/SP	X	↑ Weight and ↑ viability in newborn from mice treated with CQ/SP	↓ IEs, ↓ Hz, and normal placental histology in mice treated with CQ/SP
Lima et al., 2014	*P. berghei* *P. chabaudi*	C57BL/6	X	X	X	X	X	Accumulation of iRBCs mature forms in the placenta IEs uptake by trophoblast
Sarr et al., 2015	*P. chabaudi* AS	A/J C57BL/6	X	X	X	↑ Abortion/resorption in A/J and C57BL/6	X	↑ Tc, Bc, NKc in conceptus from infected A/J and C57BL/6 mice ↑ MΦ in the placental junctional zone of A/J mice Apoptosis present in immune cells and spongiotrophoblast
Sharma et al., 2016	*P. chabaudi* CB *P. chabaudi* AS	C57BL/6	→ To pregnancy → to CB sporozoites	→ To recrudescence → to reinfection	↑ In reinfections but not in recrudescence	↑ Stillbirth in reinfections and → to high parasitemia	Normal litter size ↓ weight and ↓ malaria susceptibility in reinfection	IEs in recrudescence No histologic alterations

(Continued)

TABLE 1 | Continued

Author(s)	*Plasmodium* strain	Murine strain	Recrudescence	Anemia	Maternal mortality	Abortion Resorption Stillbirth Preterm delivery	Litter size Newborn/fetal weight/health	Placental abnormalities/alterations
Barboza et al., 2017	*P. berghei* NK65	C57BL/6 Wildtype TLR2$^{-/-}$ TLR4$^{-/-}$ TLR9$^{-/-}$	X	X	X	X	↑ Fetal weight in infected TLR4$^{-/-}$ mice	IEs MΦ/NΦ/Dc ↑ placental vascular spaces in infected TLR4$^{-/-}$ mice
Rodrigues-Duarte et al., 2018	*P. berghei* NK65	C57BL/6 Wildtype TLR4$^{-/-}$ IFNAR1$^{-/-}$	X	X	X	↓ Stillbirth in TLR4$^{-/-}$ and IFNAR1$^{-/-}$ infected pregnant mice carrying TLR4$^{+/-}$ and IFNAR1$^{+/-}$ progeny	Wildtype and TLR4$^{-/-}$ infected mice with similar litter weight ↑ weight of IFNAR1$^{+/-}$ fetuses from infected IFNAR1$^{-/-}$ mice	X
Barboza et al., 2019	*P. berghei* NK65	C57BL/6 Wildtype MyD88$^{-/-}$	X	X	X	X	↓ Fetal weight in MyD88$^{+/-}$ progeny born from MyD88$^{-/-}$	↓ Placental vascular spaces in MyD88$^{+/-}$ placentas from MyD88$^{-/-}$

The current table content aims to summarize research conducted during the past four decades that was used to inform this review. In summary, Plasmodium and murine strains are described for each reference, as well as observations made regarding pregnancy outcomes and placental histology. Information regarding symbology is depicted as follows: Increased (↑), decreased (↓), associated (→), males (♂), females (♀), infected erythrocytes (IEs), hemozoin (Hz), leukocytes (Leu), monocytes/macrophages (MΦ), T cells (Tc), B cells (Bc), NK cells (NKc), dendritic cells (Dc), neutrophils (NΦ), chloroquine (CQ), sulphadoxine-pyrimethamine (SP).

parasite might justify why only reinfection with heterologous parasite lead to poor pregnancy outcomes. Nonetheless, exposure to heterologous *P. chabaudi* AS ensures newborn protection against postnatal infection, reliably resembling the outcomes of human MiP (Sharma et al., 2016).

Although observed in murine MiP, some studies have reported no recrudescence in protocols of treatment and immunization used in mice to control infection before pregnancy (Pathak et al., 1990; Pavia and Niederbuhl, 1991). Explanations might rely on the (1) drug efficiency to clear circulating parasite, (2) usage of less virulent strains (e.g., *P. berghei* NICD and *P. yoelii* 17X), or (3) less susceptible mice strains (e.g., Swiss Albino VRC). Nonetheless, despite some controversial experimental settings, findings associated with the aforementioned murine models have revealed some hidden biological features associated with malaria recrudescence during pregnancy that otherwise would be impossible to address.

Understanding Poor Pregnancy Outcomes Using Murine Models of MiP

A multitude of outcomes can occur as a result of developing MiP. When considering the areas of *P. falciparum* frequent and stable transmission, pregnancy-associated consequences might depend on several aspects such as infection trimester, gravidity and maternal health status that ultimately dictate the onset of maternal anemia, fetal and maternal mortality, abortion, and reduced birth weight as consequences of IUGR and/or preterm delivery (Desai et al., 2007; Umbers et al., 2011; Rogerson et al., 2018). Outcomes in human pregnancy are accessible for study; however, in addition to ethical constraints, illogical and incorrect correlations might be drawn from imprecise information given by patients enrolling in these prospective studies, such as imprecise time of infection or clinical status. Fittingly, murine models provided the opportunity to investigate MiP outcomes in a controlled experimental setting.

Maternal Anemia

Maternal anemia that develops during MiP is considered a significant risk factor for poor pregnancy outcomes and is closely associated with increased parasite burden and reduced birth weight (Rogerson et al., 2003). Accordingly, several distinct experimental murine models accurately recapitulate anemia observed during human MiP. Similarly, *P. chabaudi* AS infection potentiates the onset of anemia (reduced hematocrit percentage) in pregnant C57BL/6 mice. Anemia was therefore linked to a rapid increase in parasitemia, which peaks abruptly in pregnant mice (Poovassery and Moore, 2006; Poovassery et al., 2009; Avery et al., 2012; Sarr et al., 2012). This phenomenon was also shown to occur in different murine strains, such as BALB/c mice (Hioki et al., 1990) and Wistar rats (Desowitz et al., 1989), infected with *P. berghei* NK65 and NYU-2, respectively, in which hemoglobin and hematocrit decreased as a result of increased parasitemia. Reproducibility of anemia is expected among different experimental models since hemolysis constitutes an intrinsic step in the *Plasmodium* spp. life cycle in both rodents and humans. Nevertheless, this critical parameter has been poorly investigated in murine malaria, in which its

TABLE 2 | Compilation of references mentioning the main pathologic manifestations of malaria in pregnancy observed in humans and murine models.

	Human MiP	**Murine MiP**
Pregnancy outcome		
Recrudescence	Giobbia et al., 2005; Mayor et al., 2009; Laochan et al., 2015; Al Hammadi et al., 2017; Malvy et al., 2018	van Zon and Eling, 1980; Oduola et al., 1982; van Zon et al., 1982, 1985; Marinho et al., 2009; Megnekou et al., 2009; Sharma et al., 2016
Anemia	Brabin et al., 2001; Rogerson et al., 2003	Oduola et al., 1982; Desowitz et al., 1989; Hioki et al., 1990; Megnekou et al., 2009; Poovassery et al., 2009; Avery et al., 2012; Sarr et al., 2012; Sharma et al., 2016
Maternal mortality	Luxemburger et al., 1997; Brabin et al., 2001; Shulman et al., 2002; Nosten et al., 2004; Menéndez et al., 2008; Rogerson et al., 2018	van Zon and Eling, 1980; Oduola et al., 1982; van Zon et al., 1982; Vinayak et al., 1986; Desowitz et al., 1989; Hioki et al., 1990; Pathak et al., 1990; Pavia and Niederbuhl, 1991; Poovassery and Moore, 2006; Neres et al., 2008; Marinho et al., 2009; Megnekou et al., 2009; Poovassery et al., 2009; Rodrigues-Duarte et al., 2012; Sarr et al., 2012; Sharma and Shukla, 2014; Sharma et al., 2016
Stillbirth	Menendez, 1995; Desai et al., 2007	Oduola et al., 1982; Vinayak et al., 1986; Pathak et al., 1990; Rodrigues-Duarte et al., 2012, 2018; Sharma et al., 2016
Abortion	Desai et al., 2007	Poovassery and Moore, 2006, 2009; Neres et al., 2008; Poovassery et al., 2009; Silver et al., 2010; Avery et al., 2012; Sarr et al., 2012, 2015
Preterm delivery	Menendez et al., 2000; Desai et al., 2007; Moore et al., 2017	van Zon et al., 1982; Neres et al., 2008
Reduced newborn/fetal weight	Menendez et al., 2000; Rogerson et al., 2003; Nosten et al., 2004; Desai et al., 2007; Umbers et al., 2011	Oduola et al., 1982; Vinayak et al., 1986; Pathak et al., 1990; Neres et al., 2008; Marinho et al., 2009; Megnekou et al., 2009; Silver et al., 2010; Rodrigues-Duarte et al., 2012, 2018; Conroy et al., 2013; Barboza et al., 2014, 2017, 2019; Sharma and Shukla, 2014; Sharma et al., 2016
Congenital malaria	Rai et al., 2015; Bhatia et al., 2016	Adachi et al., 2000
Placental histology		
Parasite accumulation	Walter et al., 1982; Beeson et al., 2002; Beeson and Brown, 2004; Muthusamy et al., 2004, 2007	Oduola et al., 1982, 1986; Vinayak et al., 1986; Desowitz et al., 1989; Pathak et al., 1990; Tegoshi et al., 1992; Poovassery and Moore, 2006, 2009; Neres et al., 2008; Marinho et al., 2009; Megnekou et al., 2009; Rodrigues-Duarte et al., 2012; Sarr et al., 2012; de Moraes et al., 2013; Lima et al., 2014; Sharma and Shukla, 2014; Sharma et al., 2016; Barboza et al., 2017
Hemozoin deposition	Bulmer et al., 1993; Ismail et al., 2000	Oduola et al., 1982, 1986; Vinayak et al., 1986; Neres et al., 2008; Megnekou et al., 2009; Poovassery and Moore, 2009
Immune cells infiltrate	Walter et al., 1982; Ordi et al., 1998; Ismail et al., 2000; Rogerson et al., 2007; Othoro et al., 2008; Souza et al., 2013	Oduola et al., 1982, 1986; Tegoshi et al., 1992; Poovassery and Moore, 2006; Neres et al., 2008; Marinho et al., 2009; Poovassery et al., 2009; Barboza et al., 2017
Fibrin Necrosis	Walter et al., 1982; Ismail et al., 2000; Souza et al., 2013	Oduola et al., 1986; Tegoshi et al., 1992; Neres et al., 2008; Poovassery et al., 2009; Avery et al., 2012; Rodrigues-Duarte et al., 2012
Syncytial knots Trophoblast rupture	Ismail et al., 2000; Crocker et al., 2004; Souza et al., 2013	
Trophoblast membrane thickening	Ismail et al., 2000; Souza et al., 2013	Oduola et al., 1982, 1986; Tegoshi et al., 1992; Neres et al., 2008; Marinho et al., 2009; Rodrigues-Duarte et al., 2012; de Moraes et al., 2013; Barboza et al., 2014, 2017, 2019

Distinct pregnancy outcomes and placental histopathologic alterations are depicted. References used to inform this review are cited in the table according to their contribution in unraveling specific clinical and histologic manifestations of both human and murine malaria in pregnancy.

relationship with infection timing, gravidity, and pregnancy outcomes were never addressed as in humans (Brabin et al., 2001; Rogerson et al., 2003).

Maternal Mortality

Maternal mortality seems to be a less frequent outcome in pregnancies complicated by malaria (Menéndez et al., 2008; Rogerson et al., 2018). Maternal death associated with *Plasmodium* spp. infection tends to occur more frequently in areas of low malaria transmission due to the reduced level of premunition (Nosten et al., 2004). Nevertheless, pregnant women with malaria who live in endemic areas are also prone to die due to severe anemia (Brabin et al., 2001; Shulman et al., 2002). Likewise, both pregnant mice and rats infected with murine *Plasmodium* spp. strains were shown to die during gestation, which was dependent on the parasite strain, infection timing, and host intrinsic characteristics. Regarding parasite strains, it was demonstrated that *P. berghei* (van Zon and Eling, 1980; Oduola et al., 1982; van Zon et al., 1982; Vinayak et al., 1986; Desowitz et al., 1989; Hioki et al., 1990; Pathak et al., 1990; Neres et al., 2008; Marinho et al., 2009; Megnekou et al., 2009; Rodrigues-Duarte et al., 2012; Sharma and Shukla, 2014), *P. yoelii* (Pavia and Niederbuhl, 1991), and *P. chabaudi* (Poovassery and Moore, 2006, 2009; Poovassery et al., 2009; Sarr et al., 2012; Sharma et al., 2016) induce maternal death in a considerable variety of experimental settings. However, only one study has directly compared this outcome induced by different parasites, clearly showing increased mortality rates in pregnant C57BL/6 mice infected with *P. berghei* NK65 compared to K173 and ANKAΔpm4 strains (Rodrigues-Duarte et al., 2012). One must consider that mortality rates also depend on host susceptibility to infection since, for instance, pregnant A/J mice experience a higher risk of mortality than C57BL/6 mice when infected with *P. chabaudi* AS (Sarr et al., 2012). These findings support the notion that maternal survival is dependent on both parasite and host intrinsic features. Similarly, mortality was shown to increase in pregnant C57BL/6 and BALB/c mice infected with *P. chabaudi* (Poovassery and Moore, 2006; Poovassery et al., 2009) or *P. berghei* (Neres et al., 2008; Rodrigues-Duarte et al., 2012), respectively, when compared to their non-pregnant counterparts. This is a well-known phenomenon that also occurs in humans who are primarily in areas of unstable malaria transmission (Luxemburger et al., 1997; Nosten et al., 2004). Infection timing was also shown to be linked to maternal mortality, which primarily increases in cases of early gestation infection. This was demonstrated to occur in Wistar rats (Desowitz et al., 1989), as well as in Swiss Albino, A/J, ICR, and BALB/c mice (Oduola et al., 1982; Vinayak et al., 1986; Hioki et al., 1990) infected with *P. berghei*. However, mid-gestation infection was shown to lead to increased survival with a higher number of animals achieving parturition. Interestingly, it is unclear whether this occurs in humans and if first trimester infections lead to increased maternal mortality rates (Desai et al., 2007; Rogerson et al., 2018). Nevertheless, regardless of the infection trimester, mortality is highly dependent on gravidity and is more prevalent in primigravidae than in multiparous women (Menéndez et al., 2008). Equally, mice tend to experience the same effect, which is likely to be dependent on immunity acquired during previous gestations (van Zon and Eling, 1980; Marinho et al., 2009). Accordingly, immunity seems to be pivotal in reduced murine mortality, as ICR mice immunized with attenuated *P. yoelii* before pregnancy had improved survival rates when challenged with the lethal *P. yoelii* 17X strain (Pavia and Niederbuhl, 1991). Although a direct causality has never been proved, increased mortality observed in murine models of MiP seem to be associated with the development of systemic infection measured by increased peripheral parasitemia that correlates with decreased body weight and patent patterns of anemia (Poovassery et al., 2009; Sarr et al., 2012).

Considering the discussion above, it is clear that mortality during pregnancy depends on a wide range of factors that ultimately will affect host survival. Most of them are commonly recapitulated in rodent experimental models in which experiments have contributed with important findings in this regard. However, one must carefully consider the often-fatal outcomes of murine infections induced by highly lethal parasites that are dissimilar to the infrequent lethality observed in human MiP.

In uterus Death and Abortion

In uterus death and abortion are rather infrequent outcomes of pregnancies complicated by malaria. However, there is a clear association between MiP development and stillbirth incidence, which is noted as being higher in women with perceivable placental parasitemia (Desai et al., 2007). In contrast, the association between abortion and MiP seems to be rarely discussed, with only scarce reports of this event occurring in low transmission areas, probably resulting from early trimester infections (Menendez, 1995; Desai et al., 2007). It is important to highlight that logistical constraints imposed by cultural and socioeconomic boundaries are the reason by which it is difficult to assess this particular pregnancy outcome. Interestingly, the incidence of abortion seems to be much more frequent in murine models than in humans. Accordingly, *P. chabaudi* AS infection of pregnant C57BL/6 mice revealed a rather frequent pattern of abortion and reduced fetal viability at mid-gestation (G10-12) when the parasite was inoculated immediately after conception. This event overlaps with the peripheral parasitemia peak and massive accumulation of parasites inside the placenta (Poovassery and Moore, 2006). As expected, the parasitemia peak and abortion were frequently observed in mice having increased production of cytokines in both peripheral blood (e.g., IL-1β and IL-10) and placenta (e.g., IFN-γ) (Poovassery and Moore, 2009). In a follow-up study, it was shown that fetal loss and reduced viability in pregnant C57BL/6 mice infected with *P. chabaudi* AS were markedly influenced by IFN-γ and TNF-α production (Poovassery et al., 2009) and increased coagulopathy and impaired fibrinolysis (Avery et al., 2012). In the same extent, abortion was shown to occur concomitantly with apoptosis of inflammatory cells and spongiotrophoblast in the junctional zone (between the uterus and the labyrinth), which has been discussed to occur in a way that is dependent on TNF-α signaling (Sarr et al., 2015). Altogether, these findings established a clear link between the immune response triggered

against malaria and poor pregnancy outcomes. Moreover, models of abortion are also characterized by early infection with *P. chabaudi*, but stillbirth has also been observed in Swiss Albino (Vinayak et al., 1986; Pathak et al., 1990), BALB/c (Neres et al., 2008), and C57BL/6 (Rodrigues-Duarte et al., 2012) mice infected with *P. berghei* at mid-gestation. In these experimental models, reduced fetal viability without clear abortion observed at late gestation somewhat reflects stillbirth occurring in humans during the third gestational trimester (Desai et al., 2007).

Notably, it is important to highlight that translating these findings to human research may raise some serious controversies. Human stillbirth is often described as a dead conceptus that was expelled or removed from the womb 22 weeks after conception, being no longer considered an abortion (less than 22 weeks) (Lawn et al., 2016). In contrast, in the aforementioned murine models, there is no parallel measure at which fetal viability was assessed that considers gestational age. Vaginal secretions containing blood are normally taken as an indicative of early abortion, followed by the observation of necrotic structures (resorptions) that lack fetal or placental morphology (Poovassery and Moore, 2006; Neres et al., 2008). On the other hand, fetal viability is normally evaluated closer to term, after touching *in uterus* or delivered conceptus with pliers. Fetuses that fail to react are considered stillbirths (Neres et al., 2008). Therefore, definitions in these situations are often misleading, and conclusions should be made with extreme caution since abortion and stillbirth etiology may vary between rodent and human MiP.

Preterm Birth

Preterm birth, which encompasses every live birth that occurs before the 37th week of gestation (Goldenberg et al., 2009), is a more frequent consequence and is one of the leading causes of reduced birth weight associated with MiP (Menendez et al., 2000; Desai et al., 2007; Moore et al., 2017). Interestingly, the occurrence of spontaneous delivery in mice or rats in the context of MiP is either poorly addressed or considerably infrequent. One study reported the occurrence of preterm delivery of dead pups from pregnant BALB/c mice infected with *P. berghei* ANKA (Neres et al., 2008). However, this definition does not seem to fit the circumstance since preterm birth assumes that the progeny is born alive before term, which is unlikely to occur before G19 (reviewed elsewhere McCarthy et al., 2018). To the same extent, murine models have been questioned regarding their capacity to reproduce human preterm birth due to inaccurate measurements and non-standardized markers.

In this regard, evaluation of preterm birth in murine models of MiP is probably far from replicating human pathology. However, a better evaluation of this outcome may lead to improved disease models with more reliable translation capacities (McCarthy et al., 2018).

Progeny Outcomes and Development

Progeny outcomes and development will ultimately be severely affected as a result of the aforementioned clinical and obstetric manifestations during MiP. Often resulting from IUGR and/or preterm delivery, reduced birth weight contributes significantly to postnatal mortality and impaired child development across the malaria endemicity spectrum and is clearly more frequent in women during their first pregnancies (Nosten et al., 2004; Desai et al., 2007). It is unclear how MiP truly leads to growth restriction, yet evidences point to a multitude of factors such as angiogenic imbalance, endocrine dysregulation, deficiencies in transplacental nutrient transportation, severe inflammation and placental insufficiency (Umbers et al., 2011). During the 1980s, some studies reported the first observations of altered birth weight in mice suffering from MiP, which was found to be significantly diminished in pups from A/J, ICR (Oduola et al., 1982) and Swiss albino (Vinayak et al., 1986) litters born from mice infected with *P. berghei* NK65 and NICD, respectively. It seems that impaired progeny development is a rather well-conserved outcome of murine MiP since it was reported in BALB/c (Neres et al., 2008; Marinho et al., 2009; Megnekou et al., 2009; Silver et al., 2010) and C57BL/6 (Rodrigues-Duarte et al., 2012; Sharma et al., 2016) mice infected with a wide variety of parasite strains, suggesting the existence of conserved pathogenesis mechanisms (Rodrigues-Duarte et al., 2012). This pathologic outcome seems to be avoided with on-time administration of antimalarial drugs such as chloroquine and sulphadoxine-pyrimethamine, which were shown to abrogate systemic parasitemia, therefore improving offspring weight at delivery (Sharma and Shukla, 2014). Additionally, as it occurs in humans, progeny birth weight improved in mouse multigravidae. This trait was clearly shown to be gravidity-dependent, as pups belonging to the first litter were much smaller on average than those born from subsequent pregnancies when the corresponding dams were induced with MiP by recrudescent *P. berghei* ANKA (Marinho et al., 2009).

Further research conducted in the last decade revealed some hidden aspects of reduced birth weight etiology that would not be revealed without the wide range of genetically manipulated mice strains. Strikingly, it seems that proper fetal development is impaired upon the activation of specific components linked to innate immunity, such as complement system receptor (C5aR), Toll-like receptor 4 (TLR4), type I interferon receptor 1 (IFNAR1), and adaptor protein myeloid differentiation factor 88 (MyD88). BALB/c C5aR$^{-/-}$ (Conroy et al., 2013), C57BL/6 TLR4$^{-/-}$ (Barboza et al., 2017; Rodrigues-Duarte et al., 2018), C57BL/6 IFNAR$^{-/-}$ (Rodrigues-Duarte et al., 2018), and C57BL/6 MyD88$^{-/-}$ (Barboza et al., 2014, 2019) mice were shown to have litters with normal fetal weight, development and survival, even though being infected with *P. berghei* ANKA (in BALB/c) or *P. berghei* NK65 (in C57BL/6). These findings substantially advanced the field by strikingly implying the innate immune system on MiP-associated reduced birth weight pathogenesis, enabling future preclinical trials of anti-inflammatory drugs (e.g., the TLR4 antagonist IAXO) (Barboza et al., 2017) to be used concomitantly with antimalarial therapies to prevent MiP-associated poor pregnancy outcomes. However, progress in this field should also be done with extreme caution due to possible undesired side effects resulting from adjunctive therapies to treat severe malaria such as those observed in trials conducted with anti-TNF-α therapy

to treat cerebral malaria and others (van Hensbroek et al., 1996; Varo et al., 2018).

Moreover, the postnatal scenario of human MiP might rarely include the appearance of congenital malaria, whose onset is tightly controlled by transplacental transmission of maternal antibodies to the fetus, resulting in isolated and scarce events often diagnosed as neonatal sepsis (Rai et al., 2015; Bhatia et al., 2016). Conceptus infection might occur due to blood exchanges at delivery or during pregnancy due to loss of villi integrity and syncytium rupture (Crocker et al., 2004; Robbins and Bakardjiev, 2012; Souza et al., 2013). By contrast, the phenomenon is thought to be even rarer in rodents. In related studies, the authors have clearly stated that no congenital malaria was observed, despite an increased fetal spleen weight, which might an indicative of fetal infection (Oduola et al., 1982; Vinayak et al., 1986). However, work by Adachi et al. (2000) addressed this question under the assumption that both mice and rats rarely transmit the parasite to their offspring. Accordingly, the authors detected parasites by nested PCR in a few pups born from pregnant BALB/c mice infected with *P. berghei*. These findings support the rareness of the event due to the lack of a uniform presence of parasites, even in pups within the same litter. The hemotrichorial layer in the murine placenta presents a much thicker barrier against transplacental passage of parasites than the human hemomonochorial placenta (Crocker et al., 2004), which might explain the reduced incidence of murine congenital malaria. Nevertheless, discrepancies in existing reports contraindicate the usage of murine models in this regard, identifying them as unsuitable for studying this rare disease outcome.

In conclusion, one may face challenges when translating pregnancy outcome findings obtained in murine models to human pathology. Nevertheless, significant achievements have been made in recent years, despite the considerable diversity of experimental settings and non-standardized methodologies. Improving them will certainly lead to enriched results and more accurate and meaningful conclusions taken from murine models of MiP.

Understanding Placental Pathology Using Murine Models of MiP

Most of the aforementioned outcomes occurring in pregnancies complicated by malaria are strongly associated with the dysregulation of placental homeostasis due to a significant accumulation of parasite-infected erythrocytes inside this organ, which overlaps with the onset of PM (Sharma and Shukla, 2017). Accordingly, several cytological and histological abnormalities are easily observed in infected placentas in addition to parasite accumulation, such as the malarial pigment hemozoin, immune cells, syncytial knots, fibrin deposition, necrosis and placental barrier thickening (Walter et al., 1982; Ismail et al., 2000; Souza et al., 2013). Although despite being PM hallmarks, not all of these histopathologic features are accurately shown by murine models of MiP (schematically represented in **Figure 3**).

Parasitized Erythrocytes

Parasitized erythrocytes tend to selectively accumulate in the placenta (**Figures 1C,D**), achieving greater parasitemia levels than those observed in the peripheral blood of pregnant infected women (Beeson et al., 2002; Muthusamy et al., 2004). It is unquestionable that murine parasites also accumulate inside the placenta (**Figure 2D**) since a large number of studies of placental histopathology noted this finding for a wide range of strains (**Table 1**). Notably, Poovassery and Moore (2006) have demonstrated that *P. chabaudi* AS parasites also accumulate preferentially inside the placenta of C57BL/6 mice, leading to greater placental parasitemia than that observed in peripheral blood. Interestingly, these murine parasite populations were shown to be mainly constituted by mature forms with an almost null percentage of early ring-stage parasites (Megnekou et al., 2009), which is in line with observations made during *P. falciparum* infections (Beeson et al., 2002). Similarly, intravital microscopy studies performed in placentas from pregnant mice infected either with *P. berghei* or *P. chabaudi* also suggested that those that accumulate are indeed the mature forms of the parasite (Lima et al., 2014), which preferably adhere to the trophoblast in areas of low blood flow inside the maternal blood sinusoids (de Moraes et al., 2013). As observed for *P. falciparum* regarding its preferential adhesion to CSA and controversial binding to hyaluronic acid (HA) (Beeson and Brown, 2004; Muthusamy et al., 2007), the adhesion of *P. berghei* was also shown to be dependent on placental CSA and HA as demonstrated by adhesion assays of mice IEs to placental sections treated with chondroitinase or hyaluronidase (Neres et al., 2008; Marinho et al., 2009). These findings once again support similar pathogenesis mechanisms between human and murine PM despite the lack of known PfEMP1 homologs (Hviid et al., 2010).

The Malarial Pigment Hemozoin

The malarial pigment hemozoin, a byproduct of hemoglobin catabolism performed by *Plasmodium* spp. to detoxify free heme (Olivier et al., 2014), is frequently observed in placentas from infected pregnant women (**Figures 1E,F**) and is often used as an indicator of PM, even in the absence of detectable parasites (Bulmer et al., 1993; Ismail et al., 2000). Similarly, placental infection by murine parasites also leads to hemozoin deposition (**Figure 2F**). Oduola and coworkers first observed this phenomenon in histologic sections of placentas from different murine backgrounds infected with *P. berghei* NK65. Shortly thereafter, the pigment was exclusively observed in maternal blood sinusoids, whose concentrations increased with augmented parasite loads. Accordingly, hemozoin-containing monocytes were also frequently observed (Oduola et al., 1982, 1986). In addition, hemozoin was also detected in trophoblast giant cells, suggesting an active phagocytic process (Poovassery and Moore, 2009). The pigment is frequently detected under polarized light microscopy (Neres et al., 2008; Megnekou et al., 2009) and is used to indicate past-chronic PM in humans according to the diagnostic criteria of Bulmer et al. (1993). To our knowledge, this event was rarely observed in murine models (Oduola et al., 1986). The fact that hemozoin was rarely observed alone in infected placentas suggests that the experimental settings from current models

fail to reproduce past-chronic infections and are only able to reproduce acute PM.

Immune Cell Infiltrate

Immune cell infiltrate, which occurs as a response to parasite/hemozoin accumulation inside the placenta (**Figure 1D**), is one of the key hallmarks of the disease and is often associated with poor pregnancy outcomes (Menendez et al., 2000; Rogerson et al., 2003; Umbers et al., 2011). Monocytes/macrophages are the most abundant constituents of these inflammatory infiltrates, and recruitment is widely correlated with placental production of MIP-1α, MCP-1, I-309, and IL-8 chemokines (Abrams et al., 2003). Not surprisingly, the same event was observed in placentas from mice (**Figure 2E**) and rats infected with *P. berghei*, especially in those containing detectable placental parasites (Oduola et al., 1982, 1986; Tegoshi et al., 1992). Notably, the infiltrate, which was mainly composed of monocytes ($CD11b^{+ve}$ cells detected by cytochemistry) and macrophages ($CD11b^{+ve}$ cells detected by cytochemistry and *Cd68*- and *Mgl2*-expressing cells identified by qPCR), was linked to the production of some attractant chemokines, such as MIP-1α and MCP-1 (Neres et al., 2008; Marinho et al., 2009; Sarr et al., 2015; Barboza et al., 2017). However, the event is far from reflecting chronic intervillositis observed in humans (Ordi et al., 1998), probably due to the shorter gestational period in rodents. Additionally, some authors have commented on the scarcity of this event, especially in placentas from C57BL/6 mice infected with *P. chabaudi*, which tend to have fewer accumulated monocytes/macrophages due to reduced placental parasite burden (Poovassery and Moore, 2006; Poovassery et al., 2009). Early reports on different leukocyte populations in infected murine placentas noted the existence of mononuclear (Tegoshi et al., 1992) and polymorphonuclear (Oduola et al., 1986) cells. Molecular biology methods were later used to dissect these cell types, dividing them into dendritic cells (*Mgl2*), neutrophils (*Ncf2*), NK cells (*Klrd1*), T (*Cd3e*), and B (*Cd22*) lymphocytes (gene expression quantification by qPCR) (Marinho et al., 2009; Sarr et al., 2015; Barboza et al., 2017). Remarkably, this is in line with observations performed in human PM regarding the placental accumulation of NK cells, T lymphocytes and other non-specified polymorphonuclear cells (Ordi et al., 2001; Rogerson et al., 2007; Othoro et al., 2008). Nevertheless, despite the clear accumulation of immune cells in murine placentas, its association with poor pregnancy outcomes remains to be elucidated in experimental models that oppose current knowledge of human pathology (Rogerson et al., 2003; Umbers et al., 2011).

Placental Fibrinoid Necrosis

Placental fibrinoid necrosis normally occurs as a consequence of extensive placental tissue damage caused during *Plasmodium* spp. infection (**Figure 1B**). In fact, fibrin deposition is initiated to promote placental tissue repair but soon becomes cytotoxic, leading to necrosis (Walter et al., 1982; Ismail et al., 2000) and poor pregnancy outcomes such as premature delivery and reduced birth weight (Menendez et al., 2000; Avery et al., 2012). Likewise, pioneering studies from the 1980s revealed the presence of fibrinoid necrosis in placental sections from mice and rats infected with *P. berghei*, although without clear conclusions on its consequences (Oduola et al., 1986; Tegoshi et al., 1992). Later, fibrinoid necrosis was reported in placentas from *P. berghei*-infected BALB/c mice (**Figure 2B**), which delivered litters with a patently reduced birth weight phenotype (Neres et al., 2008), and fibrin thrombi in placentas from C57BL/6 mice infected with *P. chabaudi* that experienced spontaneous abortion (Poovassery et al., 2009). Discussion was taken to the point in which fibrin deposition in maternal blood sinusoids would significantly impair placental capability to perform physiological tasks such as respiration and nutrient exchanges, probably due to clotting, blood arrest, necrosis and trophoblast death. In a subsequent study, Avery et al. (2012) showed by western blot that fibrin deposits were increased in placentas from C57BL/6 mice infected with *P. chabaudi*, which occurred in parallel with an upregulation of coagulation-associated genes. These findings established an important association between impaired fibrinolysis and coagulation and the poor pregnancy outcomes of MiP.

Labyrinth Disarrangement

Labyrinth disarrangement will ultimately reflect murine placental dysfunction, which is characterized by particular histological alterations that have considerable differences from human PM, mostly due to the existence of a widely different villi structure (**Figures 1A,B** vs. **Figures 2A–D**). Of note, two features of human PM that have no counterparts in infected murine

BOX 1 | Suggestions to improve future murine models of MiP. Evolution has grouped murine animals and humans in similar phylogenetic clades according to some striking similarities. However, it has also separated them especially from an anatomically perspective. Some differences cannot be surpassed; yet, our understanding of some diseases and complex biological processes can be improved with the refinement of experimental design and standardization of analysis methods when using animal models such as experimental murine models of MiP. As such, our understanding of MiP could be improved by addressing some of the following points:

- Understanding the adhesion mechanism of murine parasite strains.
- Identification of VAR2CSA functional homologs in murine parasites.
- Standardization of protocols using specific species of murine parasites to differently address specific aspects of uncomplicated or severe MiP (e.g., usage of less or more virulent parasite strains, tropism for reticulocytes or mature erythrocytes, preferential tissue for accumulation).
- Standardized definitions and accurate measures for abortion, preterm delivery, stillbirth and placental malaria in the context of murine MiP.
- Investigating the possibility of congenital malaria in murine models of MiP.

placentas are syncytiotrophoblast rupture (previously discussed in the context of congenital malaria) and syncytial knots(Ismail et al., 2000; Souza et al., 2013). These protrusions of syncytial nuclear aggregates, which have been associated with hypoxia and oxidative stress in human placentas (Heazell et al., 2007), were discussed as having no similar structure in murine placentas that could be detected under light or electron microscopy (Tegoshi et al., 1992). Syncytial knots, which are considered an accumulation of degrading nuclei, was once erroneously described as a phenomenon of trophoblast hyperplasia [discussed elsewhere (Heazell et al., 2007)]. To our knowledge, the latter was never clearly discussed in the context of human PM. Accordingly, there are unclear reports of trophoblast hyperplasia occurring in the placentas of mice (Oduola et al., 1986; Vinayak et al., 1986; Pathak et al., 1990; Neres et al., 2008) and rats (Oduola et al., 1986; Tegoshi et al., 1992) infected with *P. berghei*. Nevertheless, evidence of this event includes unclear microscopy images that fail to address the apparent enlargement/swelling of tissue that occurs due to cell proliferation. However, there is a striking thickening of the trophoblast basal membrane that partially overlaps with the concept of tissue swelling. In human PM, this frequent phenomenon can be qualitatively analyzed (Ismail et al., 2000) or more accurately quantified as the distance that separates fetal capillaries from villi outer membrane (Souza et al., 2013). Regardless of the methodology used, this parameter was found to be significantly thicker during human PM and, being discussed as strongly influencing transplacental transport of vital compounds. Similarly, this was qualitatively analyzed and reported in mouse and rat placentas infected with *P. berghei*, which was hypothesized to be a consequence of the fibrotic process resulting from massive tissue repair (Oduola et al., 1986; Tegoshi et al., 1992). Later, some other works have developed methods to indirectly quantify trophoblast thickening, taking advantage of the sinusoidal nature of mouse placentas. Accordingly, maternal blood areas were quantified, and the reduction of vascular spaces in placentas from BALB/c mice infected with *P. berghei* ANKA was considered a proxy for basal membrane thickening (Neres et al., 2008). This supports the conclusion that reduced maternal blood spaces due to trophoblast membrane thickening and maternal sinusoidal remodeling (de Moraes et al., 2013) would ultimately lead to placental insufficiency and impaired transplacental transport of nutrients [discussed elsewhere (Neres et al., 2008; de Moraes et al., 2013)]. The same methodology was further used to determine that distinct parasites inflict different magnitudes of circulatory impairment and membrane thickening in placentas from C57BL/6 mice (Rodrigues-Duarte et al., 2012). The etiology of the event was further addressed in *P. berghei* NK65-infected C57BL/6 TLR4$^{-/-}$ and MyD88$^{-/-}$ KO mice, which had blood sinusoidal areas similar to those observed in non-infected pregnant mice (Barboza et al., 2014, 2017). These findings established a logical link between host innate immunity and placental pathology, once again supporting the notion that outcomes of MiP mostly result from damage and homeostatic dysregulation inflicted mostly by factors of an immunologic nature.

CONCLUSION

Clearly, the development of murine models that recapitulate traits from human MiP has definitively contributed to the current understanding of this disease. However, there are still few reports that truly reveal some of the pathogenesis mechanisms of MiP. Only in the last decade have some studies clearly unraveled some hidden molecular mechanisms of MiP, such as innate immunity activation and its contributions to poor pregnancy outcomes (Poovassery et al., 2009; Conroy et al., 2013; Barboza et al., 2014, 2017, 2019; Rodrigues-Duarte et al., 2018). Indeed, the advent of genetic engineering and the capacity to generate a wide range of knockout mice were definite turning points from which we are still benefiting. Until this point, most studies have tried to establish proper experimental settings that would ultimately validate murine models as suitable for studying MiP. First, striking similarities are observed regarding evolutionary and developmental traits between murine and human placentas, from which one might conclude that physiologically, both would behave in a particularly similar way (Georgiades et al., 2002; Wildman et al., 2006). Additionally, murine parasites exhibit a group of characteristics that support the conclusion that murine pathology would somewhat resemble human MiP, despite the well-known differences between rodent *Plasmodium* species and *P. falciparum* biology (Lamb et al., 2006; Hviid et al., 2010). Nevertheless, one might consider the patent limitations of conclusions regarding disease outcomes, which can later limit the translational capacities of observed results. As such, refinement of experimental design and standardization of methodology is necessary for the improvement of such models (**BOX 1**). Resolving these gaps will certainly enrich research in the field, possibly reaffirming the usage of murine models to address more specific and complex questions implicit in drug preclinical trials and vaccine development (Doritchamou et al., 2017). Research in the field has indeed overcome several milestones due to the usage of rodent models with much progress that is still to come.

AUTHOR CONTRIBUTIONS

AB drafted the manuscript, compiled information from the literature, and designed the figures and tables. MP drafted the manuscript and gathered information from the literature. SE supervised and reviewed the manuscript. CM supervised and reviewed the manuscript and designed the figures and tables.

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REVIEW
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Congenital Transmission of *Trypanosoma cruzi*: A Review About the Interactions Between the Parasite, the Placenta, the Maternal and the Fetal/Neonatal Immune Responses

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Chagas disease (CD), caused by the protozoan parasite *Trypanosoma cruzi*, is considered a neglected tropical disease by the World Health Organization. Congenital transmission of CD is an increasingly relevant public health problem. It progressively becomes the main transmission route over others and can occur in both endemic and non-endemic countries. Though most congenitally infected newborns are asymptomatic at birth, they display higher frequencies of prematurity, low birth weight, and lower Apgar scores compared to uninfected ones, and some suffer from severe symptoms. If not diagnosed and treated, infected newborns are at risk of developing disabling and life-threatening chronic pathologies later in life. The success or failure of congenital transmission depends on interactions between the parasite, the placenta, the mother, and the fetus. We review and discuss here the current knowledge about these parameters, including parasite virulence factors such as exovesicles, placental tropism, potential placental defense mechanisms, the placental transcriptome of infected women, gene polymorphism, and the maternal and fetal/neonatal immune responses, that might modulate the risk of *T. cruzi* congenital transmission.

Keywords: *Trypanosoma cruzi*, infection, maternal-fetal interactions, placenta, congenital chagas disease

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CHAGAS DISEASE

Trypanosoma cruzi, a protozoan parasite, is the etiological agent of CD, a neglected tropical disease (WHO, 2015; Lenk et al., 2018) endemic to Latin American countries. Affected countries extend from the United States to Argentina and Chile (Pérez-Molina and Molina, 2018). The parasite is primarily transmitted by insect vectors, but can also be transmitted through blood transfusion,

organ transplantation, consumption of parasite-contaminated food, and vertically from mother to child. In Latin America, CD is an important cause of cardiovascular morbidity and premature death (Rassi et al., 2012; WHO, 2015).

The disease evolves in two phases. The acute phase, defined by high parasitemia, lasts 2–3 months. It is often asymptomatic or involves non-specific flu-like symptoms. However, 2–12% of the infected individuals, mostly children under 3 years, die from acute myocardiopathy at times associated with a meningoencephalic compromise (Carlier and Truyens, 2015). The immune response eliminates most but not all parasites. As a consequence, the individual enters the chronic phase and remains infected for the rest of their lives. Although most patients remain asymptomatic for periods ranging in length from several months to decades, 30 – 40% of the infected people develop cardiac (mostly) and/or digestive tract (less frequent) pathologies that may lead to premature death (Rassi et al., 2012; de Oliveira et al., 2018).

CONGENITAL CHAGAS DISEASE

Most women of gestational age are chronically infected, having acquired the infection during infancy. Around 5% of them transmit the parasite to their fetus, and thus approximately 9000 newborns infected with CD in Latin America are born every year. Cases of congenital CD have also been reported outside of the naturally endemic area (WHO, 2015; Antinori et al., 2017). Congenital infection is gaining importance as a route of *T. cruzi* transmission in endemic countries, due to control programs of vectorial and transfusional transmission routes, and is now estimated to account for 22% of new cases (WHO, 2015; Picado et al., 2018). In non-endemic countries, most of the new cases result from congenital transmission (Howard et al., 2014; WHO, 2015). Importantly, since *T. cruzi* maternal-fetal transmission can be repeated at each pregnancy and observed from one generation to another, this way of transmission can easily expand in time (Messenger et al., 2017).

Congenital CD is characteristically an acute parasite infection. Though most (around 60%) congenitally infected newborns are asymptomatic at birth, they display higher frequencies of low birth weight, prematurity, and lower Apgar scores at birth compared to uninfected newborns, while some infected newborns suffer from severe symptoms that can rapidly lead to death (Torrico et al., 2004; Liempi et al., 2016). Moreover, congenitally infected infants are at risk of disabling and life-threatening chronic pathologies later in life (Cardoso et al., 2012; Requena-Méndez et al., 2014; Carlier et al., 2015; Antinori et al., 2017). It is therefore essential to prevent congenital transmission and to rapidly diagnose and treat congenitally infected newborns.

Importantly, the occurrence of congenital transmission depends on interactions between *T. cruzi*, the placenta, the maternal immune system, and the developing fetal/neonatal immune response (Carlier and Truyens, 2015; Liempi et al., 2016). Here we will discuss the factors mentioned above that modulate the risk and probability of *T. cruzi* maternal-fetal transmission.

THE PARASITE DIVERSITY

Trypanosoma cruzi belongs to the Kinetoplastida order, Trypanosomatidae family and presents a complex life cycle involving invertebrate triatomine hosts. Four main cellular forms can be identified during its life cycle: (i) the blood trypomastigote is the infective extracellular non-replicative form of the parasite, which is found in the bloodstream of the mammalian host, (ii) the metacyclic trypomastigote, also non-replicative, is present in the terminal portion of the digestive and urinary tracts of the vectors, (iii) the epimastigote is the replicative extracellular form of the parasite present in the triatomin insect vector, and (iv) the amastigote is the intracellular replicative form of the parasite in the vertebrate host (Rodrigues et al., 2014).

Trypanosoma cruzi is a paradigmatic case of predominantly clonal evolution (Tibayrenc et al., 1986) with "unequivocal evidence of genetic recombination" (Gaunt et al., 2003). Actually, the different known strains and clones of the parasite are classified into seven discrete typing units (DTUs), that are defined as a "sets of stocks that are genetically closer to each other than to any other stock and are identifiable by common molecular, genetic, biochemical, or immunological markers" (Tibayrenc, 1998). The DTUs ranged from *T. cruzi* I to *T. cruzi* VI, and lately, *T. cruzi* bat was also added (Zingales et al., 2012; Lima et al., 2015). Parasites from these DTUs are distributed differentially among insect and mammalian host species and therefore lives in different geographical areas (Zingales et al., 2012; Brenière et al., 2016). All *T. cruzi* parasites, regardless of the DTUs or where they belong, can cause CD. However, epidemiological studies suggest that *T. cruzi* I is associated with anthroponotic as well as sylvatic environments. On the other hand, *T. cruzi* II, V, and VI are mainly related to human environments and particularly chronic CD patients contrarily to *T. cruzi* III and IV which are found to be present in sylvatic habitats (Yeo et al., 2005). An association between DTUs and clinical outcomes is suspected (Messenger et al., 2015). These host and geographic specificities have been proposed to determine the probability of transmission and are related to the pathogenesis of CD. Parasites belonging to diverse DTUs have different biological properties, including growth rates in cultures, tropisms to tissue and organs, antigenicity, capacity to infect potential insect vectors, drug susceptibility, number of chromosomes, and DNA content (Macedo and Pena, 1998; Macedo et al., 2004). Characterization of the gene content and genome architecture of *T. cruzi*, as well as a whole-organism proteomic analysis of its four life cycle stages, has been reported (El-Sayed et al., 2005). Further, comparative genomics of different strains using different sequencing strategies and technologies provide data for the identification of genes associated with host tropism, pathogenicity, and modes of transmission (Berná et al., 2018; Callejas-Hernández et al., 2018; Reis-Cunha et al., 2018).

Whether all *T. cruzi* DTUs can be congenitally transmitted *in utero* is a matter of debate. There is presently no clear evidence that particular *T. cruzi* DTUs, as defined by the currently used molecular markers, would preferentially be transmitted congenitally [reviewed in Carlier and Truyens (2015), Truyens and Carlier (2017)]. We however, recently observed that congenital transmissions in Argentina and Mexico

were associated with a maternal portage of "non-TcI" parasites (we did not detect congenital cases harboring TcI DTU) (Buekens et al., 2018). Concordantly, higher congenital transmission rates were reported in Brazilian regions where *T. cruzi* DTU V predominantly circulates as compared to a country where Tc II predominates (Luquetti et al., 2015).

PARASITE TROPISM FOR PLACENTA

Tissue tropism to the placenta of different *T. cruzi* strains has been described previously, particularly in the murine model (Andrade, 1982). *T. cruzi* I (Colombiana strain) parasites present a high incidence of placental parasitism (98%) compared to *T. cruzi* II (Y strain) parasites that only infect 17% of the placentas. The same strains also present differences regarding the localization of the amastigotes in the placenta; only the Colombian strain could be observed consistently in the vascular part of the placenta. In a more recent work, chronic infected mice with two different *T. cruzi* strains, one obtained from a congenital CD patient [VD: Tc VI (Risso et al., 2004)] and the other one previously characterized as non-transmissible in mice [K98,Tc I (Solana et al., 2002)] showed, by means of DNA amplification and 18s ribosomal RNA expression studies, that VD displayed stronger placental tropism and lower parasitic loads in peripheral blood than the K98 strain (Juiz et al., 2017). On the other hand, female mice infected with the K98 strain present higher parasitemia than the ones infected with the RA strain (DTU VI). However, in RA infected females, the infected mice produced parasite-infected newborns, while K98 did not.

We also showed recently in human placental explants (HPE), as well as in a placenta-derived epithelial cell line (BeWo), that VD parasites present a higher infection capacity compared to the Y strain (Medina et al., 2018). These studies suggest that the parasite genotype plays a role in tissue tropism toward the placenta and might contribute to the probability of congenital transmission.

PARASITE EXOVESICLES AS VIRULENCE FACTORS

The persistence of *T. cruzi* parasites at a low level within specific tissues causes chronic pathogenic inflammation in some patients. How *T. cruzi* manages to persist and what factors released by the parasite influence its dynamics in tissue during chronic infection, remains poorly known (Teixeira et al., 2011). Nearly all cells can release extracellular vesicles, or exovesicles (EVs), which have been recognized as a mode of communication between cells. EVs may also participate in pathogenic processes (van Niel et al., 2006; Trocoli Torrecilhas et al., 2009; Cestari et al., 2012; de Pablos Torró et al., 2018). EVs are small membrane vesicles that are classified according to size, biogenesis, and composition, into exosomes and microvesicles (MVs). Exosomes, of 30 to 100 nm in size, are of endocytic origin, have a lipid bilayer, and are released into the extracellular compartment through the fusion of the multivesicular body (MVB) with the plasma membrane of the cell (Raposo and Stoorvogel, 2013). MVs are also referred to as ectosomes; are more heterogeneous in shape; and can vary between 100 and 300 nm in diameter. They are released as a result of the evagination toward the extracellular space from the plasma membrane. EVs differ not only in origin and size but also in lipid and protein composition. They have been identified in all biological fluids (Villagrasa et al., 2014), and their functions include intercellular communication, host-pathogen interactions, as well as the modulation of the immune response against infectious diseases or afflictions such as cancer (Schorey and Bhatnagar, 2008; Webber et al., 2015). We recently published reviews on the existence of EVs in protozoa and parasitic helminths and on the role of *T. cruzi* EVs (Marcilla et al., 2014; de Pablos Torró et al., 2018). Moreover, the induction of physiological modifications in cells by EVs derived from *T. cruzi* trypomastigotes has been recently demonstrated. These alterations involve the blocking of the cell cycle of the host cell, the permeabilization of the cells, and the disorganization of the cytoskeleton, among others (Retana Moreira et al., 2019).

EVs in *T. cruzi* were first described in 1970 (da Silveira et al., 1979) and found to be rich in glycoconjugates. Surface glycoconjugates are formed from glycoproteins (mucins) (de Lederkremer and Colli, 1995; Acosta-Serrano et al., 2001; Buscaglia et al., 2006; Mendonça-Previato et al., 2013), glycolipids (lipopeptidophosphoglycan-LPPG or glycoinosytolphospholipids-GIPL), and glycopeptides (NETNES) (Macrae et al., 2005). Electron microscopy and cryo-fracture have demonstrated that the vesicles come from the plasma membrane of the protozoan and the flagellar pocket. Recently, several surface proteins of the trans-sialidase, the cruzipain and the mucin-associated surface proteins (MASPs) families have been described in EVs, whose activity had previously been related to the modulation of the immune response by means of activating B cells and inducing a Th17 response or processes of adherence and cell invasion (Bermejo et al., 2011, 2013). Interestingly, MASPs are strongly expressed in the parasite and present a high variability between the different strains (Seco-Hidalgo et al., 2015). In recent works, we demonstrated that EVs, circulating in patients with CD, contained immature MASPs, with the presence of the C-proximal and N-terminal regions (peptide signal) (De Pablos et al., 2016; Díaz Lozano et al., 2017). MASPs are linked to the membrane through glycosylphosphatidylinositol (GPI) anchors. GPI participates in the inflammation processes that characterize CD (Almeida and Gazzinelli, 2001). The immature MASPs present in EVs (De Pablos et al., 2016; Díaz Lozano et al., 2017) are recognized by antibodies from CD patients. They help the parasite, in the presence of anti-*T. cruzi* antibodies, to evade the immune response by inhibiting the activity of C3 convertase and thereby impairing complement activation (Cestari et al., 2012). Similarly, the EVs isolated from the sera of CD patients are recognized both by the antibodies of the patients as well as by the serum of mice infected with the parasite. Therefore, it has been proposed that the immature MASPs in the outer side of the membrane of these EVs can form immunocomplexes with the IgGs of the patients (Díaz Lozano et al., 2017). In these studies, we demonstrate the involvement of EVs in the inhibition of the

complement pathway, in which the terminal regions C- and N- of the immature MASPs participate. We also show how the antigens of the C- and N- immature regions of these MASPs inhibit the complement by antigen competition, in the presence of antibodies from CD patients. Besides, we identified differences in antigen recognition of the EVs by sera from the patients, depending on the pathology manifested by those affected by CD (De Pablos et al., 2016; Díaz Lozano et al., 2017). These results allowed us to consider *T. cruzi* EVs as markers of pathology, suggesting that they play a role in the pathogeny of CD. Their role in modulating the interactions between the parasite and the placenta and the congenital transmission is under investigation. However, preliminary results show that the parasite increases placenta-derived exosomes in HPE (Castillo et al., 2017a). The placenta is a rich source of exosomes which are related to fetal-placental-maternal communication, fetal allograft survival, and resistance to specific infections, among other functions (Ouyang et al., 2014; Schorey and Harding, 2016). Therefore, it is highly probable that placenta-derived exosomes play essential roles during *T. cruzi*-host interplay.

PLACENTAL RESPONSES TO *T. cruzi*

The placenta is a temporary organ that separates the maternal and fetal compartments throughout pregnancy (Arora et al., 2017). The placenta is responsible for the metabolic exchange between mother and fetus, and fulfills endocrine and immune functions that ensure normal prenatal development of the fetus and pregnancy-related changes in the mother (Liempi et al., 2016; Arora et al., 2017). Mainly due to its regulatory role in the maternal/fetal immune response, this organ is able to protect the fetus against several pathogens (Delorme-Axford et al., 2014; Mor et al., 2017) (see below).

The human placenta is a highly invasive (hemochorial) chorioallantoic placenta. The functional units, where the placental barrier is located, are the free-floating chorionic villi formed by the trophoblast and the villous stroma. Maternal blood surrounds and contacts the trophoblast in the placental intervillous space (IVS). The trophoblast is a bi-stratified covering epithelium composed of a superficial non-proliferative syncytiotrophoblast (ST) and a proliferative germinal layer, the cytotrophoblast (CT). The trophoblast is connected to and separated from the villous stroma (VS), the fetal connective tissue by a basal lamina, a specialized structure of extracellular matrix (ECM) (Benirschke et al., 2012). Therefore, trophoblast, basal *laminae* and VS, the latter containing fetal capillaries, form the placental barrier that must be crossed by *T. cruzi* in order to infect the fetus during trans-placental transmission (**Figure 1**; Duaso et al., 2010; Carlier et al., 2012; Liempi et al., 2016). Of note, *T. cruzi* displays a high tropism for the decidual part of the placenta as compared to the heart, that is likely related to the local immune environment [see further and (Mjihdi et al., 2002)]. Also, in the placenta of mothers of congenitally infected infants, only a few amastigotes can be found in stromal cells such as macrophages, fibroblasts, and giant cells while they are not or scarcely found in the trophoblast, except in case of severe mortal cases of congenital infection (Carlier and Truyens, 2015).

Since the trophoblast is the first placental tissue in contact with the maternal blood, the ST is the first fetal cell layer exposed to the parasite. It has been demonstrated that a high concentration of *T. cruzi* trypomastigotes induces the destruction of the trophoblast in *ex vivo* infected HPE (Duaso et al., 2010). However, low parasite concentration induces cellular proliferation (Liempi et al., 2016; Droguett et al., 2017) and differentiation (Liempi et al., 2014, 2016) in the same placental tissue (see below). The basal *laminae*, located between trophoblast and the fetal connective tissue and around the fetal vessels are other structures that *T. cruzi* needs to overcome (Duaso et al., 2010). *T. cruzi* can bind to ECM molecules such as glycosaminoglycans, fibronectin, and laminin (Lima et al., 2002) using surface molecules such as gp85 (Maeda et al., 2014) and gp83 (Nde et al., 2006). The VS, the placental fetal connective tissue, is also an important obstacle for the parasite. The parasite presents several proteases, among which the cruzipain has been proposed to be responsible for collagen I destruction and disorganization in placentas from mothers with chronic CD or in *ex vivo* infected HPE (Scharfstein and Morrot, 1999; Castillo et al., 2012; Duaso et al., 2012; Maeda et al., 2014). Matrix metalloproteinases (MMPs) such as MMP-2 and MMP-9 are activated, and their expression levels are increased in the presence of the parasite. Interestingly, if these MMPs are inhibited, the parasite-induced damage in the placental tissue is prevented, and the presence of parasite DNA is decreased in *ex vivo* infected HPE (Castillo et al., 2012). Importantly, the ECM forms a complex tridimensional network with different types of elastic fibers and collagen molecules including collagen I fibers, glycoproteins, and proteoglycans (Duaso et al., 2010; Theocharis et al., 2019). Collagen I fibers are the main components of the ECM, and if these molecules are disorganized or destroyed, the 3D network of the ECM is also disrupted, a fact that could facilitate the parasite's motility in the tissue, and its entrance into cells (Duaso et al., 2010). Moreover, *in vitro* studies have shown that during parasite-ECM interactions, the parasite changes its cytoskeletal conformation, the state of protease activation and adapts its metabolism (Mattos et al., 2019). In addition, parasite-induced ECM changes modulate the presence of cytokines and chemokines, allowing *T. cruzi* to manipulate and escape the innate and adaptive immune responses (Marino et al., 2003). Studies performed in CD patients with cardiac alteration have shown that *T. cruzi* derived-antigens promote a differential expression of MMP-2 and MMP-9. There is a positive correlation between MMP-2 and the immunomodulating cytokine IL-10, and a negative one with the pro-inflammatory cytokine IL-1β, whereas MMP-9 showed a negative correlation with IL-10. Therefore, it has been suggested that MMPs and cytokines produced in the myocardium in patients with CD are essential contributors to cardiac remodeling (Medeiros et al., 2017). It would be interesting to study the interactions between parasite-modulated MMPs and cytokines in the placenta.

Another placental response to the parasite is the trophoblast epithelial turnover. The epithelial turnover is part of the innate immune system. Pathogens adhere to the plasma membrane

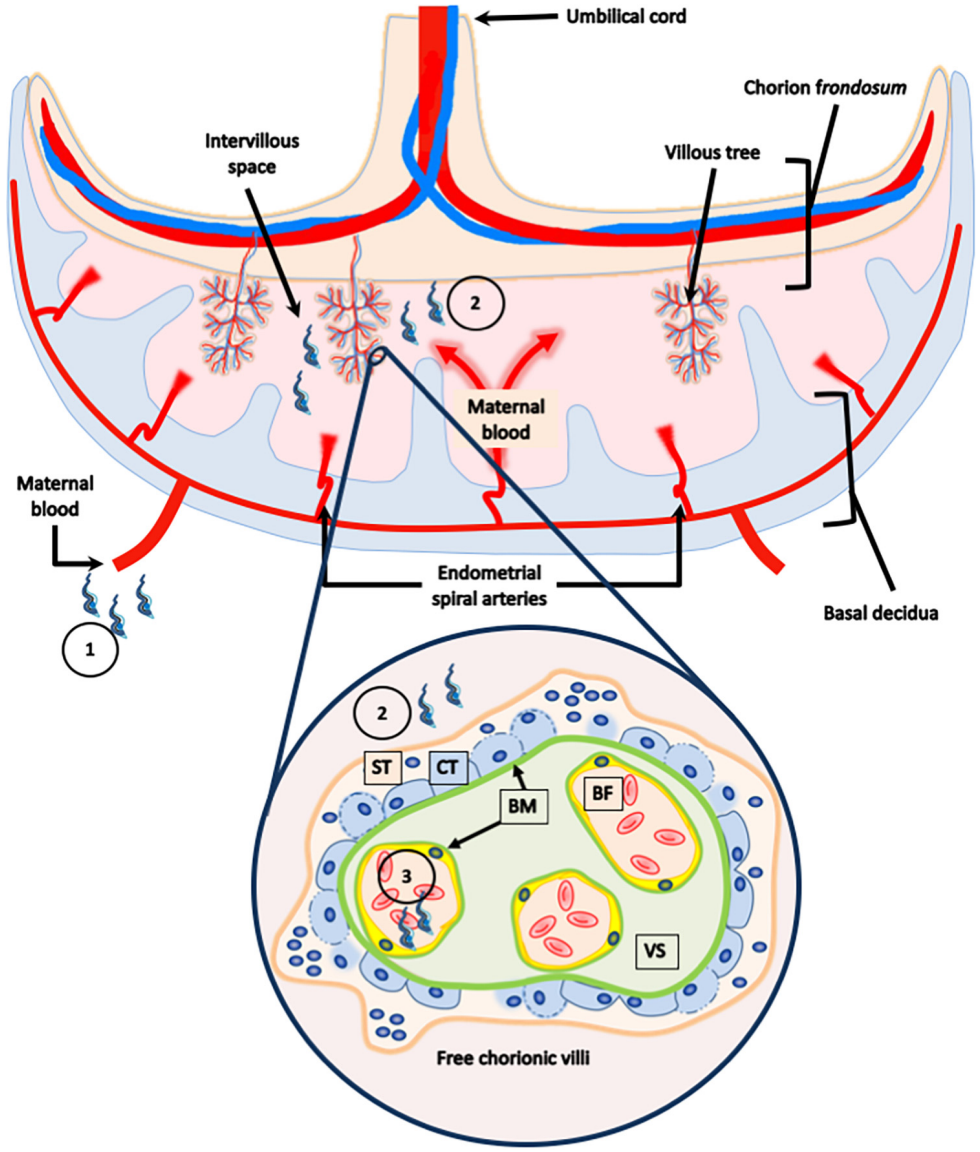

FIGURE 1 | Human placental barrier. The human placenta is classified as a hemochorial chorioallantoic placenta. The placenta is composed of a fetal portion, developed from the chorion frondosum, and a maternal portion, or basal decidua, which originates from the endometrium. The functional units, were the placental barrier is located, are the free-floating chorionic villi formed by the trophoblast, and the villous stroma. Maternal blood surrounds and contacts the trophoblast in the placental intervillous space. The trophoblast is a bi-stratified covering epithelium composed of a superficial non-proliferative syncytiotrophoblast (ST), and a proliferate germinal layer, the cytotrophoblast (CT). The trophoblast is connected to and separated from the villous stroma (VS), the fetal connective tissue, and by a basal lamina. The parasite present in the maternal blood (1), that comes in contact with the trophoblast in the intervillous space (2), must cross the placental barrier in order to reach the fetal capillaries (3), and infect the fetus during transplacental transmission.

before invading host cells and, in case of a lining epithelium, they adhere to the cells of the superficial cell layer which is continuously removed. Therefore, the attached pathogens are removed with the superficial epithelial cells (Liempi et al., 2016).

The trophoblast turnover implies precise orchestration of different cellular processes: (i) cell proliferation of the CT, (ii) cellular differentiation (referring to the incorporation of CT cells into a non-replicative ST), and (iii) cell death by forming apoptotic bodies (knots) in the ST that counterbalance the proliferation of the CT cells. The apoptotic knots are released into the IVS where the maternal blood circulates (Benirschke et al., 2012; Mayhew, 2014; Liempi et al., 2016).

We have previously shown that *T. cruzi* induces the above-mentioned cellular processes related to the epithelial turnover in the trophoblast in both *ex vivo* infected HPE and *in vitro* infected BeWo cells (trophoblastic cell line) (Duaso et al., 2011; Liempi et al., 2014, 2016; Carrillo et al., 2016; Droguett et al., 2017). Thus, *T. cruzi* increases DNA synthesis as well as cellular proliferation markers such as PCNA (proliferating cell nuclear antigen) (Liempi et al., 2016). In BeWo cells, the parasite

also increases the percentage of cells in the S and G_2/M cell cycle phases and other commonly used proliferation markers (AgNORs and Ki67) (Droguett et al., 2017). It is noteworthy that PCNA modulates other cellular processes including DNA repair, cell cycle, survival, and gene expression (Wang, 2014), and that an increase of PCNA expression might occur in response to *T. cruzi*-induced damage. Regarding trophoblast differentiation, the parasite increases, in HPE and BeWo cells, the expression and or secretion of the main biochemical markers, including β-human chorionic gonadotropin (β-hCG) and syncytin (Liempi et al., 2014, 2016). Additionally, in BeWo cells *T. cruzi* induces cell fusion as demonstrated by the analysis of the re-distribution of desmoplakin (intercellular adhesion protein) and by a two-color fusion assay (Liempi et al., 2014). The parasite also activates the ERK1/2 MAPK pathway (Castillo et al., 2013b), one of the MAPK signaling pathways that mediate trophoblast differentiation (Forbes and Westwood, 2010). Finally, the parasite induces apoptotic cell death as part of trophoblast turnover (Liempi et al., 2016). Indeed, *T. cruzi* activates in HPE a caspase 3 like activity, followed by DNA fragmentation and pyknosis (Duaso et al., 2011). These data indicate that *T. cruzi* can boost all steps of the turnover of chorionic trophoblasts. Interestingly, a relation exists in trophoblasts between the cellular processes related to apoptosis and their differentiation (Gauster et al., 2009a,b). This is through caspase 8, a caspase operating upstream the pro-apoptotic caspase 3. Caspase 8 is transiently activated in CT cells just before their fusion into ST and is an essential protein in the process of formation of the ST barrier (Huppertz and Borges, 2008). We observed that *T. cruzi* infection increases the expression and activation of caspase 8 in BeWo cells as well as in HPE (Duaso et al., 2011; Carrillo et al., 2016). This fact might favor the integrity of the ST barrier as well as limit parasite entrance into ST (by favoring its turnover). Also, we showed that caspase 8 can slow down intracellular parasite multiplication (Carrillo et al., 2016), pinpointing caspase-8 activity as part of the trophoblast cell defense mechanisms against *T. cruzi* infection. Altogether, these results suggest that the trophoblastic response to *T. cruzi* may be a mechanism that reduces the risk of congenital transmission of the parasite. On the other hand, caspase 8 has recently been disclosed to display pro-inflammatory effects, by optimizing the cytokinic response to TLRs and by being able to activate the cytokine IL-1β (Lawlor et al., 2017). We speculate that such pro-inflammatory action causes placental lesions, favoring the trans-placental passage of *T. cruzi*.

As mentioned above, the placenta is an immune regulatory organ that modulates fetal as well as maternal immune responses (Mor et al., 2017). Pathogens are recognized by pattern recognition receptors (PRRs), including *Toll*-like receptors (TLRs). Activation of those PPRs results in the secretion of cytokines and chemokines aiming to fight infections (Janeway and Medzhitov, 2002). The human trophoblast expresses all ten of the known functional TLRs (Koga et al., 2009). *T. cruzi* is recognized by TLR-2 and TLR-4 (present at the cell surface) as well as the endosomal TLR-7 and TLR-9 (Tarleton, 2007; Gravina et al., 2013; Castillo et al., 2017b). We have shown, in HPE, that *T. cruzi* infection induces protein expression and activation of TLR-2, but not of TLR-4 and TLR-9 (Castillo et al., 2017b). *T. cruzi* induces the secretion of IL-1β, IL-6, IL-8, IL-10, and TNF-α in HPE (Castillo et al., 2017b). Of note is that secretion of IL-1β, IL-6, and TNF-α have been associated, within the trophoblast, with cellular proliferation, and differentiation (Haider and Knöfler, 2009; Hamilton et al., 2012). Interestingly, the inhibition of TLR-2 impairs trophoblast turnover and increases parasite infection (Castillo et al., 2017c). However, to date we do not know if cells other than trophoblasts, such as macrophages in the VS, are also involved in TLR activation. However, our results allow us to propose TLR-2 initiated cytokine profile as a local placental defense mechanism.

TRANSCRIPTOMIC STUDIES IN PLACENTAS IN RESPONSE TO *T. cruzi* INFECTION

The global placental transcriptomic response to *T. cruzi* infection has also been studied. This approach allows us to study the gene expression profiles during infection, allowing the identification of new genes and/or pathways implicated in the establishment of the infection and pathogenesis. The RNA-seq study has been carried out in placentas obtained from term deliveries in *T. cruzi*-infected and non-infected women. Forty-two differentially expressed genes (DEGs) were identified, and gene-set association analysis was performed to detect pathways linked to parasite infection. It showed, not surprisingly, that the inflammatory and the immune responses were upregulated in infected placentas while the anti-inflammatory cytokine IL-38 (formerly IL-1 family member 10-IL1F10) (Garraud et al., 2018) was under-represented (Juiz et al., 2018). Placental inflammation is in line with other reports showing that: (i) newborns of *T. cruzi*-infected mothers are prone to produce higher levels of pro-inflammatory cytokines in comparison to those born to non-infected mothers (Vekemans et al., 2000), (ii) *T. cruzi* infection of pregnant women affects the developing immune system of fetuses independently of congenital infection (Dauby et al., 2009), which might be related to placental inflammation (Jennewein et al., 2017), and (iii) the parasite induces, as mentioned above, in *ex vivo* infected HPE, a highly significant increase of pro-inflammatory and immunomodulatory cytokines (Castillo et al., 2017b,c).

Analysis of DEGs also suggests that the placental inflammation might be counter-regulated in infected mothers. For instance, placentas from infected women displayed lower expression of genes related to exocytosis pathways and neutrophil degranulation, probably limiting damages associated with inflammation. Moreover, the expression of the immunomodulatory HLA-G was increased (Juiz et al., 2018). HLA-G is responsible for the generation of immunological tolerance during gestation by inducing regulatory T cells and tolerogenic dendritic cells (Carosella, 2011; Ferreira et al., 2017) and might increase susceptibility to infections. Hence, in malaria, increased levels of HLA-G in infected mothers are associated with an increased risk to acquire malaria during infancy (Sadissou et al., 2014). S100A14 is another interesting parasite-modulated gene, that is downregulated in placentas from women with CD. This gene codes for a member of the S100 protein family that

regulates cell cycle progression, cellular differentiation, and triggers an inflammatory response by engaging the receptor for advanced glycation end products (RAGE) (Jin et al., 2011). Interestingly, this same protein, S100A14, is reported to increase the expression of the collagenase MMP2 (Chen et al., 2012; Qian et al., 2016), which as explained above regulates parasite infection. This might be in line with the observation that mutations on the MMP2 gene favor congenital *T. cruzi* transmission (see hereunder). On the other hand, PRG2 a gene that encodes the precursor form of the eosinophil major basic protein (proMBP) is an upregulated DEG in *T. cruzi*-infected placentas. MBP can participate in the extracellular killing of parasites in the absence of antibodies (Nakhle et al., 2018). Another important function of MBP in the placenta is the inhibition of the pregnancy-associated plasma protein A (PAPPA) (Weyer and Glerup, 2011), whose low levels are associated with intrauterine growth restriction (Albu et al., 2014). Therefore, overexpression of the PAPPA inhibitor in the placenta of women with CD might also play a role in the congenital transmission of *T. cruzi*.

Other, DEGs observed in placentas from seropositive women and related to pregnancy were the genes CGB5 and KISS1, which encode for hCG and kisspeptin, respectively. Both genes were downregulated, and low serum levels of both proteins have been proposed as markers of miscarriage (Jayasena et al., 2014). Low KISS1 expression is associated with recurrent pregnancy loss as well as preeclampsia and intrauterine growth restriction (Armstrong et al., 2009; Park et al., 2012). In contrast, the DEG TAC3 gene [encoding neurokinin B (NKB)] was upregulated in placentas from seropositive women. NKB overexpression in the trophoblast is associated with a decreased blood flow to the placenta and increased vasoconstriction in the endometrium and therefore related to preeclampsia (Familari et al., 2017). Interestingly, NKB and kisspeptin have a role in hCG placental expression in response to estradiol (Oride et al., 2015) and hCG, as described above, is induced by *T. cruzi* in HPE and BeWo cells (Liempi et al., 2014).

A microarray-based transcriptomics study carried out in *ex vivo* infected HPE (Castillo et al., 2018), corroborates most data obtained by RNA-seq in whole placentas (Juiz et al., 2017, 2018) as well as the *T. cruzi*-invasion mechanisms in HPE. Genes that are involved with ECM remodeling are upregulated, in concordance with our previous findings that demonstrate the parasite-induced expression and activation of MMP-2 and MMP-9 (Castillo et al., 2012). The changes in gene expression regarding signal transduction pathways were also confirmed (Castillo et al., 2013b, 2017b). Genes involved in innate immunity were overexpressed, including CD46 and C1q that regulate or form part of the complement system. Upregulation of C1q might have an impact since it binds the *T. cruzi* calreticulin, thereby enhancing the infection (Castillo et al., 2013a). On the other hand, TLR-7 and TLR-8 are mainly increased while TLR-2, whose inhibition favors infection and tissue damage (Castillo et al., 2017c), appeared not to be upregulated in the microarray analysis. Another contradictory observation was that a high *T. cruzi* concentration decreased IL-6 expression more than 60-fold as compared to control non-infected explants, whereas no changes were observed when HPE were infected with a low parasite concentration. On the other hand, mRNA expression analysis showed a higher level of transcription, suggesting that regulation of IL-6 could occur at post-transcriptional stages (Castillo et al., 2017c).

Furthermore, similar to the study in whole placentas, the majority of overexpressed genes were related to fetal development and pregnancy-related processes, particularly *GH2*, *CSH1*, and *CSH2* genes that encode the pregnancy-specific beta-1-glycoproteins growth hormone 2, chorionic somatomammotropin hormone 1 and 2, respectively.

Another microarray-based study was done in placental tissues from C57Bl/6J mice chronically infected with two distinct *T. cruzi* strains (VD and K98, see above) (Juiz et al., 2017) A total of 247 DEGs were identified between infected and non-infected mice: 140 and 107 genes were up- and genes downregulated, respectively, compared to non-infected mice. Placentas infected with the VD parasites showed a higher number of DEGs compared to the K98 infected ones (211 vs. 89 DEGs). There were DEGs common to both infected groups and specific ones for each infected group; while 69% (59/89) of genes were downregulated by K98 infection, VD infection produced this response only in 39% (83/211) of genes. Analysis of DEG networks by GeneMANIA showed that the "Secretory Granule" pathway was downregulated in both infected groups, whereas "Response to Interferon-gamma" as well as "Innate Immune Response" pathways were upregulated only in placental tissues infected with VD. This is interesting since, as said above, the K98 strain displays poor placental tropism and no congenital transmissibility, contrary to the VD strain. It suggests the role of placental inflammation in the process of congenital transmission. Another analysis that detects small changes in predetermined gene sets (Gene-Set Enrichment Analysis algorithm) showed downregulation of genes involved in transcription, macromolecular transport, and metabolism in infected placentas and upregulation of genes regulating signal transduction pathways. Genes regulating apoptotic cell death were downregulated in the murine placentas infected with both parasite strain.

These transcriptomic analyses identify a gene expression profile in the placentas from *T. cruzi*- seropositive mothers that are different from those from seronegative women. It globally shows that affected genes are involved in cell adhesion, cellular proliferation and differentiation, apoptosis, vesicle transport processes, ECM organization, lipid and protein metabolism, and the inflammatory/immune responses.

FAMILY CLUSTERING OF CONGENITAL TRANSMISSION OF *T. cruzi* INFECTION AND SNPs

It is still unknown why some infected mothers transmit the infection in successive gestations to their babies while others do not, leading to family clustering of congenital transmission. A case-control study of single-nucleotide polymorphisms (SNPs) located within human loci encoding different proteins expressed in placental tissues was performed using genomic DNA

from clinical samples of 116 non-infected children born to seropositive women and children with congenital *T. cruzi* infection. Logistic regression analysis showed that susceptibility of congenital infection was associated with SNPs in sites rs243866, rs17859821, and rs2285053from MMP2 gene and rs11244787 and rs1871054 sites from the ADAM 12 locus. In case of MMP2 rs243866 and rs17859821 positions, one or both copies of the mutant allele (Adenine) increased the likelihood of congenital infection, whereas, for rs2285053, both T alleles are needed for susceptibility to infection. Mutation in rs2285053 interrupts a CCACC box promoter site (Sp1-type) causing a weaker activity of the promotor. The rs243866 position is located upstream of a half-palindromic potential estrogen receptor binding site (Harendza et al., 2003). Both rs243866 and rs2285053 mutant alleles reduce the transcription activity of MMP2, which in turn modulate the ECM-remodeling and immune responses, and thus the susceptibility to infection. ADAM12 belongs to the ADAM protein family; it is a membrane-bound MMP-protease with a role in cell-cell and cell-matrix interactions that are associated with muscle development, fertilization, neurogenesis inflammatory, and immune responses. In preeclampsia (Grill et al., 2009) and ectopic pregnancy (Rausch and Barnhart, 2012), among other disorders related to pregnancy, ADAM 12 plasmatic concentrations are altered. Also, ADAM 12 promotes the transforming growth factor β (TGF-β) signaling activation leading to transcriptional activation. Importantly, TGF-β activation increases host cell susceptibility to *T. cruzi* infection (Ming et al., 1995; Hall and Pereira, 2000).

Various other SNPs have been related to the susceptibility to *T. cruzi* infection and progression of CD. Particularly, genes encoding cytokines/chemokines involved in inflammatory and immune response have been proposed as biomarkers (Calzada et al., 2009; Torres et al., 2010; Pissetti et al., 2011; Flórez et al., 2012; Nogueira et al., 2015; Furini et al., 2016; Ferreira et al., 2018). Thus, TGF-β1 polymorphism, mainly CT and TT genotypes at position – 509 of the TGFB1 gene have been associated with the susceptibility of acquiring CD in a Brazilian population (Ferreira et al., 2018). The same genotype, in addition to the genotypes −988 C/A; −800 G/A; −10 T/C; and 263 C/T has been studied in the Colombian and Peruvian populations. In this study, the genotype 10 C/C was increased in the group of CD patients of both populations and has been proposed to be involved in differential susceptibility to *T. cruzi* infection (Torres et al., 2010). Another important pro-inflammatory cytokine involved in CD is TNF-α. The TNF- α gene is located in the MHC locus, and its polymorphism is associated with many infectious diseases, including parasitic ones. TNF-α is increased in the hearts of patients with chronic chagasic cardiomyopathy and is associated with tissue damage. An association was observed between the absence of the TNF-238A allele and negative serology for CD. Seropositive individuals carrying the TNF-238A allele produced significantly higher TNF-alpha levels than healthy ones and therefore the TNF-α polymorphism at position −238 has also been associated to the susceptibility to *T. cruzi* infection and CD progression (Pissetti et al., 2011). Other SNPs in genes encoding for cytokines and molecules involved in the immune response has been related to CD, including interferon (IFN)-γ (-874 T/A) (Torres et al., 2010) and IL-18 (rs2043055 polymorphism) (Flórez et al., 2012; Nogueira et al., 2015), RANTES, and chemokine receptors CCR2 and CCR5 (Flórez et al., 2012). It would thus be worthwhile to also investigate such polymorphisms in the context of congenital transmission of *T. cruzi.*

MATERNAL *T. cruzi* SYSTEMIC IMMUNE RESPONSE, GESTATION, AND CONGENITAL INFECTION

The immune system during pregnancy is characterized by a subtle balance between immune tolerance and activation. Regulatory T cells are increased during pregnancy in order to avoid fetal rejection. Indeed, the maternal immune cells recognize paternal antigens expressed by the trophoblast and other fetal cells crossing the placenta to the maternal circulation. The mechanisms leading to this regulatory environment have been largely described elsewhere (Aluvihare et al., 2004; Racicot et al., 2014). Maternal immune tolerance is fundamental to a successful pregnancy. It does, however, not mean that the pregnant women are unable to mount immune responses, and trophoblasts possess multiple ways to escape immune attack (Straszewski-Chavez et al., 2004). Besides, immune responses during gestation are, except for the periods of implantation, placentation, and parturition, physiologically oriented toward a Th2-type. It likely aims to protect the uteroplacental unit and the fetus against harmful inflammation (Mor et al., 2017). Whereas the regulatory and Th2-biased environments are particularly pronounced at the level of the UPU, it may also impact peripheral, and systemic immune responses (Reinhard et al., 1998).

Trypanosoma cruzi infection induces a complex immune response. The control of *T. cruzi* infection relies on diverse effector mechanisms needed to fight both extracellular trypomastigotes and intracellular amastigotes. Therefore, IFN-γ, cytotoxic cells, and antibodies (Abs) all play a role in the control of the infection [reviewed in Truyens and Carlier (2017)]. Antibodies potentially participate in extracellular parasite elimination mainly by inducing complement-dependent and independent lysis of parasites and phagocytosis of opsonized parasites. IFN-γ, initially produced by NK cells, then by both CD4 + Th1 and CD8 + T cells, is a significant player in the immune responses against intracellular pathogens (Kak et al., 2018). It activates among other the microbicidal properties of phagocytes, mainly macrophages, allowing them to limit and/or kill the intracellular parasites. Infected cells can be killed by cytotoxic CD8 + T cells while the cytotoxic action of NK cell on *T. cruzi*-infected cells is likely not significant (Truyens and Carlier, 2017). The cellular response to *T. cruzi* is thus mainly Th1-oriented. Our studies in mice show that this inflammatory immune response has serious harmful effects on gestation when the host is in the acute phase of infection. Indeed, acute infection impedes reproduction, related to either infertility induced by the infection (inhibition of cell division of the embryo before implantation) or fetal loss occurring along gestation, due to massive placental invasion by parasites. Chronic maternal infection does not affect reproductive capacity but induces

reversible fetal growth retardation (Carlier et al., 1987). Such deleterious effects did not rely on congenital infection but were associated with increased production of TNF-α (Rivera et al., 1995; Mjihdi et al., 2002). In humans, most women of gestational age have a chronic infection (see above). We have similarly observed that *T. cruzi*-infected women give birth to newborns with low birth weight, in the absence of congenital infection more frequently (Torrico et al., 2004).

Besides, in the absence of congenital transmission, several data indicate bidirectional and opposite interactions between the immune response to *T. cruzi* and the immune environment of gestation. On one side, pregnancy dampens and modifies the immune response to *T. cruzi*. Indeed, we found that blood cells from chronically infected pregnant women released, on average, two to threefold less IFN-γ and IL-2 in response to non-specific lymphocyte stimulants (PHA and LPS) than non-pregnant women (Hermann et al., 2004). They also produced, on average, sevenfold less IL-10. Accordingly, lower levels of IL-10 and IFN-γ were detected in the serum of pregnant infected women than in non-pregnant infected ones (Cardoni et al., 2004). This shows that pregnancy reduces the global capacity of their lymphocytes to be activated (though it is not completely inhibited), likely as a result of the relative immunosuppression associated with gestation. Also, the cytokine profile moved toward a more inflammatory environment in infected pregnant women, since the capacity to produce the inflammatory cytokine IFN-γ was less inhibited during pregnancy than the capacity to produce IL-10 (an anti-inflammatory cytokine), being in line with the inflammatory transcriptome detected in the placenta of *T. cruzi*-infected women [see above (Juiz et al., 2018)]. On the other hand, Egui et al. recently observed in a murine experimental *T. cruzi* infection model that chronic infection reduced the expression of gestation-induced inhibitory receptors on T cells (CTLA4 on CD4 + and CD8 + T cells and CD160 on CD8 + T cells), suggesting that *T. cruzi* chronic infection counteracts the immunosuppression associated with gestation but does not impact the gestation outcome (Egui et al., 2017).

Our previous studies in pregnant chronically infected women showed that gestation also reduced the specific IFN-γ response to parasite antigens as compared to the response of non-pregnant infected ones (Hermann et al., 2004). This was the case for women that transmitted (T mothers) as well as those that did not transmit (NT mothers) the parasite to their fetus. Strikingly, this specific IFN-γ response was markedly lower in T than NT mothers and was associated with less activated monocytes (Hermann et al., 2004). On the other hand, we and others have reported that the circulating parasite load is slightly increased during the second and third trimester of pregnancy (Brutus et al., 2010; Siriano et al., 2011). Moreover, T mothers displayed higher parasitemia than NT ones (Hermann et al., 2004; Kaplinski et al., 2015). IFN-γ is well known as a key factor in the control of *T. cruzi* infection (Truyens and Carlier, 2017). These data strongly suggest that the increase of parasite burden in T mothers results from a reduced control of the infection and points to a central role of parasite burden as a risk factor of congenital transmission. In line with this, several studies underline that congenital transmission occurs mostly in mothers displaying blood parasites amounts that are detectable by PCR (i.e., PCRs are generally negative in NT mothers). The microbial burden is also a key factor of congenital transmission of other pathogens (Lilleri and Gerna, 2017).

The increase of parasite burden during gestation likely results from the relative immunosuppression associated with gestation, while the increased parasite load in T mothers probably relates to their more depressed IFN-γ response. The question is, thus, why T mothers display such feature? Several factors may be considered, concerning the parasite, the host, or epidemiological parameters. Living in endemic countries where vectorial transmission occurs may lead to frequent reinfections. Multiple infections, though leading to transiently increased parasite levels, result in lower parasitemia in the long term, the hypothesis being that repeated antigen exposure reinforces the Th1 response that controls the parasite (Machado et al., 2001; Bustamante et al., 2007; Kaplinski et al., 2015). In line with this, it was observed that higher vector exposure of women living in endemic countries decreases the risk of congenital transmission (Sánchez Negrette et al., 2005; Kaplinski et al., 2015). Congenital transmission rates are also lower in non-endemic countries (2.7%), free of vectorial reinfections, than in endemic countries [2.7% vs. 5%, respectively (Howard et al., 2014)]. Parasite load may also vary according to the parasite genotype/strain (Moreira et al., 2013).

Our data also suggest that host factors modulate the risk of congenital transmission of *T. cruzi*. Genetic and epigenetic factors may influence the efficacy of immune responses against pathogens and consequently modulate the microbial load (Möller et al., 2018). For instance, polymorphism of genes encoding proteins of the axis IL-12-IFN-γ and genetic variations affecting the modulatory action of long non-coding RNAs or micro-RNAs are reported to impact diseases (Vannberg et al., 2011; Duval et al., 2017; Gao and Wei, 2017; Ellwanger et al., 2018). We compared the ability of T and NT women to produce IFN-γ to *T. cruzi* and mitogens several months after they had given birth, i.e., in the absence of the immunosuppression of pregnancy (Hermann et al., 2004). We found that T women still displayed a more depressed ability to produce this cytokine than NT women, suggesting that inherent host factors involved might be genetic. Likewise, we have recently reported that several genes related to the immune system were differentially expressed in the placenta of T and NT mothers as well as an association between congenital *T. cruzi* transmission and the presence of polymorphism among some gene expressed in the placenta (see above) (Juiz et al., 2016, 2018). Of note, differential immune responses associated with the congenital transmission of other pathogens have also been recently reported (Lilleri and Gerna, 2017; Wujcicka et al., 2018). Besides, epigenetic modulation may also have occurred in some women in relation either with the *T. cruzi* strains present in the host or to their history of infections with other pathogens (Gomez et al., 2013; Smith and Denning, 2014).

Finally, we and others noticed that T mothers were younger and had a lower mean number of previous pregnancies than the NT mothers (Hermann et al., 2004; Kaplinski et al., 2015), which might be related to the immuno-enhancing effect of multiparity on the maternal immune system (Skowron-Cendrzak et al., 1999).

NEONATAL IMMUNE RESPONSE TO *T. cruzi*

Immune responses in early life are physiologically different from adult responses. Similarly, to what occurs during pregnancy, the neonatal immune system is characterized by a certain degree of immunodeficiency and weak Th1 but excessive Th2 responses, contributing to high susceptibility to pathogens and the development of suboptimal immune responses to vaccines administered in early life. However, the mechanisms leading to immunodeficiency and Th2 bias are completely different from those activated during pregnancy and have been reviewed elsewhere (Levy, 2007; PrabhuDas et al., 2011; Elahi et al., 2013). Mechanisms responsible for the regulatory environment comprises, among others, impaired signaling pathways downstream of *Toll-like* receptors 3 and 4, the presence of higher circulating levels of adenosine, the presence of higher numbers of regulatory T cells, and the presence of particular inhibitory "erythroid cells." The Th2 bias results from the hypermethylation of the *IFNG* gene, while the Th2 locus is hypomethylated. Also, the proliferation of Th1 T cells in early life is strongly hindered due to the expression of a particular receptor for IL-4 produced by Th2 T cells. Engagement of this receptor induces apoptosis of Th1 cells.

Our studies in humans, performed in infants from chagasic mothers, point out that *T. cruzi* infection triggers neonatal type 1 immune responses, overcoming the physiological immune deficiency associated with early life (Hermann et al., 2002). Indeed, CD8 + T cells, and CD4 + T cells to a lesser extent, were activated in *T. cruzi* congenitally infected new-borns to an adult-like level and produced IFN-γ. Besides, their NK cells display phenotypic and functional alterations suggestive of a previous *in utero* activation when parasites were transmitted from the mother (Hermann et al., 2006). It also pinpoints the imprinting of the maternal *T. cruzi* infection on the neonatal immune system, revealing an immuno-stimulatory/adjuvant property of the parasite, as both congenitally infected and uninfected infants from chagasic mothers responded more strongly to vaccines directly administered during the first 6 months of life, like those against BCG, hepatitis B, tetanus, and diphtheria (Dauby et al., 2009). In trying to decipher the mechanisms allowing the parasite to induce type 1 immune responses in early life, we showed that the parasite could strongly activate neonatal NK cells to produce IFN-γ, known to drive Th1 type responses rapidly. NK cell activation by *T. cruzi* is indirect and depends on cross-talk with monocytes (and not with dendritic cells), on IL-12 synthesis and engagement of TLR2, 4, 7, and 9 (Guilmot et al., 2013). The parasite also very efficiently licensed neonatal dendritic cells to activate CD4 + and CD8 + T cells. Interestingly, DC and monocyte activation by *T. cruzi* is reinforced in the presence of maternal IgG isolated from cord blood samples from neonates born to NT infected mothers, i.e., carrying *T. cruzi*-specific IgG (Rodriguez et al., 2012). These observations allow us to make the hypothesis that monocytes, likely activated in fetuses from infected mothers combined with the maternally transmitted *T. cruzi* – specific antibodies might allow the fetus to fight parasites and maybe in some cases to eliminate them if only a few parasites were transmitted.

FINAL REMARKS

Some relevant questions regarding the role of parasite diversity, host genetic response, and host immune responses deserve further investigations. The study of gene expression profiles during infection constitutes a powerful tool to analyze global responses of several kinds of cells and tissues, allowing the identification of new genes and/or pathways implicated in the establishment of the infection and pathogenesis as well as possible local tissue responses. Other aspects such as the role of maternal microbiomes in the likelihood of vertical transmission of *T. cruzi* have not been investigated yet.

AUTHOR CONTRIBUTIONS

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

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The Impact of Infection in Pregnancy on Placental Vascular Development and Adverse Birth Outcomes

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Healthy fetal development is dependent on nutrient and oxygen transfer *via* the placenta. Optimal growth and function of placental vasculature is therefore essential to support *in utero* development. Vasculogenesis, the *de novo* formation of blood vessels, and angiogenesis, the branching and remodeling of existing vasculature, mediate the development and maturation of placental villi, which form the materno-fetal interface. Several lines of evidence indicate that systemic maternal infection and consequent inflammation can disrupt placental vasculogenesis and angiogenesis. The resulting alterations in placental hemodynamics impact fetal growth and contribute to poor birth outcomes including preterm delivery, small-for-gestational age (SGA), stillbirth, and low birth weight (LBW). Furthermore, pathways involved in maternal immune activation and placental vascularization parallel those involved in normal fetal development, notably neurovascular development. Therefore, immune-mediated disruption of angiogenic pathways at the materno-fetal interface may also have long-term neurological consequences for offspring. Here, we review current literature evaluating the influence of maternal infection and immune activation at the materno-fetal interface and the subsequent impact on placental vascular function and birth outcome. Immunomodulatory pathways, including chemokines and cytokines released in response to maternal infection, interact closely with the principal pathways regulating placental vascular development, including the angiopoietin-Tie-2, vascular endothelial growth factor (VEGF), and placental growth factor (PlGF) pathways. A detailed mechanistic understanding of how maternal infections impact placental and fetal development is critical to the design of effective interventions to promote placental growth and function and thereby reduce adverse birth outcomes.

Keywords: infection, pregnancy, placenta, vascular development, adverse birth outcomes

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INTRODUCTION

Each year an estimated 20 million infants are born low birth weight (LBW) (<2,500 g) and 14.9 million are born preterm (Lee et al., 2013). Preterm birth (PTB) is the leading direct cause of under 5 mortality, responsible for more than 1 million deaths per year (Liu et al., 2016). According to the Global Burden of Disease Study, the disability-adjusted life years attributable

to PTB is 77 million, comparable to the estimates for HIV or malaria (Murray et al., 2012). While these adverse birth outcomes predominantly occur in low- and middle-income countries (Blencowe et al., 2012), rates are increasing globally, and have been consistently linked with increased risks of long-term health consequences for offspring including cardiovascular disease, diabetes, obesity, and neurodevelopmental disorders (Bilbo and Schwarz, 2009; Calkins and Devaskar, 2011). The burden of infectious diseases in pregnancy (e.g., malaria, HIV, sexually transmitted infections) is also highest in low- and middle-income countries, and a growing body of evidence indicates that these prevalent infections contribute to poor birth outcomes by inflammation-mediated disruption of placental development and function (Watson-Jones et al., 2002; Chico et al., 2017; Conroy et al., 2017; McDonald et al., 2018, 2019).

Fetal development is governed by tightly regulated processes at the materno-fetal interface. Placental vasculogenesis and angiogenesis mediate placental vascular development, which is critical to nutrient and oxygen delivery to the developing fetus. These processes are primarily regulated by mediators in the vascular endothelial growth factor (VEGF) and angiopoietin families (Geva et al., 2002). Dysregulation of these factors is associated with inadequate placental vascularization, leading to hemodynamic placental insufficiency, inadequate delivery of nutrients and oxygen to the fetus, and consequently adverse birth outcomes (Kaufmann et al., 2003). Inflammatory and angiogenic pathways are interdependent and co-regulatory, suggesting that the host response to maternal infection could dysregulate pathways essential for placental vascular development. Here, we review the impact of systemic maternal infections resulting in immune activation at the materno-fetal interface – and its subsequent impact on placental vascularization – adverse birth outcomes, and later-life neurocognitive deficits in offspring.

PLACENTAL DEVELOPMENT: VASCULOGENESIS AND ANGIOGENESIS

The placenta forms the primary interface between mother and fetus, and a healthy functioning placenta is essential for a successful pregnancy. The placenta is a multi-function organ, acting as the site of materno-fetal nutrient, oxygen and waste exchange; producing hormones and growth factors critical for pregnancy progression and maintenance; and acting as a barrier to protect the fetus from maternal immune attack, toxins, and infectious pathogens (Wang and Zhao, 2010). These functions all rely on proper vascularization and perfusion of the placenta, and disruptions to placental vascular development and adaptation are associated with adverse pregnancy outcomes including preeclampsia, small-for-gestational age (SGA), PTB, and stillbirth (Kingdom, 1998; Gagnon, 2003; Wang and Zhao, 2010; Romero et al., 2011; Conroy et al., 2013; Morgan, 2016; Silver, 2018).

Placental vascular development begins early in pregnancy and undergoes adaptations across gestation. On the maternal side, uteroplacental circulation is established by the end of the first trimester (Wang and Zhao, 2010). Maternal vascular adaptation involves remodeling of the uterine spiral arteries by invasive fetal-derived extravillous trophoblasts to enable low-resistance blood flow into the intervillous space of the placenta (Wang and Zhao, 2010; Pollheimer et al., 2018). On the fetal side, primary placental villi begin to develop around day 13 post-conception, and fetoplacental vascularization of villi begins around 21 days post-conception (Kingdom et al., 2000; Kaufmann et al., 2004; Demir et al., 2006). The tertiary villi around which maternal blood flows in the intervillous space act as the functional units of the materno-fetal interface. Fetal-derived syncytiotrophoblasts are the primary mediators of exchange, protein-production, and defense at the materno-fetal interface.

Vascularization of placental villi involves the sequential processes of vasculogenesis and angiogenesis. Vasculogenesis is the *de novo* formation of blood vessels *via* differentiation of mesenchymal cells to hemangiogenic stem cells and then endothelial precursors (Demir et al., 2007). The VEGF family of ligands and receptors are heavily involved in the regulation of both vasculogenesis and angiogenesis. VEGF and its receptors (VEGFR-1 and -2) are expressed very early in placental development, and the production of VEGF by cytotrophoblasts and Hofbauer cells is thought to drive early placental vasculogenesis and subsequent angiogenesis (Kaufmann et al., 2004; Demir et al., 2006, 2007).

Angiogenesis begins at approximately 32 days post-conception (Kaufmann et al., 2004). From this point until term, the placental vascular network needed to support the rapidly growing fetus is built predominantly *via* branching and non-branching angiogenesis. The molecular mediation of angiogenesis requires tight temporal and spatial coordination and interaction between VEGF and angiopoietin protein family signaling (Ahmed and Perkins, 2000; Carmeliet, 2000; Yancopoulos et al., 2000; Geva et al., 2002; Charnock-Jones et al., 2004; Kaufmann et al., 2004; Benirschke et al., 2012). VEGF, placental growth factor (PlGF), and their inhibitor soluble fms-like tyrosine kinase-1 (sFlt-1) are produced by trophoblasts, and their balance is critical to healthy placental vascular development (Ahmed and Perkins, 2000; Kingdom et al., 2000; Charnock-Jones et al., 2004; Kaufmann et al., 2004). The timing and ratio of angiopoietin-1 (Ang-1) and its antagonist angiopoietin-2 (Ang-2) signaling through their receptor Tie2 is also essential for placental vascularization. Ang-1 promotes vascular maturation and stability, whereas Ang-2 allows for the destabilization and endothelial plasticity required for VEGF to drive angiogenesis and vascular remodeling (Carmeliet, 2000; Yancopoulos et al., 2000; Geva et al., 2002). These functions are reflected in the longitudinal dynamics of Ang-1 and -2 in healthy pregnancies: Ang-1 is initially low and increases across pregnancy as placental vasculature becomes more established, while Ang-2 decreases across pregnancy (Geva et al., 2002). Several groups have hypothesized that the tightly regulated longitudinal dynamics

Abbreviations: Ang-1 and -2, Angiopoietin-1 and -2; cART, Combined antiretroviral therapy; CMV, Cytomegalovirus; dNK, Decidual natural killer cells; IL, Interleukin; INF, Interferon; LBW, Low birth weight; NO, Nitric oxide; PlGF, Placental growth factor; PTB, Preterm birth; sFlt-1, Soluble fms-like tyrosine kinase-1; SGA, Small-for-gestational age; TNF, Tumor necrosis factor; VEGF, Vascular endothelial growth factor.

of VEGF and PlGF, as well as Ang-1 and -2, provide a molecular basis for the temporal transition from vasculogenesis to branching and then non-branching angiogenesis that underlies placental vascular development (**Figure 1**; Ahmed and Perkins, 2000; Geva et al., 2002; Kaufmann et al., 2004).

VEGF, PlGF, and VEGFR-1 are expressed on extravillous and villous trophoblasts, as well as Hofbauer cells in human placentas (Charnock-Jones et al., 1994; Ahmed et al., 1995; Clark et al., 1996; Khaliq et al., 1996; Vuorela et al., 1997). Their signaling has a role in trophoblast function including proliferation, differentiation, and nitric oxide (NO) production (Charnock-Jones et al., 1994; Ahmed et al., 1997; Athanassiades et al., 1998; Athanassiades and Lala, 1998; Khaliq et al., 1999). Ang-1 and -2 and their receptor Tie2 are also expressed in villous and

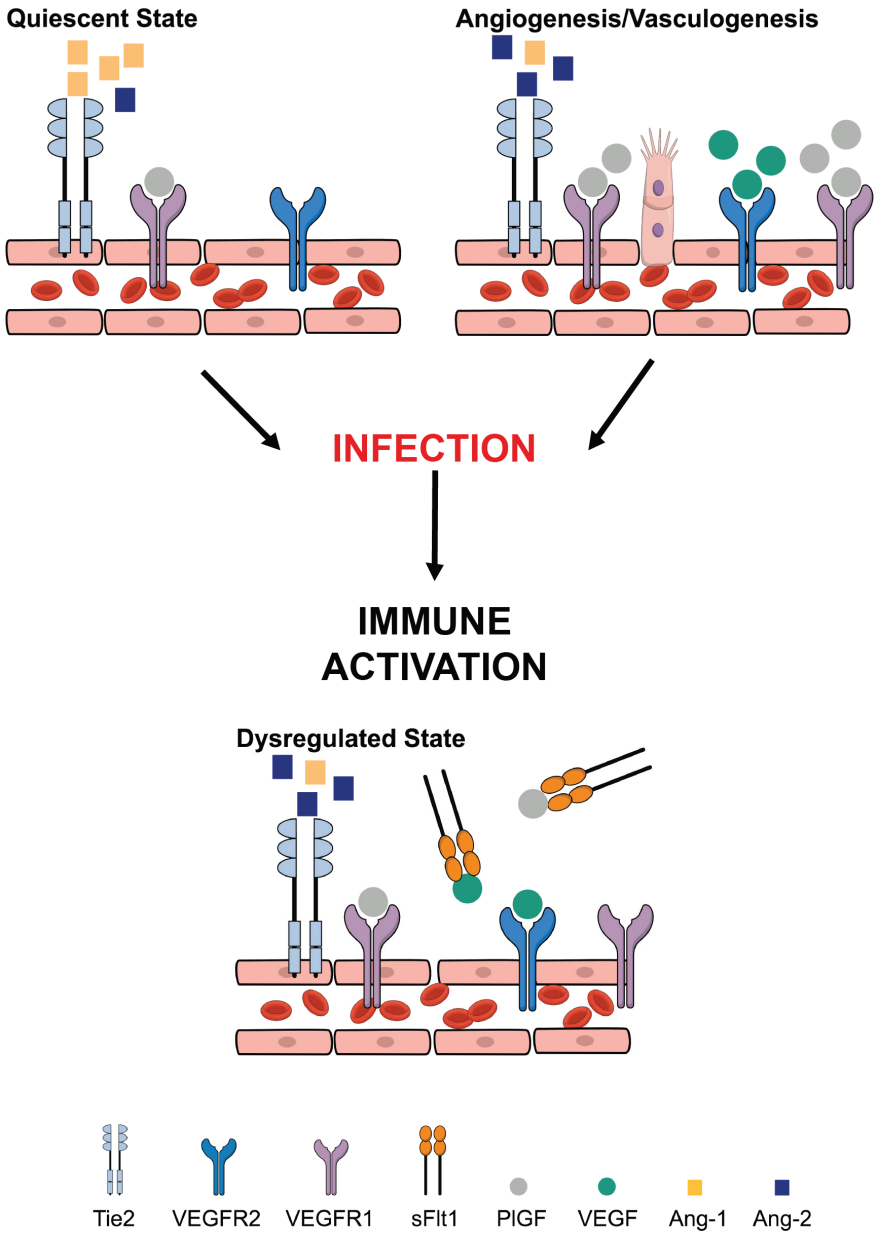

FIGURE 1 | Overview of key angiogenic and vasculogenic factors mediating placental function and how they may be disrupted in the context of maternal infection. Placental vasculogenesis and angiogenesis are processes that are vital for placental vascular development and function. These processes depend on a fine balance between pro-angiogenic and anti-angiogenic pathways. The vascular endothelial growth factor (VEGF) family of proteins (including PlGF – placental growth factor) are pro-angiogenic mediators. VEGF and PlGF bind VEGF receptor 1 [fms-like tyrosine kinase (Flt-1)] to induce vessel proliferation and sprouting. Alternative splicing of Flt-1 results in soluble Flt-1 (sFlt-1) that is anti-angiogenic. Angiopoietin-1 (Ang-1) binds its tyrosine-kinase receptor Tie2 inducing vessel maturation, whereas angiopoietin-2 (Ang-2) promotes vessel destabilization and angiogenesis. Tight control of these pathways is essential for proper vascular development, remodeling, robust placental function, and healthy birth outcomes. Maternal infection (e.g., malaria, HIV-1) can result in immune activation and inflammation which dysregulates these tightly regulated processes, contributing to poor birth outcomes.

extravillous trophoblasts in specific cell-type and temporal patterns across pregnancy, and *in vitro* studies reported a role for Ang/Tie2 signaling in trophoblast NO production and migration (Dunk et al., 2000; Goldman-Wohl et al., 2000; Seval et al., 2008).

Collectively, these data support critical roles for the VEGF and angiopoietin pathways in both fetoplacental vascularization (i.e., vasculogenesis and angiogenesis in the villi) and trophoblast function, as well as uteroplacental remodeling (i.e. trophoblast-mediated maternal spiral artery remodeling). With such diverse and interdependent roles for angiogenic factors across placental development, it is not surprising that their dysregulation has been associated with pathologic pregnancies and adverse birth outcomes.

PLACENTAL DEVELOPMENT: A ROLE FOR INFLAMMATORY MEDIATORS

In a healthy pregnancy, the maternal immune system adapts to protect the semi-allogeneic fetus and placenta. Circulating levels of both cytokines [e.g., interferon (IFN)-γ, tumor necrosis factor (TNF), etc.] and components of the complement system (e.g., C3a, C5a, etc.) are altered across normal pregnancy (Kraus et al., 2010; Regal et al., 2015). Several cell types in the placenta including maternal and fetal-derived placental cells, as well as specialized immune cells like decidual natural killer cells (dNK), produce, express and/or secrete inflammatory cytokines and complement regulatory proteins at the materno-fetal interface in a healthy pregnancy (Bowen et al., 2002; Weckman et al., 2018). These inflammatory mediators play a dual role in immunity and processes of normal placental development including extravillous trophoblast proliferation and invasion necessary for uterine spiral artery remodeling (Albieri et al., 1999; Bowen et al., 2002; Hanna et al., 2006; Bulla et al., 2008; Pollheimer et al., 2018). Furthermore, dNKs play an important role in placental vascular development *via* the production of angiogenic factors including VEGF, PlGF, Ang-1, and Ang-2 (Hanna et al., 2006; Le Bouteiller, 2013). Trophoblasts also increase VEGF production in response to cytokine stimulation, and monocytes will increase production of sFlt-1 in response to complement activation (Choi et al., 2002; Girardi et al., 2006; Conroy et al., 2009). Inflammatory and angiogenic systems are interdependent and tightly regulated across pregnancy. Together, their regulation is critical to placental vascular development. Therefore, disruption of either system could lead to a cascade of downstream events with negative impacts on placental vascular development and birth outcomes.

ABNORMAL PLACENTAL VASCULAR DEVELOPMENT UNDERLIES PREGNANCY COMPLICATIONS

There is abundant evidence for defective maternal spiral artery remodeling (Khong et al., 1986; Hustin et al., 1990; Pijnenborg et al., 1991; Kingdom et al., 2000; Romero et al., 2011; Fisher, 2015; Burton and Jauniaux, 2018; Pollheimer et al., 2018), abnormal villous development and vascularization (Jackson et al., 1995; Kingdom and Kaufmann, 1997; Kingdom et al., 2000; Vedmedovska et al., 2011; Burton and Jauniaux, 2018; Silver, 2018; Travaglino et al., 2019), and impaired umbilical blood flow (Trudinger et al., 1985; Salafia et al., 1997; Ferrazzi et al., 2000; Zhu et al., 2016) in a range of pregnancy outcomes with placental pathologies including preeclampsia, SGA, PTB, spontaneous abortion, and stillbirth.

Dysregulation of specific angiogenic mediators has also been associated with placental insufficiency and adverse pregnancy outcomes. The balance between angiogenic and anti-angiogenic factors, and the resulting alterations to placental vasculature, is modulated by multiple factors including oxygen homeostasis, external agents (e.g., drugs), infection, and inflammation (Khaliq et al., 1999; Kaufmann et al., 2004; Girardi et al., 2006; Conroy et al., 2013; McDonald et al., 2018; Mohammadi et al., 2018). Seminal preclinical studies established the importance of a precise angiogenic balance *in utero*, as both an absence and excess of VEGF and the angiopoietins were associated with abnormal embryonic development or lethality (Carmeliet et al., 1996; Suri et al., 1996; Maisonpierre et al., 1997; Miquerol et al., 2000). These findings have been extended to human studies, where multiple adverse pregnancy outcomes have been linked to dysregulation of circulating levels of angiogenic mediators critical for normal placental development including VEGF, PlGF, and sFlt-1, Ang-1 and -2, and soluble endoglin (Sharkey et al., 1996; Levine et al., 2004, 2006; Venkatesha et al., 2006; Chaiworapongsa et al., 2009; Romero et al., 2010; Conroy et al., 2013, 2017; Darling et al., 2014; McDonald et al., 2015b, 2018; Straughen et al., 2017).

As discussed above, there is a tight interrelationship between inflammatory pathways and angiogenic balance during placental development. Consequently, adverse pregnancy outcomes including spontaneous PTB and SGA have been associated with simultaneous dysregulation of both pathways (Girardi et al., 2006; Conroy et al., 2013; Darling et al., 2014; McDonald et al., 2015b). These data strongly support a role for disrupted placental development, due to inflammatory and angiogenic dysregulation at the materno-fetal interface, as a common pathway in the pathobiology of adverse birth outcomes.

MATERNAL INFECTION DISRUPTS PLACENTAL VASCULAR DEVELOPMENT

An important contributor to disruption of the inflammatory and angiogenic environment at the materno-fetal interface is maternal infection. Infections due to the TORCH pathogens [*Toxoplasma gondii*, others, rubella virus, cytomegalovirus (CMV), and herpes simplex virus] may result in adverse pregnancy outcomes *via* vertical transmission to the fetus (Coyne and Lazear, 2016). However, even in the absence of congenital infection, maternal infections such as malaria and

HIV have been linked with adverse birth outcomes including PTB, LBW, SGA, and stillbirth (Desai et al., 2007; Wedi et al., 2016; Rogerson et al., 2018). Despite increased coverage of treatment for infections in pregnancy such as malaria and HIV, rates of adverse birth outcomes remain high (Madanitsa et al., 2016; Santosa et al., 2019), and a better understanding of the pathophysiology underlying infection-induced adverse birth outcomes is needed. Increasing evidence suggests placental vascular pathology may be an important contributor to the link between maternal infection and adverse birth outcomes.

A growing body of evidence has linked maternal infection with abnormal placental pathology and altered maternal and fetal hemodynamics. Histopathological examination of human placentas have reported abnormal placental villous architecture and maternal vascular malperfusion in the context of viral, bacterial and parasitic maternal infections (Carmona-Fonseca et al., 2013; Ahmed et al., 2014; Chaikitgosiyakul et al., 2014; Kim et al., 2015; Kalk et al., 2017; Ribeiro et al., 2017; Moeller et al., 2018). Preclinical studies have also demonstrated significantly altered placental vascularization, vascular remodeling, and oxygen transport in response to maternal infection (Tabata et al., 2012; Conroy et al., 2013; Hirsch et al., 2018; McDonald et al., 2018; Phillips et al., 2018). In humans, maternal infections including influenza, *Helicobacter pylori*, malaria, and HIV have been associated with impaired maternal and fetal hemodynamics (e.g., high arterial resistance) (Dorman et al., 2002; Griffin et al., 2012; Hernandez-Andrade et al., 2014; McClure et al., 2014; Di Simone et al., 2017; Ome-Kaius et al., 2017). The available evidence implicates abnormalities in placental vascularization and function as a common driver behind infection-induced adverse birth outcomes, even in non-congenital infections.

Mechanistically, dysregulation of angiogenic mediators including sFlt-1, Ang-1, and -2, soluble endoglin, and PlGF and altered placental vascular structure and function has been reported in the context of maternal infections (e.g., malaria, HIV, CMV, and acute pyelonephritis) associated with adverse birth outcomes (Chaiworapongsa et al., 2010; Silver et al., 2010, 2011; Conroy et al., 2013, 2017; Ataíde et al., 2015; Gustafsson et al., 2015; McDonald et al., 2018). Inflammatory mediators including cytokines [e.g., interleukin (IL)-1, INF-γ, and TNF] and the complement system exhibit regulatory cross-talk with angiogenic factors critical to placental development (Naldini and Carraro, 2005; Fiedler and Augustin, 2006; Girardi et al., 2006; Conroy et al., 2009, 2013). There is evidence for systemic inflammation in bacterial, viral, and parasitic maternal infections (Horton et al., 2008; Conroy et al., 2013; Cérbulo-Vázquez et al., 2014; Romero et al., 2016; Fried et al., 2017; Wilkinson et al., 2017; Harjunmaa et al., 2018; McDonald et al., 2019), and statistical modeling suggests a hierarchical relationship between dysregulated inflammation, angiogenesis, and adverse birth outcomes (Conroy et al., 2013). Collectively, these studies suggest inflammation-mediated dysregulation of the tight angiogenic balance required for placental development as a shared mechanism underlying adverse birth outcomes in maternal infection (**Figure 2**).

FETAL DEVELOPMENT IS IMPACTED BY DYSREGULATION OF THE INFLAMMATORY-ANGIOGENIC AXIS IN MATERNAL INFECTION

Many pathways critical to placental vascular development are mirrored in fetal vascular development. Normal vasculogenesis, angiogenesis, and materno-fetal hemodynamics are critical to organ growth *in utero*. Mounting evidence supports the hypothesis that disruption of these processes *via* maternal immune activation may cause defects in development of the fetal lungs, heart, and brain, with long-term consequences for offspring (Bilbo and Schwarz, 2009; McAdams et al., 2012; Burton and Jauniaux, 2018). The nature of the disruption to fetal development may depend on timing of infection. For example, studies suggest disruption to placental development and hemodynamics, especially in the first month of pregnancy, could be reflected in fetal cardiac abnormalities and congenital heart disease (Linask, 2013; Linask et al., 2014; Burton and Jauniaux, 2018). Dysregulation of inflammatory and angiogenic factors (i.e., *via* maternal infection) during a critical period of lung development is associated with bronchopulmonary dysplasia and increased neonatal mortality in the first 28 days of life (Thébaud and Abman, 2007; McAdams et al., 2012; Procianoy et al., 2015). Furthermore, a large and rapidly growing body of evidence has linked maternal immune activation and dysregulation of angiogenesis with impaired fetal neurodevelopment and long-lasting neurocognitive and neuropsychiatric sequelae for offspring (Knuesel et al., 2014; Estes and McAllister, 2016; Brown and Meyer, 2018).

Neurodevelopment is an intricate and strictly orchestrated process. Proper molecular signaling during critical gestational and postnatal periods is required for the establishment of effective neural networks (Stiles and Jernigan, 2010). Immune cells and cytokines such as microglia, complement, and IL-6 play an integral role in mediating these signals, and are tightly regulated at the materno-fetal interface (Billiards et al., 2006; Smith et al., 2007; Bilbo and Schwarz, 2009; Gallagher et al., 2013; McDonald et al., 2013; Graham et al., 2018; Prins et al., 2018; Rudolph et al., 2018; Spann et al., 2018; Rasmussen et al., 2019). In response to bacterial, viral, and parasitic infections, the maternal immune system is activated, exposing the fetus to cytokines and immune cells that are capable of passing through the immature fetal blood brain barrier and potentially impacting fetal neurodevelopment (Dammann and Leviton, 1997; Prins et al., 2018).

Evidence of the association between maternal immune activation and neuropsychiatric disorders, including schizophrenia (SZ), autism spectrum disorder (ASD) and bipolar disorder (BD), has accumulated in both epidemiological studies and preclinical models (Knuesel et al., 2014; Estes

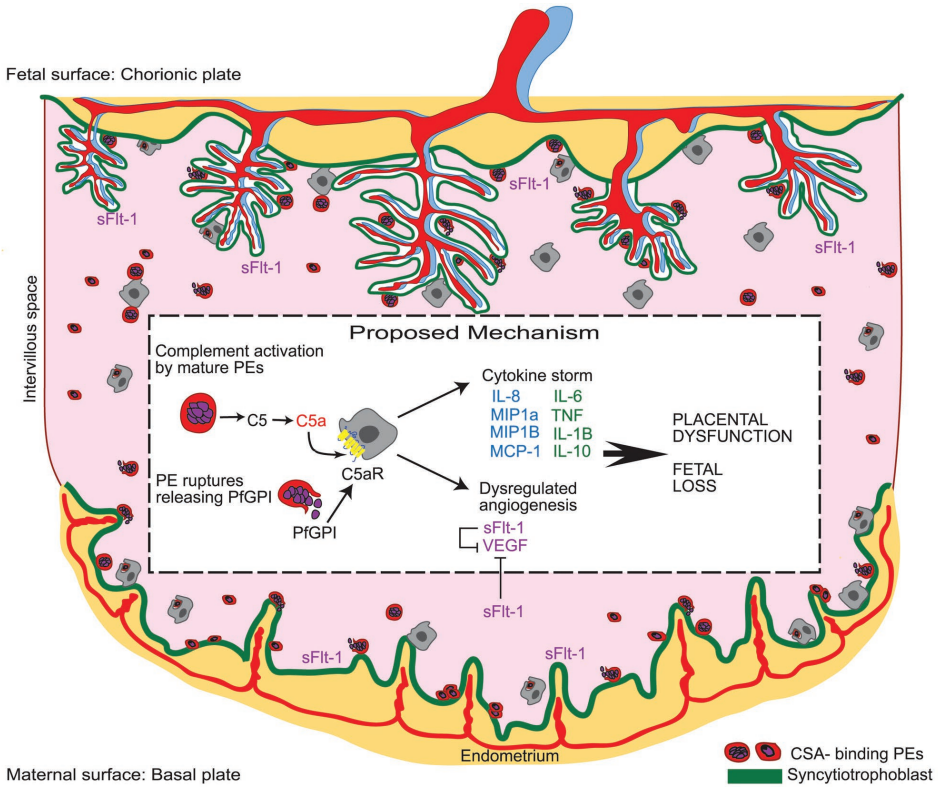

FIGURE 2 | Malaria in pregnancy as a model for infection-driven dysregulation of placental vascular development as a common mechanism underlying adverse birth outcomes. During malaria in pregnancy, parasitized erythrocytes (PE) activate the maternal complement cascade. Complement component C5a acts synergistically with circulating parasite-associated bioactive molecules (i.e., *Plasmodium falciparum* glycosylphosphatidylinositol anchor molecules; *Pf*GPI) to trigger an inflammatory response at the materno-fetal interface. This inflammatory host response dysregulates the tight balance of angiogenic factors required for placental vascular development leading to aberrant placental vascularization, placental dysfunction, and adverse birth outcomes. Existing evidence for other infections during pregnancy supports this model as a common mechanism underlying adverse birth outcomes in maternal infection [reproduced from Conroy et al., 2009].

and McAllister, 2016; Brown and Meyer, 2018). Maternal immune activation *via* individual cytokines (e.g., IL-6 and IL-2), viral/bacterial mimics [e.g., polyinosinic:polycytidylic acid, poly(I:C); lipopolysaccharides, LPS], and actual infections (e.g., malaria, influenza, urinary tract infections) have been associated with long-term behavioral consequences for exposed offspring (Cai et al., 2000; Ponzio et al., 2007; Smith et al., 2007; Giovanoli et al., 2013; Knuesel et al., 2014; McDonald et al., 2015a; Choi et al., 2016; Brown and Meyer, 2018). Altered expression of proinflammatory mediators with dual roles in inflammation and neurodevelopment have been implicated in this link. Proinflammatory cytokine IL-6, which induced psychiatric behavioral outcomes in a seminal preclinical study of maternal immune activation (Smith et al., 2007), also has roles in neurogenesis, synapse formation, white matter development, and dendritic spine architecture (Wei et al., 2012; Gallagher et al., 2013; Rasmussen et al., 2019). Complement components including C5a, C3, and C1q also have well-characterized dual roles in maternal response to infection and neurodevelopment (i.e., synaptic pruning), and dysregulation of complement has been implicated in maternal infection-associated neurocognitive deficits in offspring (Stevens et al., 2007; Schafer et al., 2012; McDonald et al., 2015a; Weckman et al., 2018).

Furthermore, evidence points toward a direct connection and cross-talk between pathways critical to angiogenesis and neurodevelopment; proteins including VEGF family members have important roles in both processes (Carmeliet, 2003). Interestingly, VEGF participates in signaling required for the intricate growth and patterning of nerves and blood vessels alongside one another in the developing brain (Carmeliet, 2003). This indicates that disruption of the angiogenic environment (possibly downstream of maternal infection and inflammation) at the materno-fetal interface could also impact neurocognitive development with potential long-term neurological consequences for the offspring. In support of this contention, micro-CT images of malaria-exposed murine offspring showed alterations to fetal neurovasculature that was C5a-C5aR signaling-dependent (McDonald et al., 2015a). Since C5a activation seems to be upstream of angiogenic dysregulation in malaria in pregnancy (**Figure 2**; Conroy et al., 2009, 2013), these preclinical data support a model whereby maternal host response to infection in pregnancy could induce disruptions to fetal neurodevelopment *via* both angiogenic and inflammatory mechanisms.

ABNORMAL PLACENTAL DEVELOPMENT AS A DRIVER OF INFECTION-INDUCED ADVERSE BIRTH OUTCOMES HAS IMPLICATIONS FOR TREATMENT STRATEGIES

An increased understanding of the impact of maternal infections on placental vascular development has implications for new intervention strategies to reduce adverse birth outcomes. For example in maternal HIV infection, despite antiretroviral treatment women exhibited dysregulation of angiogenic factors (i.e., increased circulating soluble endoglin and decreased PlGF concentrations, resulting in an anti-angiogenic state) that were associated with PTB, SGA and stillbirth (Conroy et al., 2017). Women receiving antiretrovirals and cotrimoxazole that were co-infected with HIV and malaria exhibited systemic inflammation (i.e. increased soluble TNF receptor-2) that was also associated with PTB (McDonald et al., 2019). Persistent dysregulation of inflammatory and angiogenic pathways critical to placental vascular development could explain why rates of adverse birth outcomes remain high even in the face of appropriate antimicrobial therapy and indicate that alternative strategies, including modifying host response pathways, may be necessary to reduce poor birth outcomes.

Considering the early stage at which placental vasculature is established, the timing of infection during pregnancy has important implications for prevention/treatment strategies. Increasing evidence suggests that early infection with malaria in pregnancy increases the risk of adverse birth outcomes including SGA *via* disruption to placental development (Griffin et al., 2012; Moeller et al., 2018). Since current malaria drug-based prevention strategies are not implemented until the second trimester, this could represent an important gap in the prevention of infection-induced dysregulation of placental development and resulting adverse birth outcomes (Huynh et al., 2015). The use of combined antiretroviral therapy (cART) in pregnancy has also been associated with dysregulated angiogenesis, compromised placental vascular development, and adverse birth outcomes, especially when initiated early in pregnancy (Mohammadi et al., 2018). In pre-clinical studies, supplementing cART-treated mice with progesterone prevented placental abnormalities, indicating that this addition to current treatment strategies might improve pregnancy outcomes by targeting dysregulated placental vascular development. In support of this contention, a study in a large cohort of women in Papua New Guinea demonstrated the ability of sulphadoxine-pyrimethamine and azithromycin to improve adverse birth outcomes by regulating inflammatory (e.g., C-reactive protein) and angiogenic factors (e.g., soluble endoglin, sFlt-1) critical to placental vascular development (Unger et al., 2019).

CONCLUSIONS

Despite Millennium and Sustainable Development Goals to improve maternal-child health, the global burden of adverse birth outcomes remains high. This is in part due to a critical knowledge gap in our understanding of the mechanisms underlying adverse birth outcomes, and consequently limited or ineffective strategies. Several lines of evidence suggest that infection-driven dysregulation of inflammation and angiogenesis at the materno-fetal interface is a common mechanism underlying inadequate placental and fetal development, and adverse birth outcomes. Placental vascular development and vascular adaptation requires tight temporal and spatial regulation of cytokines, the complement system, and angiogenic factors including the VEGF and angiopoietin families. Dysregulation of those systems in the context of maternal infection leads to aberrations in spiral artery remodeling, placental vascularization and villous architecture, and deleterious materno-fetal hemodynamics that are associated with adverse birth outcomes. Current interventions for infections in pregnancy including malaria and HIV target only the pathogen and not the host response that may drive poor birth outcomes. This may explain, at least in part, why rates of adverse birth outcomes remain high. Further, dysregulation of inflammatory and angiogenic factors at the materno-fetal interface can lead to impairments in fetal neurodevelopment including neurogenesis and neurovascular development, with long term cognitive and behavioral sequelae for offspring. Considering the importance of the relationship between infection-induced dysregulation and adverse birth outcomes, future research should focus on therapeutics that target early placental development as a strategy to reduce the global burden of adverse birth outcomes.

AUTHOR CONTRIBUTIONS

CM and KK managed project conception and oversight. AW, MN, JW, and CM contributed to research, analysis, and writing of the manuscript. All authors contributed to manuscript revisions and approved the final manuscript.

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Sequelae of Fetal Infection in a Non-human Primate Model of Listeriosis

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Listeria monocytogenes (Lm) is a common environmental bacterium that thrives on vegetation and soil matter, but can infect humans if contaminated food products are ingested, resulting in severe disease in immunosuppressed populations, including pregnant women and newborns. To better understand how the unique immunological milieu of pregnancy increases susceptibility to infection, we study listeriosis in cynomolgus macaques, a non-human primate that closely resembles humans in placentation and in the physiology, and immunology of pregnancy. Non-human primates are naturally susceptible to Lm infection, and spontaneous abortions due to listeriosis are known to occur in outdoor macaque colonies, making them ideal models to understand the disease pathogenesis and host-pathogen relationship of listeriosis. We have previously shown that Lm infection in the first trimester has a high rate of miscarriage. This study expands on our previous findings by assessing how the quantity of Lm as well as stage of pregnancy at the time of exposure may influence disease susceptibility. In the current study we inoculated a cohort of macaques with a lower dose of Lm than our previous study and although this did not result in fetal demise, there was evidence of *in utero* inflammation and fetal distress. Animals that were reinfected with an equivalent or higher dose of the same strain of Lm resulted in approximately half of cases continuing to term and half ending in fetal demise. These cases had inconsistent bacterial colonization of the fetal compartment, suggesting that Lm does not need to directly infect the placenta to cause adverse pregnancy outcomes. Timed surgical collection of tissues following inoculation demonstrated that transmission from mother to fetus can occur as soon as 5 days post-inoculation. Lastly, third trimester inoculation resulted in pregnancy loss in 3 out of 4 macaques, accompanied by characteristic pathology and Lm colonization. Collectively, our studies demonstrate that common laboratory culture tests may not always recover Lm despite known maternal ingestion. Notably, we also find it is possible for maternal infection to resolve in some cases with no discernible adverse outcome; however, such cases had evidence of a sterile intrauterine inflammatory response, with unknown consequences for fetal development.

Keywords: listeriosis, pregnancy, histopathology, cytokines, non-human primate, fetal infection

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INTRODUCTION

Listeriosis remains a public health concern for pregnant individuals, despite efforts in surveillance, reporting, public awareness campaigns, and implementation of safety measures by industry and agriculture (Tappero et al., 1995; Pohl et al., 2019). A foodborne disease caused by the bacterium *Listeria monocytogenes* (Lm), listeriosis can result in miscarriage, stillbirth, preterm birth, or neonatal infection (Craig et al., 2019). Lm is able to survive in a wide range of environments, including at refrigeration temperature. Common sources of infection include unpasteurized dairy products and ready-to-eat foods, although less common items such as caramel-coated apples have been responsible for human outbreaks (Glass et al., 2015). It can lead to a particularly insidious disease because symptoms may be mild, mistaken for the flu, or masked by morning sickness, resulting in delayed diagnosis and treatment. The United States' Centers for Disease Control and Prevention (CDC) reports that 1 in 6 cases of listeriosis in the U.S. is associated with pregnancy (Centers for Disease Control and Prevention, 2019) and that nearly one quarter of pregnancy-associated cases result in fetal loss or death of the newborn (Centers for Disease Control and Prevention, 2016). Most data concerning the course and nature of human infection come from retrospective clinical cases, identified based on symptoms, positive bacterial cultures, or adverse pregnancy outcomes. The first prospective clinical study, the Multicentric Observational NAtional Study on LISteriosis and ListeriA (MONALISA), found that the disease burden of listeriosis is higher than previously estimated: particularly, that more than 80% of infected mothers experienced major fetal or neonatal complications, and that the rate of fetal loss was significantly greater at <29 weeks of gestation (Charlier et al., 2017). Underreporting of pregnancy-associated listeriosis is very likely due to undiagnosed asymptomatic illness, unreported/unrecognized early miscarriage, misdiagnosed stillbirths, and difficulty in positively identifying Lm by standard laboratory culture, and Gram stain (Kylat et al., 2016).

Our current study addresses these limitations using a macaque model of infection, which allows for experimental manipulation, including a predetermined dose and timing of exposure, in a system highly relevant to the unique structure and physiology of human pregnancy (Lamond and Freitag, 2018; Lowe et al., 2018). Previously we found that dams given an inoculum of 10^7 CFUs Lm in the first trimester of pregnancy resulted in rapid infection and fetal demise, accompanied by diffuse bacterial colonization of the maternal-fetal interface (Wolfe et al., 2017). The objectives of the current study were to determine how a milder exposure changes the disease trajectory and outcome, and if increasing gestational age or previous infection influences susceptibility. We gave dams a single dose of 10^6 CFUs Lm in the first trimester. Exposure to $<10^7$ CFUs Lm in a single dose did not result in acute pregnancy loss. In subsequent pregnancies, we gave dams an equivalent or higher dose of Lm than previously administered. We find that re-exposure following a previous infection resulted in a lower rate of bacterial colonization, but could still induce adverse pregnancy outcomes associated with a sterile inflammatory response in the placenta and fetus. Because the maternal-fetal interface is not in a static state as pregnancy progresses, we next assessed third trimester infection to determine if increasing gestational age may enhance or mitigate Lm virulence. Our results demonstrate that infection with 10^7 CFUs Lm in late gestation results in a lower bacterial burden and slightly higher rate of fetal survival compared to the same dose in early gestation (33 vs. 20%, respectively), but does not mitigate against adverse pregnancy outcomes, which remain severe and can occur in as few as 4 days following exposure.

Abbreviations: CFUs, colony forming units; GD, gestational day; Lm, *Listeria monocytogenes;* MFI, maternal-fetal interface; PCA, principle component analysis; WNPRC, Wisconsin National Primate Research Center.

MATERIALS AND METHODS

Ethics Statement

The cynomolgus macaques (*Macaca fascicularis*) in this study were cared for by the staff at the Wisconsin National Primate Research Center (WNPRC) according to the regulations and guidelines of the University of Wisconsin Institutional Animal Care and Use Committee which approved this study, and adheres to principles described in the National Research Council's Guide for the Care and Use of Laboratory Animals.

Animals and Breeding

Adult female cynomolgus monkeys maintained at the WNPRC were cohoused with compatible males and observed daily for menses and copulation. Pregnancy was detected by ultrasound examination of the uterus approximately 18 to 20 days following the predicted day of ovulation. The day of gestation when pregnancy was detected was estimated based on previous experience and published data describing cynomolgus macaque fetal size during gestation (Tarantal and Hendrickx, 1988). Ultrasound examination of the uterus was done weekly or biweekly to monitor placental and fetal growth until the day of Lm inoculation, and every 2–3 days or daily after inoculation to assess fetal vital signs.

Inoculation With *L. monocytogenes*

At varied days of gestation in the first trimester (between days 36 to 50) or third trimester (between days 110 to 135 [full term is day 165]), monkeys were sedated, the uterus was examined by ultrasound to confirm a viable pregnancy, and a single dose between 10^6 and 10^8 colony forming units (CFUs) log-phase cells of strain LM2203 (serotype 4b, derived from an outbreak of listeriosis among pregnant women in Winston-Salem, North Carolina in the year 2000 MacDonald et al., 2005), were administered in 10 ml of whipping cream via a soft intragastric feeding tube as previously described (Smith et al., 2003). For each inoculation, Lm was cultured at 37°C in Tryptic Soy Broth (Becton Dickinson, Sparks, MD). 500 ul of the inoculum was diluted in phosphate-buffered saline (PBS; Catalog #P5368, Sigma-Aldrich, St. Louis, MO), plated on Trypticase soy agar with 5% sheep blood (Becton Dickinson, Sparks, MD), and

incubated at 37°C to confirm the dose given to each animal as CFUs of Lm per milliliter. Six monkeys were given a whipping cream inoculum without Lm in an identical manner to be included as uninfected controls.

Fecal Shedding

Before and after inoculation, fecal samples were collected from cage pans to monitor fecal shedding of Lm. Schedules of sample collection are described in the legend to **Figure 1**. Serial dilutions

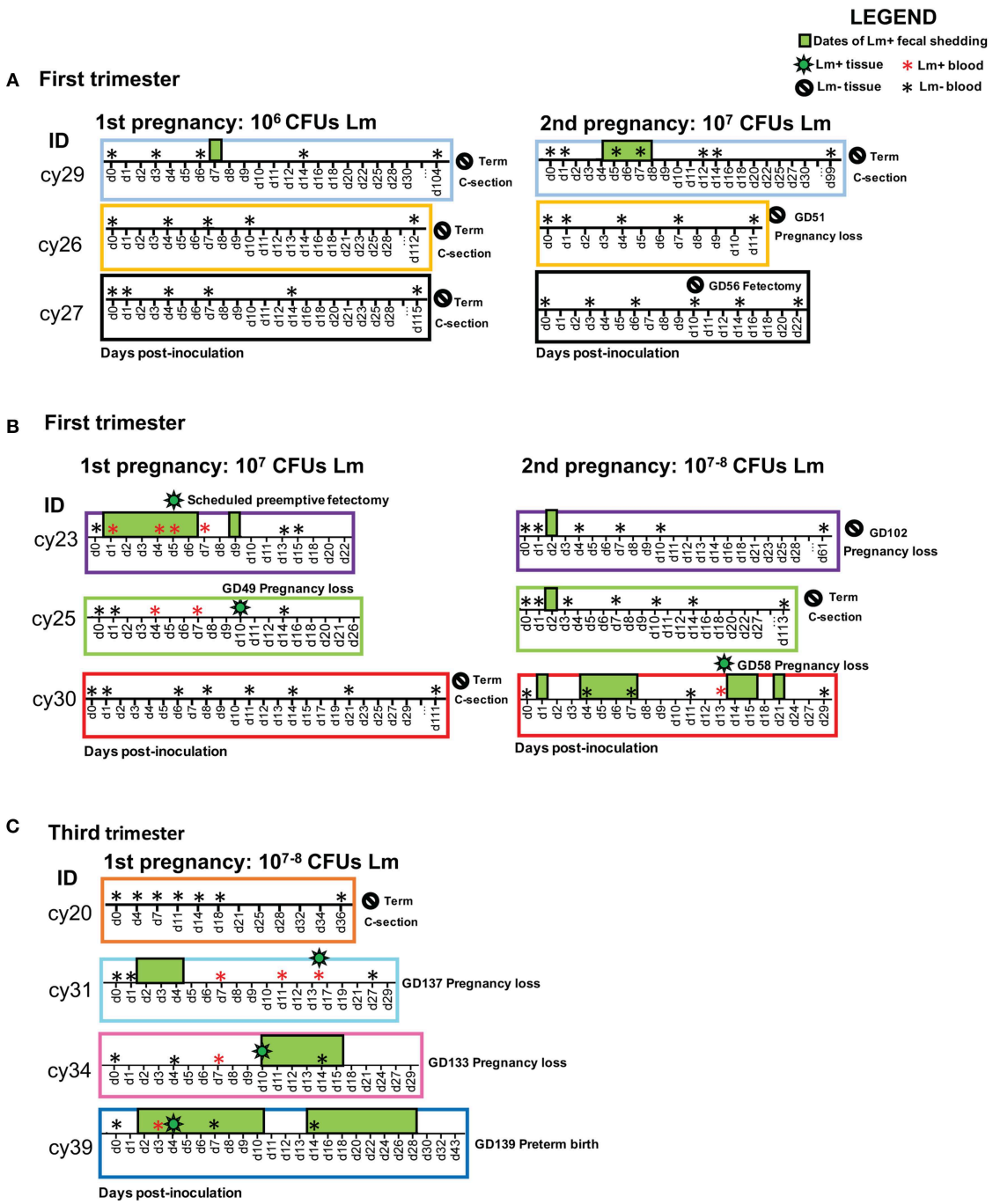

FIGURE 1 | Experimental timelines for each cohort showing animal ID, sample collections, and pregnancy outcome. The x-axis displays post-inoculation days. The following symbols indicate events on the timeline: positive blood culture (red asterisk), negative blood culture (black asterisk), positive tissue culture (green bacteria), negative tissue culture (negative sign), and positive fecal culture (green box). Fecal samples were collected on each date shown on the timeline; dates without a green box denote negative fecal cultures, and absent dates indicate that no samples were collected. Overlapping symbols indicate concurrent events. **(A)** First trimester cohort: 10^6 CFUs Lm initial dose; 10^7 CFUs Lm reexposure. **(B)** First trimester cohort: 10^7 CFUs Lm initial dose; 10^7 CFUs Lm reexposure. **(C)** Third trimester cohort: 10^7–10^8 CFUs Lm dose.

in PBS of fecal samples were plated in duplicate on modified Oxford agar plates (Kang and Fun, 1999) and the number of colonies was determined using ImageJ colony-counting software (https://imagej.nih.gov/ij/plugins/colony-counter.html) after 24 to 48 h of incubation at 37°C.

Bacteremia Monitoring

Peripheral blood samples were collected periodically for aerobic and anaerobic cultures to detect bacteremia as previously described (Lancaster, 2015) and processed on a BD Bactec 9,050 blood culture system (Becton Dickinson Diagnostic Systems, Sparks, MD) in the Clinical Pathology Laboratory at the University of Wisconsin—Madison School of Veterinary Medicine. Bactec Peds Plus/F blood culture bottles aseptically inoculated with 3 ml sample per bottle were incubated at 35°C until a positive signal was observed or for a maximum of 5 days. Bottles that did not signal positive at the end of 5 days were Gram-stained and sub-cultured to two chocolate agar plates (Remel, Lenexa, KS). Subcultures were incubated for 24–48 h aerobically at 35°C in 5% CO2 and anaerobically in an anaerobic chamber (SHEL LAB, Cornelius, OR) at 35°C in 80% N2, 5% H2, and 5% CO2 for verification of true or false negativity. Positive blood cultures were Gram-stained and subcultured to a blood agar plate supplemented with 5% sheep blood, chocolate agar, eosin methylene blue agar, and Columbia nalidixic acid agar (Remel, Lenexa, KS), and incubated at 35°C for 24–28 h in 5% CO2. An anaerobic subculture was also performed and incubated in an anaerobic chamber for 24–48 h as indicated above. Recovered isolates were identified by matrix-assisted laser desorption-ionization time-of-flight (MALDI-TOF) mass spectrometry (Bruker Daltonics, Billerica, MA). Sample extraction and strain identification was performed following the manufacturer's instruction. A score of >2 indicated secure genus and probable species identification.

Surgery and Tissue Processing

Ultrasound examination of the uterus was done 1 to 3 times per week after Lm dosing to monitor fetal well-being and confirm fetal heartbeat and umbilical blood flow. When fetal demise was indicated by absence of heartbeat, fetal and maternal tissues were promptly collected at laparotomy. The entire conceptus (decidua, placenta, fetal membranes, umbilical cord, amniotic fluid, and fetus) was removed by uterotomy. These were survival surgeries for the dams. The fetus was dissected into ∼4-mm coronal segments, and alternating segments were fixed and embedded for histology (see below), or homogenized for bacteriological analysis on blood agar plates as previously described (Poulsen et al., 2013).

Histology and Histopathological Scoring

Dissected tissues were fixed in 2 to 4% paraformaldehyde for 24 to 72 h, rinsed in PBS, and stored in 70% ethanol until processed and embedded in paraffin. Paraffin sections were stained with hematoxylin and eosin (H&E) and Gram-stained using standard methods. Lm was confirmed by chromogenic immunohistochemistry with *Listeria* O antiserum (Difco Laboratories' rabbit polyclonal antibody against the somatic O antigen of *Listeria* serogroup 4). Stained sections were then examined and scored by WNPRC veterinary pathologists. Gram-stained slides were digitally scanned by the Wisconsin State Lab of Hygiene and analyzed using Aperio eSlide Manager with ImageScope 12.4 software (Leica Biosystems Inc. Buffalo Grove, IL). Scatterplot graphs of histological scores were prepared using GraphPad Prism version 8.0 (GraphPad Software, La Jolla, CA).

Cytokine/Chemokine Expression

Homogenized tissue samples were analyzed for cytokine expression by Luminex multiplex assay (Luminex Corporation, Austin, TX) with an NHP-validated kit containing CCL5/Regulated on Activation, Normal T cell Expressed and Secreted (RANTES), interleukin-1 beta (IL1b), IL-1 receptor antagonist (IL1ra), IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-13, IL-15, IL-16, IL-17 alpha, IL-18, IL-21, IL-23, IL-33, fibroblast growth factor 2 (FGF-2), granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage-colony stimulating factor (GM-CSF), granzyme A, interferon gamma (IFN-g), monocyte chemoattractant protein (MCP)-1, monocyte inflammatory protein (MIP)-1alpha, MIP-1beta, perforin, soluble CD40 Ligand (sCD40L), soluble FAS ligand (sFASL), tumor necrosis factor alpha (TNFa), and vascular endothelial growth factor (VEGF) (Milliplex Non-Human Primate Cytokine and Chemokine Panel, Millipore, Billerica, MA). Cytokine and chemokine concentrations were quantified using the Bioplex 200 system (Biorad, Hercules, CA). Concentrations were normalized to the protein levels in each homogenate using a Thermo Scientific Pierce Micro BCA Protein Assay Kit (Catolog #23235, Thermo Fisher Scientific, Waltham, MA). Values were log-transformed and hierarchical clustering of principle components was performed using ClustVis (Metsalu and Vilo, 2015).

RESULTS

Pregnancy Outcomes With 10^6 Lm

All three dams continued to term with no evident fetal infection and no evident colonization of the maternal reproductive tract (**Table 1**; **Figure 1A**). There was sporadic maternal fecal shedding, but none of the dams showed signs of illness such as inappetence, fever, or bacteremia. Tissues collected at cesarean section appeared grossly normal in all cases. Microscopically, however, all cases had histologic signs of inflammation, including decidual fibrinoid necrosis, neutrophilic villitis with villous necrosis, neutrophilic chorioamnionitis, fetal hepatitis, and increased numbers of intra-alveolar squamous cells and cellular debris consistent with fetal distress *in utero* (**Figures 2A,B**). The placenta from case cy29.1 also had evidence that basal plate infarction and bleeding into the intervillous space had occurred earlier in gestation, with focal but extensive areas of organized fibrin and hemosiderin deposition. Cases cy29.1 and cy26.1 had moderate numbers of lymphocytes and plasma cells within the basal plate and placental villi, which is indicative of a chronic inflammatory response. After grading for extent and severity of histopathology, all cases from this cohort scored higher than age-matched controls (**Figure 3**: Lower dose vs. Control). Consistent with this trend, placenta samples from this cohort had increased levels of several pro-inflammatory cytokines, including RANTES,

TABLE 1 | Overview of treatment groups and outcomes.

Treatment	Trimester	Cohort	Outcome	ID	Gestation at inoculation	Maternal age	Maternal weight (kg)	Confirmed total dose Lm	Dose by weight (Lm/kg)	Peak maternal temperature (°F)	Gestation at collection	Duration	Fetal sex	Fetal weight (kg)
Lm	1	10^6, first exposure	Fetal survival	cy29.1	44	6	3.74	1.64E+06	4.39E+05	101	148	104	M	0.32
Lm	1	10^6, first exposure	Fetal survival	cy26.1	40	7	4.2	2.80E+06	6.67E+05	100.5	152	112	F	0.34
Lm	1	10^6, first exposure	Fetal survival	cy27.1	39	7	4.5	2.60E+06	5.78E+05	101.4	154	115	F	0.45
Lm	1	10^7, first exposure	Pregnancy loss	cy19.1	40	9	5.74	2.47E+07	4.30E+06	102.1*	48	8	x	0.004
Lm	1	10^7, first exposure	Pregnancy loss	cy21.1	36	6	3.35	1.23E+07	3.67E+06	101.4	50	14	x	0.019
Lm	1	10^7, first exposure	Pregnancy loss	cy23.1	41	7	5.03	1.52E+07	3.02E+06	101	46	5	x	(–)
Lm	1	10^7, first exposure	Pregnancy loss	cy25.1	39	6	3.32	1.60E+07	4.82E+06	102.9*	50	11	x	(–)
Lm	1	10^7, first exposure	Fetal survival	cy30.1	43	13	3.45	1.65E+07	4.78E+06	101.1	154	111	M	0.33
Lm	1	10^7, re-exposure	Fetal survival	cy29.2	47	7	4.66	1.34E+07	2.88E+06	101	145	98	F	0.33
Lm	1	10^7, re-exposure	Pregnancy loss	cy26.2	40	8	4.78	2.00E+07	4.18E+06	98.9	51	11	x	(–)
Lm	1	10^7, re-exposure	Timed fetectomy	cy27.2	46	8	4.44	1.13E+07	2.55E+06	98.6	56	10	x	0.0101
Lm	1	10^7, re-exposure	Pregnancy loss	cy23.2	41	8	5.2	1.20E+07	2.31E+06	101.2	102	61	x	0.098
Lm	1	10^7, re-exposure	Fetal survival	cy25.2	39	7	3.9	2.00E+07	5.13E+06	99.7	153	114	M	0.34
Lm	1	10^8, re-exposure	Pregnancy loss	cy30.2	45	14	4.16	1.40E+08	3.37E+07	99.1	58	13	x	0.0115
Lm	3	10^7, first exposure	Fetal survival	cy20.1	110	5	5.92	1.60E+07	2.70E+06	100.3	146	36	M	0.34
Lm	3	10^7, first exposure	Pregnancy loss	cy31.1	123	6	4	1.60E+07	4.00E+06	101	137	14	M	0.27
Lm	3	10^7, first exposure	Pregnancy loss	cy34.1	123	9	5.34	1.12E+07	2.10E+06	96.6	133	10	M	0.21
Lm	3	10^8, first exposure	Pregnancy loss	cy39.1	135	5	3.86	2.33E+08	6.04E+07	101.2	139	4	M	0.25
None	1	Control	Normal	cy24c	42	8	4.38	n/a	n/a	99.3	57	15	x	0.008
None	1	Control	Normal	cy27c	41	6	4.14	n/a	n/a	99.9	51	10	x	0.006
None	1	Control	Normal	cy35c	46	5	2.68	n/a	n/a	99.5	51	5	x	0.003
None	1	Control	Normal	cy26c	38	6	3.86	n/a	n/a	99.7	55	17	x	0.02
None	3	Control	Normal	cy35c	141	6	3.54	n/a	n/a	98.6	154	13	M	0.31
None	3	Control	Normal	cy33	124	10	8.15	n/a	n/a	97.9	152	28	F	0.34
None	3	Control	Normal	cy38	115	5	4.03	n/a	n/a	99.9	137	22	F	0.31
None	3	Control	Normal	cy31c	133	9	4.36	n/a	n/a	98.1	137	4	M	0.296

Cynomolgus macaque females reach maturity around age 4, are fertile until approximately age 24, and have an average lifespan of 30 years.

** Maternal fever.*

x, too early in gestation to determine.

(–), not recorded.

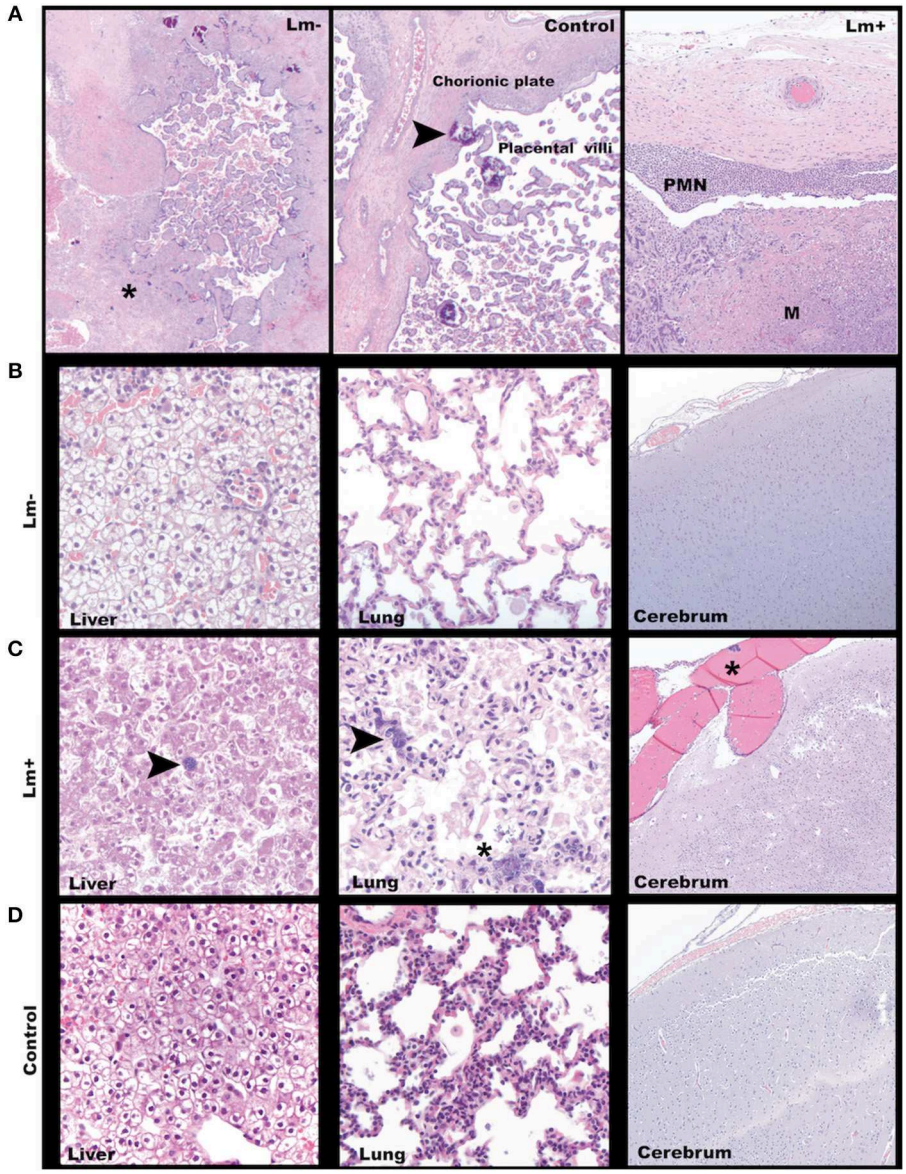

FIGURE 2 | Third trimester histology. **(A)** Maternal-fetal interface. Left: A focus of avascular villi entrapped in fibrin (asterisk) in a placenta from a dam exposed to Lm but without discernible infection. Cy29.1 H&E, 2x. Center: Normal placenta with incidental multifocal mineralization (arrowhead). Cy33. H&E, 40x. Right: Lm-infected placenta with acute subchorionic neutrophilic (PMN) inflammation and intervillous microabscesses (M). Cy31. H&E, 2x. **(B)** Representative tissue findings in Lm-inoculated animals who did not show clinical signs of infection. Left: Fetal liver with mild diffuse subcapsular neutrophil infiltration, intravascular and perivascular neutrophils. Cy27.1. H&E 40x. Center: Fetal lung with rare squamous cells in alveoli. Cy26.1. H&E 40x. Right: Fetal cerebrum with no significant findings Cy26.1. H&E, 10x. **(C)** Representative tissue findings in Lm-infected animals. Left: Lm+ Fetal liver with intralesional bacteria (arrow). Cy31. H&E, 10x. Center: Lm+ fetal lung with intravascular bacteria (arrow) and alveolus with luminal bacteria, neutrophils, squamous cells, and cellular debris. Cy31. H&E, 40x. Right: Lm+ fetal cerebrum with an intravascular bacterial embolus (asterisk). Cy31. H&E, 10x. **(D)** Representative tissue findings in control animals. Left: Control fetal liver. Cy33. H&E, 40x. Center: Control fetal lung. Occasional interalveolar squamous cells are an incidental finding commonly seen in cesarean deliveries. Cy33. H&E, 40x. Right: Control fetal cerebrum. Cy33. H&E, 10x.

perforin, and granzyme A. Anti-inflammatory IL-1RA, which antagonizes IL1b, was highest in controls and lowest with Lm infection. Placentas from pregnancies that received Lm but continued to term C-section had levels of IL-1RA higher than samples from Lm+ cases of pregnancy loss, but lower than age-matched controls (**Figure 4**).

Pregnancy Outcomes Following Re-infection

Inoculation with the same strain of Lm in a subsequent pregnancy resulted in fetal demise in 3 of 5 dams: cy26.2 at gestation day (gd)51 (11 days post-inoculation), cy30.2 at gd58 (13 days post-inoculation), and cy23.2 at gd102 (61 days

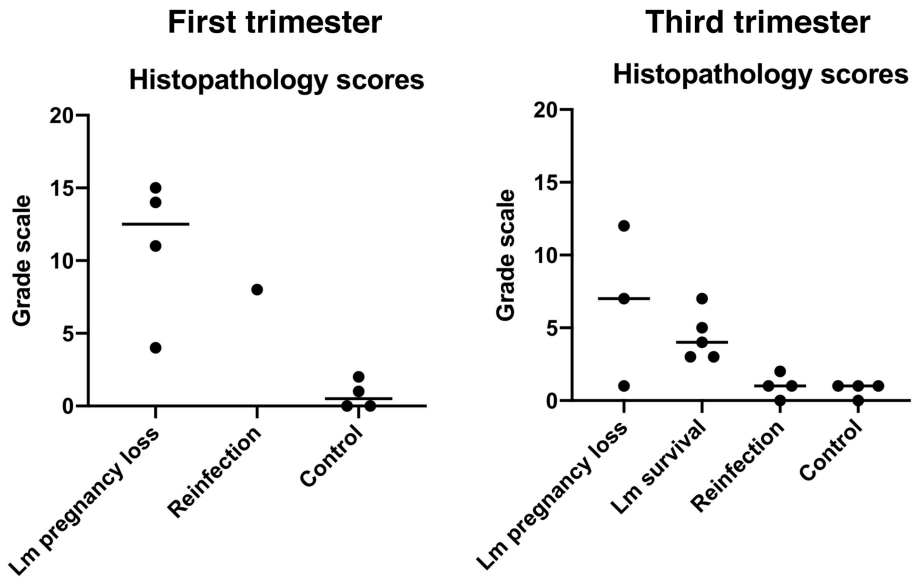

FIGURE 3 | Histopathology scores. Each dot represents the final score from a single case. Final scores were calculated from the sum of abnormal histological findings noted in both fetal and maternal-fetal interface tissues. Central black lines indicate the average score of a group.

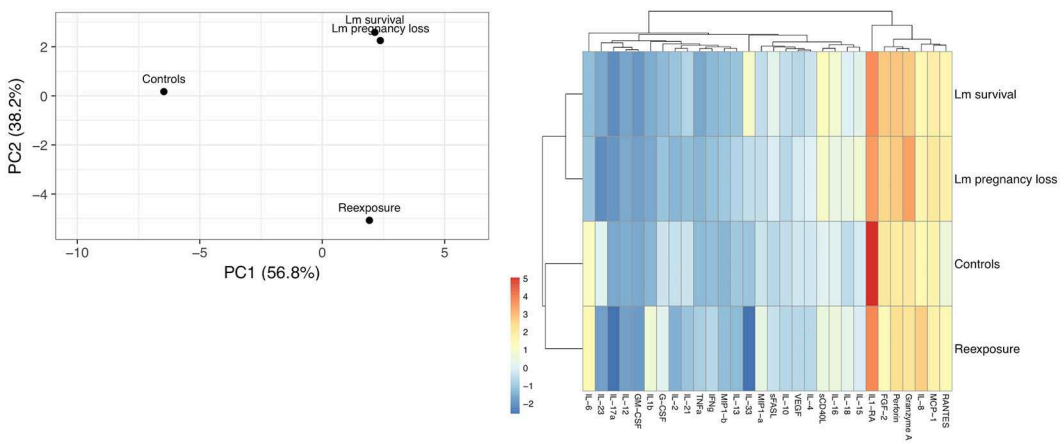

FIGURE 4 | Cytokine cluster analysis of third trimester placenta samples. **(A)** Principle component analysis based on the variation between subgroups. Original values were ln(x + 1)-transformed. Singular value decomposition with imputation was used to calculate principal components. X and Y axes show principal component 1 and principal component 2 that explain 56.4 and 37.2% of the total variance, respectively. **(B)** Heat map showing relative expression of all 30 cytokines assayed. Original values were ln(x + 1)-transformed. Pareto scaling was applied to rows. Both rows and columns were clustered using Manhattan distance and average linkage. Dendrograms indicate tightness of clusters.

post-inoculation) (**Figure 1B**). In this cohort, two received an Lm dose equivalent to their first exposure (cy26.2, cy29.2), and three received a higher dose (cy23.2, cy25.2, cy30.2) (**Table 1**). We were unable to culture bacteria (Lm) from the maternal-fetal interface in two cases (cy26.2 and cy23.2), and one of the dams (cy26.2) had no detectable Lm shedding or signs of infection prior to first trimester miscarriage. In this case (cy26.2), placental and fetal tissues were not available for analysis due to maternal placentophagy following spontaneous abortion. Cy23.2 had Lm-positive maternal fecal shedding and miscarried in the second trimester. Tissues examined from cy23.2 demonstrated mild histopathology, with multifocal neutrophilic villitis in the placenta and diffuse lymphoplasmacytic deciduitis. The fifth dam (cy30.2) received a higher dose in her second pregnancy (10^8 CFUs) and spontaneously aborted at gd58 (13 days post-inoculation), accompanied by fecal shedding, maternal bacteremia, and tissue colonization and histopathology characteristic of listeriosis (**Figures 1B**, **5**, **6**). The remaining two cases, cy29.2 and cy25.2, continued uneventfully to term. On histological examination, the placenta from cy29.2 showed basal plate infarcts with hemosiderin, indicating prior hemorrhage, and the placenta from cy25.2 had focal villous infarcts with

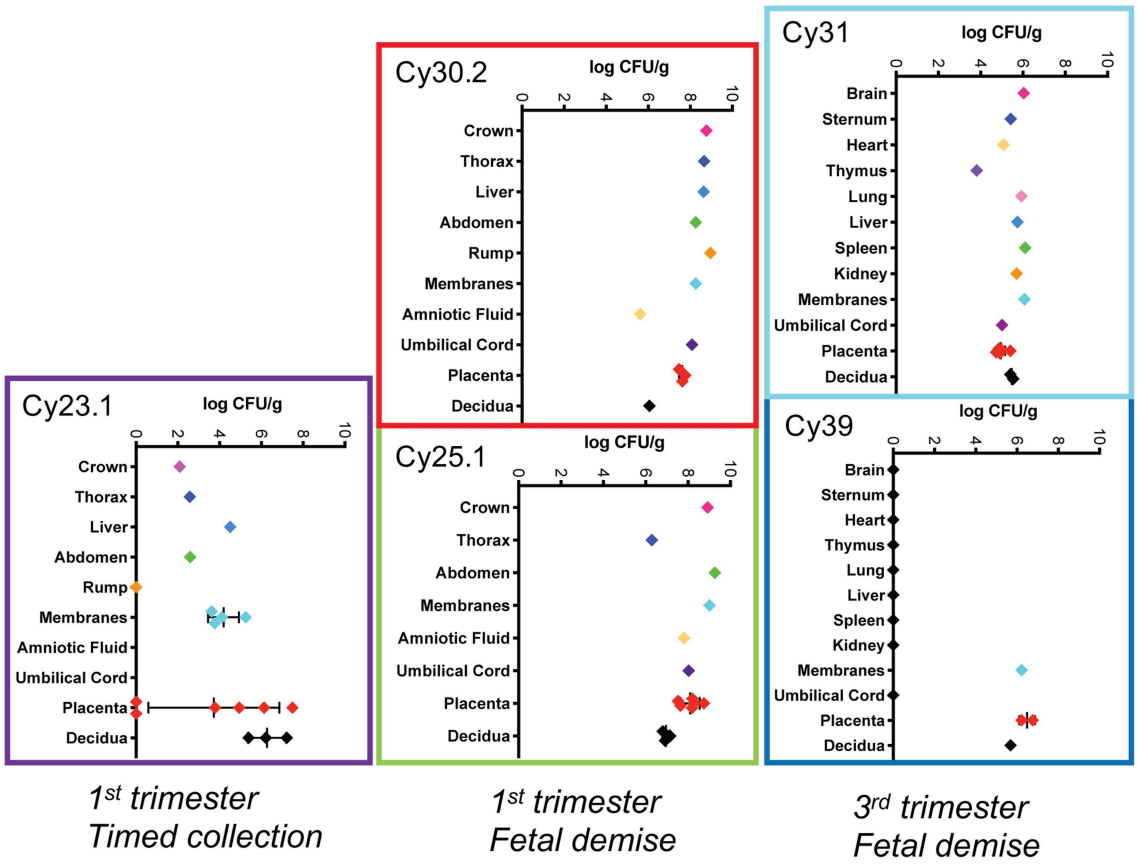

FIGURE 5 | Tissue bacterial burden Bacterial burden of Lm from individual pregnancies. The y-axis shows tissues and the x-axis shows the bacterial burden in colony forming units per gram (CFUs/g) in log scale for each tissue. Central black lines indicate the mean CFUs/g for replicate samples.

lymphoplasmacytic deciduitis. Interestingly, placenta samples from this cohort did not cluster with control samples by PCA plot and showed a shift in cytokine expression distinct from the other cohorts (**Figure 4**).

Timed Surgical Collection Following Inoculation to Assess Timing of Colonization

We performed pre-emptive surgery and sample collection shortly following inoculation to assess early events at the maternal-fetal interface. We assessed first time exposure (cy23.1) and re-exposure (cy27.2). Tissues collected at post-inoculation day 5 (cy23.1) showed that replicate decidua biopsies had a consistently high bacterial burden (5–7 log CFUs/g) whereas replicate placenta biopsies had more variable colonization (0–8 log CFUs/g). Of these, 2 of 6 replicates yielded no culturable Lm. There was moderate colonization of the amniotic membranes (3–6 log CFUs/g), and no culturable bacteria in the amniotic fluid (**Figures 1B**, **5**), suggesting that surgery had interrupted colonization of the fetal compartment. Once the placenta has been breached, Lm characteristically colonizes highly vascular tissues of the fetus including developing bone, lung, spleen, and liver (**Figure 7**). The most heavily infected fetal organ in case cy23.1 was liver, with a bacterial burden equivalent to that of the amniotic membranes (**Figure 5**). All fetal tissues in this case had a bacterial burden several logs lower than cases which had fetal death *in utero* and an incubation duration longer than 5 days (1st trimester timed collection vs. 1st trimester fetal demise). Consistent with interrupted infection at this early timepoint, there was no discernable histopathology or inflammation in the fetus (data not shown). Tissues at the maternal-fetal interface demonstrated necrosuppurative arteritis in the decidua and multifocal suppurative necrosis of placental septa and anchoring villi, with a laterally dissecting hemorrhage and focal abscessation of a basal vein. Of note, cy23.1 tested positive for Lm in both fecal and blood cultures the day after inoculation. In contrast, cy27.2 had no maternal fecal shedding or bacteremia, and tissue samples at day 10 post-inoculation yielded no positive cultures and no evident histopathology (**Figure 1A**; **Table 2**).

Third Trimester Infection

We assessed third trimester pregnancy outcomes following infection with the same clinical strain of Lm. Two of three monkeys given 10^7 CFUs Lm in the third trimester of pregnancy had a stillbirth between 10 and 14 days post-inoculation. A fourth monkey received 10^8 CFUs and gave birth prematurely

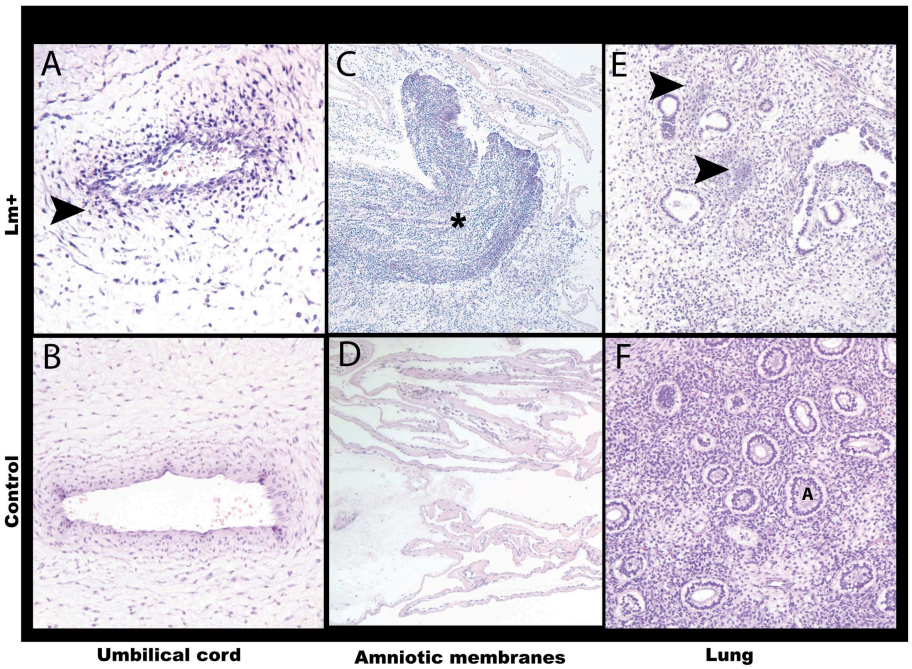

FIGURE 6 | Histology of the first trimester Lm infection. **(A)** Umbilical cord. Neutrophilic umbilical arteritis. Arrow points to clustered neutrophils. Cy30.2. H&E, 20x. **(B)** Control umbilical cord. Cy26c H&E, 20x. **(C)** Amniotic membranes with Gram-positive bacteria and neutrophilic infiltrate (asterisk). Cy30.2. H&E, 4x. **(D)** Control amniotic membranes. Cy26c H&E, 20x. **(E)** Fetal lung with listerial infiltration of pulmonary interstitium between alveoli. Arrows point to bacterial foci. Cy30.2 H&E, 20x. **(F)** Control fetal lung. A indicates alveolus. Cy26c H&E, 20x.

at 4 days post-inoculation. All three of these cases had Lm+ maternal fecal shedding, bacteremia, and tissue colonization (**Figures 1C**, **5**). The most severe pathologic changes were seen in the stillbirth cases, characterized by neutrophilic deciduitis, suppurative villitis, and chorioamnionitis at the maternal-fetal interface, and fetal hepatitis and pulmonary congestion with neutrophils (**Figure 2C**). The preterm infant had Lm-positive cultures only within the amniotic membranes, placenta, and decidua, and no discernible inflammatory reaction or pathologic changes in the fetus (**Table 2**). The lack of fetal colonization is likely due to the occurrence of preterm birth before Lm could traffic to the fetal compartment, consistent with our observation that cy23.1 had a comparatively low fetal bacterial burden at post-inoculation day 5 (**Figure 5**). One dam (cy20) showed no evidence of maternal infection and continued to term uneventfully, but examination of tissues after surgical collection demonstrated villous necrosis of the placenta as well as fetal hepatitis, neither of which have been noted in age-matched control tissues. All third trimester placentas had variable mineralization, also seen in healthy controls, and the degree of mineralization most strongly associated with increasing gestational age. Focal placental infarction with few scattered neutrophils and focal areas of decidual necrosis is also an incidental finding in the third trimester (**Figure 2D**). Areas of infarction and mineralization are a normal consequence of cell death and ischemia due to the nature of the placenta as a temporary fetal organ that undergoes senescence as it nears parturition.

DISCUSSION

It is known that listeriosis causes adverse pregnancy outcomes, including miscarriage, stillbirth, and neonatal infection (Hof, 2003; Lamont et al., 2011; World Health Organization, 2018). Our research aims to clarify the course of infection, as well as the histological and cellular sequelae, which cannot be readily studied in human pregnancy. We have previously described the course of infection following a first-time exposure in the first trimester of pregnancy in a non-human primate model, resulting in acute infection and loss of the pregnancy between 1 and 2 weeks post-inoculation (Wolfe et al., 2017). Here we employed the same clinical isolate of Lm to determine how a lower initial dose impacts disease pathogenesis and pregnancy outcomes, if the virulence of our strain of Lm depends on gestational age, and whether or not prior exposure influences the immune response with reinfection in a subsequent pregnancy. Our previous study showed that the first trimester of pregnancy is keenly sensitive to infection, which caused fetal demise in 4 out of 4 monkeys following inoculation with 10^7 CFUs Lm. With the lower dose given in this study, we hypothesized that we would observe a long incubation period followed by acute fetal infection, which is thought to occur in human outbreaks based on retrospective surveys, clinical cases and estimated exposure dates (Goulet et al., 2013). However, we found that inoculation with 10^6 CFUs Lm in the first trimester did not result in latent maternal infection, nor in acute or chronic fetal infection. All 3 monkeys progressed to term C-section without

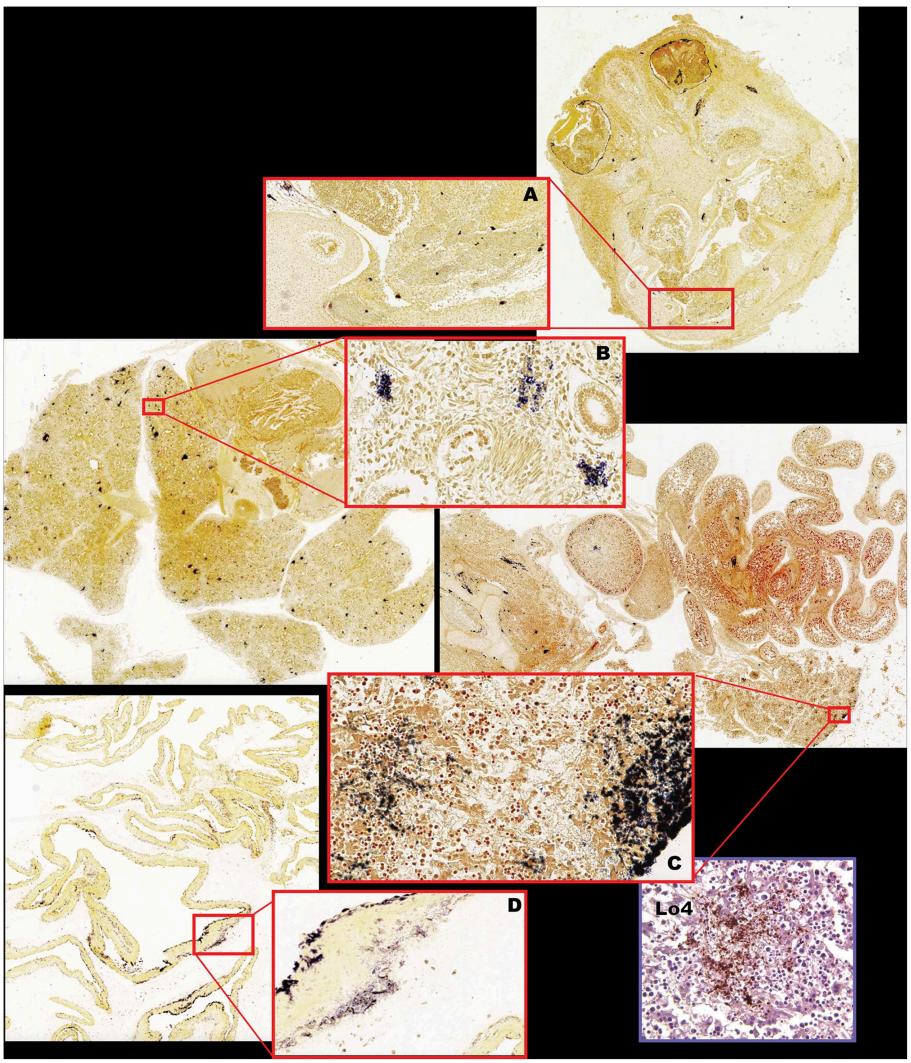

FIGURE 7 | Fetal bacterial distribution with Lm infection in the first trimester (Cy25.1). **(A)** Fetal cranium with large clusters of Gram-positive rods within the ocular musculature, diffusely within the nasal sinus and nasal cartilage, multifocally within the neural tissue, periosteum of the skull, and within the dermis, and subcutaneous tissues. Gram stain, 2x (inset 10x). **(B)** Fetal lung with multiple foci of intravascular bacteria. Gram stain, 2x (inset 40x). **(C)** Fetal liver, vertebrae, intestines, kidney, and adrenal gland, with multiple foci of bacterial colonization of the kidney, adrenal gland, intestinal serosa, hepatic capsule and interstitium, periosteum, and perichondrium. Gram stain, 2x (inset liver 40x). **(D)** Fetal membranes (chorioamniotic membranes) with multiple foci of Gram-positive bacteria. Gram stain 2x (inset 10x). Inset (Lo4): Immunohistochemical staining of fetal liver confirming Lm-positive foci.

adverse events. Infant weights remained comparable to controls, and there were no gross abnormalities. None of the sampled tissues had Gram-positive stained or culturable Lm. Interestingly, however, histological examination of tissues following surgical collection revealed lesions consistent with chronic inflammation and intrauterine bleeding, not found in gestational age-matched control samples, as well as a shift in the placenta toward a pro-inflammatory cytokine profile.

We next examined the impact of gestational age on infection susceptibility and pregnancy outcomes. Following the same experimental paradigm as before, we observed an adverse outcome in 3 out of 4 monkeys given 10^7-10^8 CFUs Lm in the third trimester. Two spontaneously aborted at 10- and 14-days post-inoculation with significant inflammation of the maternal and fetal tissues, a tissue bacterial burden comparable to that seen in the first trimester, and histological evidence of fetal hepatitis and pneumonia. The third (cy39.1) delivered a premature infant 4 days post-inoculation. Although the fourth (cy20.1) continued to term C-section, there was evidence of fetal distress *in utero* and abnormal placental necrosis. At 10^7 CFUs Lm, our combined studies show a survival rate of 1/3 in the third trimester and 1/5 in the first trimester. Our findings of fewer stillbirths yet still severe outcomes in later pregnancy are consistent with reports that fetal survival odds increase with gestational age, potentially due to an improved fetal immune response (Wadhwa and Smith, 2017). It will be intriguing for future studies to assess the development

TABLE 2 | Histopathology score chart.

Treatment (Tissue +/–)	Trimester	ID	Maternal-Fetal Interface						Fetal					TOTAL
			Lymphocytic and neutrophilic deciduitis	Decidual fibrinoid necrosis	Placental villous necrosis	Neutrophilic villitis	Neutrophilic chorio-amnionitis	MFI score	Funisitis	Hepatitis	Splenitis	Pulmonary congestion with neutrophils	Fetal score	
Control	1	cy24c						0					0	0
Control	1	cy27c						0					0	0
Control	1	cy26c	x					1					0	1
Control	1	cy35c	x		x			2					0	2
Lm (+)	1	cy23.1	x		x	xx		4					0	4
Lm (+)	1	cy30.2	xxx			xxx	xx	8					0	8
Lm (+)	1	cy21.1	xx	x	x	xx	x	7		xxx		x	4	11
Lm (+)	1	cy25.1	xx	xx	x	xx	xxx	10	x	xxx			4	14
Lm (+)	1	cy19.1	xx	xx	xxx	xxx	x	11	x	xx		x	4	15
Lm (–)	1	cy27.2						0					0	0
Lm (–)	1	cy26.2	x	x	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Lm (–)	2	cy23.2	x*			x		2					0	2
Lm (–)	3	cy26.1	x*			x	x	3					0	3
Lm (–)	3	cy29.1	xx*		xx			4			x		1	5
Lm (–)	3	cy27.1	xx	xx	xx			6		x			1	7
Lm (–)	3	cy29.2	x					1					0	1
Lm (–)	3	cy25.2	x*					1					0	1
Lm (+)	3	cy39.1				x		1					0	1
Lm (–)	3	cy20.1			x			1		x			1	2
Lm (–)	3	cy30.1			xx	xx		4				x	1	5
Lm (+)	3	cy34.1	xx	xx		xx		6		x			1	7
Lm (+)	3	cy31.1	xxx			xx	xxx	8		x		xxx	4	12
Control	3	cy35c						0					0	0
Control	3	cy33c		x				1					0	1
Control	3	cy38c		x				1					0	1
Control	3	cy31c		x				1					0	1

*Histological scores for samples from the maternal-fetal interface and fetal tissues are presented as the sum of discrete pathologies noted by WNPRC board-certified veterinary pathologists on examination of H&E stained sections. Cases are organized by cohort and trimester at the time of tissue collection. ID ending in "c" indicates control, "1" indicates first pregnancy, and "2" indicates second pregnancy. Case cy26.2 had no identifiable uterine contents following spontaneous abortion, and n/a indicates these samples could not be analyzed for discrete histopathology. *Presence of plasma cells (indicative of a chronic inflammatory response).*

Score criteria are as follows:

X, mild (rare single scattered cells throughout the histologic section or multiple clusters of 4–10 cells; findings impact 0–25% of thesection).

XX, moderate (1–2 clusters of 10–20 cells or diffuse cell infiltration; findings impact 26–50% of thesection).

XXX, severe (1–2 clusters of more than 20 cells; findings impact more than 50% of thesection).

and adult health of infants known to have survived a pregnancy impacted by listeriosis.

Despite moderate to severe inflammation at the maternal-fetal interface, there was frequently minimal to absent fetal immune response detected by histologic examination (**Table 2**: cy23.1, cy30.1, cy34.1, cy26.1, cy26.1, cy27.1). The capacity for a fetal immune response emerges as pregnancy progresses with the development of hematopoietic and lymphoid organs (Chougnet, 2018). Differentiated immune cells, including neutrophils, macrophages, B cells, and T cells, can be observed by the late second to early third trimester, but these cells appear to be geared toward immunosuppression rather than defense against non-self-entities, and evidence suggests that the fetal immune system functions distinctly differently from that of adults as well as newborns, given its *in utero* niche and need to coexist with the maternal immune system (Makori et al., 2003; McGovern et al., 2017). An absence of fetal inflammatory response in the first trimester can be attributed to early gestational age and a lack of infiltration by maternal immune cells. At later stages of pregnancy, this may be due to fetal death or expulsion of uterine contents occurring before tissue-level changes caused by a cellular defense response can be observed. Similar to our early pregnancy study, we observed a fairly short incubation period of 4–14 days, which may be strain-dependent.

To determine if prior exposure to Lm confers protection or causes sensitization to subsequent exposures, we re-inoculated dams with an equivalent or higher dose of the same clinical isolate of Lm at approximately the same gestational day during a second pregnancy. We hypothesized first-time exposure would elicit a maternal immune response that would provide subsequent protection. Re-exposure resulted in a lower rate of pregnancy loss than first-time exposure, which is consistent with observations in livestock (Fensterbank, 1987), however we did not identify a clear association between initial response and secondary response that would have allowed us to predict the pregnancy outcomes of specific individuals. For example, cy25 lost her first pregnancy to Lm infection and then carried her second pregnancy to term despite reinfection, suggesting protection. In contrast, cy23 also had fulminant tissue colonization and maternal bacteremia in her first pregnancy, yet lost her second pregnancy to Lm infection. Cy30 and cy26 each had successful first pregnancies but miscarried their second, while cy29 successfully carried both pregnancies to term despite culture-positive maternal Lm infection each time. In total, re-exposure resulted in fetal demise in 3 out of 5 cases. Two of these cases (cy30.2 and cy23.2) could be diagnostically attributed to listeriosis. We did not recover Lm from the third case (cy26.2). While this case cannot be diagnostically attributed to listeriosis because all bacterial cultures and histologic stains for Lm were negative, it is possible that Lm does not need to directly infect the reproductive tract to induce adverse pregnancy outcomes. Peripheral immune cell activation following maternal infection may induce a signaling cascade of pro-inflammatory cytokines or other cellular responses contributing to the incidence of spontaneous abortion and preterm birth (Rowe et al., 2012).

The rate of miscarriage in this study exceeds the rate of spontaneous abortion at WNPRC's breeding colony of macaques, which were not used in these experimental studies. The breeding colony at WNPRC serves as an excellent control for infection studies because these animals are housed indoors and protected from environmental exposures. According to colony management records, the rate of spontaneous abortion in the colony per year is around 1 in 25 pregnancies. Less than 1% of these could be attributed to spontaneous ascending bacterial infection associated with fecal or vaginal flora. Prior to initiation of this study, there were no positive Lm culture results in the WNPRC database, which spans 35+ years of clinical and pathological results. All WNPRC animals and aborted tissues receive gross and histologic evaluation with appropriate bacteriological and viral testing. Additionally, our studies on listeriosis to-date have included a total of 10 designated control monkeys who underwent the same treatments and procedures as our experimental animals, except that they did not receive Lm in the inoculum. None of the control pregnancies in our current or previous studies have resulted in an adverse outcome, which suggests that maternal exposure to Lm, even without detectable reproductive tract colonization, has a detrimental impact on pregnancy health.

A preliminary assessment of cytokine expression in placenta samples from our cohorts shows that exposure to Lm alters the balance of pro- vs anti-inflammatory mediators at the maternal-fetal interface. Principle component analysis (PCA) can be used to visualize the degree of variability between sample sets and is particularly useful in demonstrating general relationships among subgroups based on multivariate data (Helmy et al., 2012). Because cytokines are not expressed in isolation, rather many are pleiotropic, act upon one another, and have the capacity to be pro-inflammatory or anti-inflammatory depending on the context and presence other cytokines, we therefore wanted to perform an exploratory cluster analysis to evaluate how much variation between our cohorts could be identified based on their overall pattern of cytokine expression. On PCA plot, Lm samples cluster apart from control samples. Given the inflammatory tissue response induced by Lm infection, this confirms expectations. Notably, Lm pregnancy loss samples had reduced IL-1RA expression, which has been linked to an impaired primary immune response and increased susceptibility to Lm infection (Irikura et al., 1999). Placenta samples from dams who received Lm yet went on to have uneventful pregnancies (Lm survival) clustered more closely to Lm pregnancy loss samples than to controls. Intriguingly, Lm survival samples had greater IL-1RA expression than Lm pregnancy loss samples, yet lower than controls. This suggests that the histopathology observed in the Lm survival cohort may be underpinned by responsive cell signaling. On the same plot, placentas from dams re-exposed to Lm in a subsequent pregnancy clustered apart from all other groups, suggesting a distinct secondary response. Hierarchical clustering based on cytokine expression demonstrates that exposure to Lm alters the chemical milieu of the placenta even in cases without adverse pregnancy outcomes. It will be enlightening to confirm these trends pending a larger study with additional samples, and to further evaluate this dataset in relation to cytokine signaling from the maternal decidua and fetal amniotic membranes. We cannot say if the Lm survival

cohort's sterile yet inflammatory intrauterine environment would have an impact on infant development, behavior, or health in later life, as this study was not designed to explore that question. Retrospective studies on the long-term morbidity and neurodevelopment of survivors of prenatal listeriosis are mixed (Evans et al., 1984; Bortolussi and Mailman, 2011) and more definitive studies assessing prenatal programming from a pregnancy impacted by maternal Lm infection would be valuable.

Diagnosing Lm infection prior to an adverse event such as stillbirth or preterm birth continues to be difficult and inconsistent based on patient presentation and laboratory results (Charlier et al., 2014). A retrospective study at a hospital in Israel found that listeriosis is simultaneously underdiagnosed and overtreated (Fouks et al., 2018): over the course of five years, more than one hundred pregnant women who received a diagnosis of suspected listeriosis did not subsequently have a Lm+ culture, while 7 patients who had culture-confirmed Lm had not initially received a diagnosis of suspected listeriosis. One of the advantages of our model is that we can assess infection outcomes given a known bacterial quantity and timing of exposure. Although we directly administered a known amount of Lm to the maternal gastrointestinal tract, we note inconsistent fecal shedding of bacteria, clinical signs, and disease course. In some cases, we were unable to grow colonies on blood agar plates even though microscopy showed Gram-positive rods in tissues, and in other cases we did not observe Gram-positive rods despite Lm-positive cultures. In several cases, we had Lm-positive maternal samples, yet did not recover Lm from fetal or maternal-fetal interface samples through assay, culture, or cold enrichment. It has been reported that Lm is capable of entering a viable but non-culturable (VBNC) state (Highmore et al., 2018) which could complicate diagnostics, and Gram-staining can yield false results if artifacts are introduced or errors are made in specimen collection and processing (Samuel et al., 2016). Another cause for discrepancy with Lm detection across samples may be the characteristically multifocal pattern of listerial colonization: within the same tissue, we observe infiltration of some regions but not others. As we collect biopsies, it is possible that one contains a focus of Lm while another does not. In cases with maternal bacteremia and extensive tissue colonization, we had no difficulty identifying Lm; rather it was the cases in which there was light maternal fecal shedding and minimal colonization of maternal organs that proved more ambiguous. In these cases, we hypothesize that Lm was restricted from the intrauterine compartment, and that histologically-evident placental and fetal pathologies resulted from an immune response to infection, as opposed to direct infection. Maternal bacteremia was our strongest predictor of an adverse outcome and associated fetal histopathology. The pregnancies of dams who became bacteremic consistently ended in acute fetal demise shortly after initial detection of bacteremia, and all of these cases had bacterial colonization of the maternal-fetal interface, in contrast to dams with positive fecal cultures but no bacteremia, indicating that hematologic trafficking is critical for Lm to reach the reproductive tract. Identifying accurate and reliable markers of early infection that can be tested *in utero* remains an important goal to prevent the unnecessary use of antibiotics while at the same time ensuring that patients genuinely at risk of pre- and perinatal listeriosis can be promptly recognized and treated.

Based on our findings, prior maternal exposure does not appear to be predictive of disease susceptibility or pregnancy outcome, nor does the timing or duration of maternal shedding of Lm. Interindividual variation in response to transmissible disease is notable in humans, and no doubt factors into the range of outcomes observed in nonhuman primates as well (Casadevall and Pirofski, 2018). Confounding factors such as environment, diet, exercise, comorbidities and prior medical treatment plausibly may be ruled out given the controlled conditions of indoor housing and colony management at WNPRC facilities. We documented fetal sex and maternal age, weight, and body temperature, and these do not appear to correlate with pregnancy outcome. Maternal temperatures were, on average, mildly elevated following Lm inoculation, and two dams had clinical fever, but this was neither pathognomonic nor predictive of infection. We took into account the range of maternal body size by calculating the Lm dose per kilogram, and individual dams proved susceptible to different doses. Some, like cy34.1, had pregnancy loss with 2^6 Lm/kg, whereas cy30.1 tolerated 4^6 Lm/kg without pregnancy loss. Like humans, monkeys are genetically diverse and it is possible that disease resilience is driven by genomic differences or microbiome composition (Namasivayam et al., 2019), or that social dynamics play a role in immune programming (Snyder-Mackler et al., 2016). Individual determinants of disease remain an important area of study.

Although we observed fewer adverse pregnancy outcomes in our cohort of monkeys that received a lower dose of Lm, we cannot conclude and do not suggest that we have found any safe level of exposure to Lm. It is evident that different bacterial strains, environmental conditions, and individual susceptibility can alter Lm virulence and infection kinetics (Vázquez-Boland et al., 2001; Smith et al., 2008). Our study instead offers evidence that maternal Lm infection which spares the fetus still impacts the placenta, creating a more pro-inflammatory intrauterine environment, and reiterates that Lm is a pathogen of serious consequence. Ongoing studies from these same cohorts examining the maternal and fetal cellular response to listeriosis, including transcriptomic changes, post-transcriptional modifications, and host microbiome shifts, aim to shed light on the determinants of disease susceptibility vs. resilience.

DATA AVAILABILITY

WNPRC has a policy of sharing data and materials when scientifically relevant. The datasets for the current study are available upon reasonable request. Requests to access the datasets should be directed to TG (golos@primate.wisc.edu) and to HS (hsimmons@primate.wisc.edu).

ETHICS STATEMENT

The animal study was reviewed and approved by University of Wisconsin Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

BW, CC, and TG contributed to the conception and design of the study. BW organized experiments, quantified bacterial burden, produced figures, and wrote the first draft of the manuscript. HS and AM collected, read, and scored histological specimens. HS photographed H&E sections. AK curated, scanned, and analyzed Gram-stained specimens. BW, HS, and TG wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Full-Term Human Placental Macrophages Eliminate *Coxiella burnetii* Through an IFN-γ Autocrine Loop

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The intracellular bacterium *Coxiella burnetii* is responsible for Q fever, an infectious disease that increases the risk of abortion, preterm labor, and stillbirth in pregnant women. It has been shown that *C. burnetii* replicates in BeWo trophoblast cell line and inhibits the activation and maturation of decidual dendritic cells. Although tissue macrophages are known to be targeted by *C. burnetii*, no studies have investigated the interplay between placental macrophages and *C. burnetii*. Here, $CD14^+$ macrophages from 46 full-term placentas were isolated by positive selection. They consisted of a mixed population of maternal and fetal origin as shown by genotype analysis. We showed that *C. burnetii* organisms infected placental macrophages after 4 h. When these infected macrophages were incubated for an additional 9-day culture, they completely eliminated organisms as shown by quantitative PCR. The ability of placental macrophages to form multinucleated giant cells was not affected by *C. burnetii* infection. The transcriptional immune response of placental macrophages to *C. burnetii* was investigated using quantitative real-time RT-PCR *on* 8 inflammatory and 10 immunoregulatory genes. *C. burnetii* clearly induced an inflammatory profile. Interestingly, the production by placental macrophages of interferon-γ, a cytokine known to be involved in efficient immune responses, was dramatically increased in response to *C. burnetii*. In addition, a clear correlation between interferon-γ production and *C. burnetii* elimination was found, suggesting that macrophages from full-term placentas eliminate *C. burnetii* under the control of an autocrine production of interferon-γ.

Keywords: *Coxiella burnetii*, inflammation, interferon-γ, macrophages, multinuclear giant cells, placenta

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INTRODUCTION

The placenta is a unique chimeric organ made of fetal (chorion) and maternal (decidua) tissues at the materno-fetal interface. It is essential for nutritional exchanges and the immune tolerance of the fetus (Houser, 2012; Pinhal-Enfield et al., 2012). The latter is maintained by placental immune cells that prevent fetus rejection and protect it from infections. These immune cells mainly consist of natural killer (NK) cells and macrophages whereas T and B lymphocytes, dendritic cell,s and mast cells are less represented (Bulmer et al., 2010; Ben Amara et al., 2013; Gorvel et al., 2014; Mezouar et al., 2018, 2019c).

Placental macrophages represent 20–30% of the leukocytes found in all compartments of the placenta and their proportion increased throughout pregnancy (Bulmer et al., 2010). The phenotypic characterization of placental macrophages relies on the expression of CD68, a myeloid marker and CD14, a marker of circulating monocytes that is lacking in tissue macrophages other than placental macrophages (Ben Amara et al., 2013; Mezouar et al., 2019a). Placental macrophages include two populations of different origin. While Hofbauer cells are of fetal origin, decidual macrophages are of maternal origin (Pinhal-Enfield et al., 2012). These two populations are usually distinguished by their location within the placenta: Hofbauer cells are found in the fetal chorionic villi and decidual macrophages are found in decidua basalis (Pinhal-Enfield et al., 2012). Unfortunately, no specific phenotypic marker allows the distinction of these two populations. The role of placental macrophages in pregnancy is plural: they are involved in tissue remodeling, angiogenesis (Pongcharoen et al., 2007; Fong, 2008; Loegl et al., 2016), antigen presentation (Heikkinen et al., 2003, 2004), and maternal-fetal tolerance (Wang et al., 2018). They spontaneously form *in vitro* multinuclear giant cells (MGCs) even if the precise role of these MGCs remains to be elucidated (Ben Amara et al., 2013). It is well known that the activation status, also called polarization, of macrophages govern their biological activities: M1 macrophages are inflammatory and microbicidal whereas M2 macrophages are immunoregulatory and non-microbicidal. The M1/M2 status of macrophages is dependent on their microenvironment, especially on cytokines. Indeed, inflammatory cytokines such as tumor necrosis factor (TNF) and interferon (IFN)-γ induce a M1 polarization whereas immunoregulatory cytokines such as interleukin (IL)-10 induce a M2 polarization of macrophages (Benoit et al., 2008b; Mantovani et al., 2013). As reported for other macrophages, placental macrophages have been classified in either the M1 or M2 group. The M2 profile of placental macrophages is found at the beginning of pregnancy whereas the M1 profile is found at the end of pregnancy (Zhang et al., 2017). An inappropriate polarization of placental macrophages is associated with pregnancy complications such as abortion or miscarriage (Mezouar and Mege, 2018). Chorioamnionitis, a placental infection, interferes with placental macrophage polarization (Brown et al., 2014; Mezouar and Mege, 2018), and consists in an altered inflammatory response including decreased production of IL-10 (Ben Amara et al., 2013). For others, maintenance of the M2 polarization profile was observed (Joerink et al., 2011).

Coxiella burnetii, an intracellular gram-negative bacterium, is the causative agent of Q fever, a widespread zoonosis. *C. burnetii* infection leads to two major clinical forms. The primary infection is most often (60% of cases) asymptomatic and is usually spontaneously resolved. *C. burnetii* infection may by persist in the host for a minority of infected patients (less than 5%) leading to endocarditis or vascular infection (Raoult et al., 2005). *C. burnetii* infection of pregnant women is associated with specific problems (Carcopino et al., 2007) mainly due to the absence of clinical signs characteristic of the acute Q fever. The risk of pregnancy complications such as abortions and prematurity is high when the infection occurs during the first trimester. Malformations, stunting, or death *in utero* have also been reported (Eldin et al., 2017). Animal models of *C. burnetii* infection show that approximately 10^9 bacteria are present in 1 g of placental tissue (Sobotta et al., 2017). An imbalance of cytokine production is also observed in pregnant goats infected by *C. burnetii* (Roest et al., 2012). In *C. burnetii*-infected women, it has been suggested that abortions are related to infected and inflammatory placentas (Shinar et al., 2012).

The cellular reservoirs of *C. burnetii* in placenta are likely diverse. When human BeWo trophoblast cell line is infected with *C. burnetii*, the bacteria replicate within acid phagosomes (Ben Amara et al., 2013). The analysis of the transcriptional signature of these trophoblasts reveals the up-modulation of genes associated with inflammation pathways (Ben Amara et al., 2010). We also showed that *C. burnetii* infects placental dendritic cells and prevents their maturation and likely their ability to present antigens to the adaptive immune system (Gorvel et al., 2014). To our knowledge, the effect of *C. burnetii* infection on the functional activity of placental macrophages is unknown. In this study, we showed that isolated $CD14^+$ placental macrophages were able to eliminate *C. burnetii* within 9 days. The ability of these macrophages to spontaneously differentiate within MGCs was not affected by *C. burnetii* infection. In contrast, placental macrophages exhibited an inflammatory profile with an unexpected upregulated production of IFN-γ correlated with *C. burnetii* elimination.

MATERIALS AND METHODS

Placenta Collection

The study was approved by the "Comité d'Ethique d'Aix-Marseille Université" (number 08-012). Forty-six full-term placentas were collected at the Gynecology-Obstetrics Department of the "Hôpital de la Conception" (Marseille, France) after informed consent of the mothers. Women were devoid of pathologies, with a mean age of 34 years (21–42 years), a gestational age of 39 weeks (36–42 weeks) with main vaginal delivery (44 vaginal deliveries *versus* 2 caesarean deliveries). The placentas did not show any lesions or inflammation by macroscopically observation.

Bacteria

Coxiella burnetii Nine Mile strain (RSA496) was cultured as previously described (Ka et al., 2016). Briefly, L929 cells were infected for 8 days, sonicated, and centrifuged 10 min at 300 ×*g*.

Bacteria were collected after centrifugation at 10,000 ×*g* for 10 min, then washed and stored at −80°C. The concentration and the bacterial viability were assessed using the LIVE/DEAD BacLight bacterial viability kit (Life Technologies). In some experiments, organisms were labeled with the membrane fluorescent marker 4-(4-(dihexadecylamino)styryl)-N-methylpyridinium *iodide* (DID, Thermo Fisher Scientific) for 20 min at 37°C in phosphate buffered saline (PBS).

Isolation of Placental Macrophages

Placental macrophages were isolated as previously described (Mezouar et al., 2019a). Briefly, entire placenta tissue was digested in Hank's Balanced Salt Solution (HBSS), DNase I 2.5 mM, and 2.5% trypsin (Life Technologies). Cell suspension was filtered through 100-µm pores and deposited on a Ficoll cushion and centrifuged at 700 ×*g* for 20 min to collect mononuclear cells. Placental macrophages were isolated using magnetic beads coated with anti-CD14 antibodies (Miltenyi Biotec). The purity of isolated $CD14^+$ placental macrophages was assessed by flow cytometry and was higher than 98%.

Flow Cytometry Phenotyping of Isolated $CD14^+$ Placental Cells

The phenotype of isolated $CD14^+$ placental macrophages was assessed as follows. Cells (1×10^6 cells per assay) were stained using mice IgG1 anti-human CD14-APC (Allophycocyanin) and anti-human CD68-FITC (Fluorescein isothiocyanate) antibodies or isotype controls in PBS containing 5% Fetal Bovine Serum (FBS) for 30 min at 4°C. Cells were washed, fixed with 4% paraformaldehyde, centrifuged at 600 ×g for 5 min, and then diluted in PBS. Stained cells were then analyzed by flow cytometry (10,000 events/acquisition) using a BD FACS Canto II flow cytometer (BD biosciences). The results of flow cytometry were analyzed with FlowJo software.

Placental Macrophage Genotyping

To evaluate the maternal or fetal origin of isolated macrophages, the placentas of male fetuses were genotyped using a commercial kit containing probes for detection of specific centromeric regions of X and Y chromosomes, and chromosome 18 was used as control, according to the manufacturer's instructions (Fast Fish prenatal enumeration probe kit, Cytocell), as previously described (Huang et al., 2017). Briefly, after fixation using methanol and glacial acetic acid mix (3:1), placental macrophages were deposited onto a slide for fluorescence *in situ* hybridization including denaturation and hybridization of probes, and addition of Hoechst 33342 to DNA labeling. Slides were analyzed with a fluorescence microscope and the proportion of cells with maternal and fetal origin was determined.

Formation of Multinuclear Giant Cells

Placental macrophages (2×10^5 cells per assay) were incubated in 24 well plates containing glass coverslips in Dulbecco's Modified Eagle Medium (DMEM)-F12 supplemented with 10% FBS, 100 U/ml penicillin, and 50 µg/ml streptomycin (Life Technologies) for 9 days, as previously described (Ben Amara et al., 2013). Every 3 days, the presence of MGCs was determined by DNA staining and labeling of filamentous actin (F-actin) with Hoechst 3342 and phalloidin-488 (Life Technologies), respectively. The formation of MGCs was then analyzed by confocal microscopy using an LSM 800 Airyscan confocal microscope (Zeiss). The number of MGCs was determined and the results expressed in percentage of cells presenting at least two nuclei.

Lactate Dehydrogenase Assay

Isolated macrophages were incubated or not with *C. burnetii* (bacterium-to-cell ratio of 50:1) for 4 h and extensively washed to remove free bacteria (time designed as day 0). Cells were additionally cultured for 9 days, and the culture supernatants were collected at various time points. Lactate dehydrogenase release was quantified photometrically using Roche/Hitachi cobas c501 systems.

Microbicidal Activity of Placental Macrophages

Isolated macrophages (2×10^6 cells per assay) were cultured in DMEM-F12 supplemented with FBS and antibiotics. They were then incubated with *C. burnetii* (bacterium-to-cell ratio of 50:1) for 4 h and extensively washed to remove free bacteria (time designed as day 0). Placental macrophages were additionally cultured for 9 days and the uptake of bacteria was studied according two different approaches. First, macrophages were incubated with DID-labeled bacteria, stained with Hoechst 3342 and phalloidin-488, and the intracellular localization of bacteria was studied by confocal microscopy. Second, the uptake of bacteria was studied by qPCR. Briefly, DNA was extracted using a DNA Mini Kit (Qiagen), and infection was quantified using quantitative real time PCR (qPCR), as previously described (Ka et al., 2016). qPCR was performed with SYBR Green Fast Master mix (Roche Diagnostics) and the CXF Touch Real-Time PCR Detection System (Bio-Rad) using F (5′-GCACTAT TTTTAGCCG-GAACCTT-3′) and R (5′-TTGAGGAGAAAA-ACTGGATTGAGA-3′) primers that amplified a 225-bp fragment of the *C. burnetii com1* gene. A standard curve was generated using serial dilutions from a known concentration of *C. burnetii* DNA.

Inflammatory Response of Placental Macrophages

Two different approaches were used to study the inflammatory response of placental macrophages. First, the transcriptional response of 8 M1 genes and 10 M2 genes was studied by real-time quantitative PCR (qRT-PCR). For that purpose, placental macrophages (3×10^5 cells per assay) were incubated with *C. burnetii* (bacterium-to-cell ratio of 50:1), or 1 µg/ml lipopolysaccharide (LPS, Sigma-Aldrich) as control, for 6 h. Total RNA was extracted using RNeasy Mini Kit (Qiagen) and DNAse I treatment to eliminate DNA contaminants, as previously described (Mezouar et al., 2019b). The quantity and the quality of RNA were evaluated using a Nanodrop spectrophotometer (Nanodrop Technologies). Reverse transcription of isolated RNA was performed using a Moloney murine leukemia virus-reverse

transcriptase kit and oligo(dT) primers (Life Technologies). qRT-PCR was performed using SYBR Green Fast Master mix (Roche Diagnostics) and a CFX Touch real-time PCR Detection System (Bio-Rad) using specific primers (Ben Amara et al., 2013) listed in **Table 1**. The results were normalized using the housekeeping *actb* gene encoding β-actin endogenous control and were expressed as relative quantity (RQ) using the following formula: $RQ = 2^{-\Delta Ct}$, where $\Delta Ct = (Ct_{Target} - Ct_{Actin})$. The threshold cycle (Ct) was defined as the number of cycles required to detect the fluorescent signal. Data were analyzed using Clustvis software.

Second, the release of IFN-γ by placental macrophages was studied using specific immunoassay kits (R & D Systems). Briefly, placental macrophages (3×10^5 cells) were stimulated or not with *C. burnetii* (bacterium-to-cell ratio of 50:1) for 6 h. The supernatants were centrifuged at 1,000 × g for 10 min and frozen at −80°C. In some experiments, placental macrophages (1×10^6 cells) were incubated or not with *C. burnetii* for 4 h and, after washing to remove free bacteria (time designed as day 0), they were cultured for 9 days. Every 3 days, supernatants were collected and the release of IFN-γ was quantified. The sensitivity of the assays was 1.0 pg/ml.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism software version 7.0a (GraphPad Software, La Jolla California USA, www.graphpad.com). *In vitro* data were analyzed using the Mann-Whitney *t* test, one-way ANOVA, or ANOVA Kruskal-Wallis test. Correlations were evaluated using the non-parametric Spearman test. The results are presented as the mean score of at least three independent experiments, and $p < 0.05$ was considered statistically significant.

RESULTS

Characterization of Placental Macrophages

To ensure that placental $CD14^+$ cells from healthy donors were macrophages distinct from maternal circulating monocytes, we used flow cytometry to assess the expression of CD68, a canonical macrophage marker, by $CD14^+$ cells. $CD14^+$ cells also expressed CD68 (**Figure 1A**), demonstrating that they are placental macrophages. Then, we evaluated the fetal or maternal origin of isolated $CD14^+$ placental macrophages using fluorescence *in situ* hybridization technique and fluorescent probes targeting the X and Y chromosomes (**Figure 1B**). The study of five different placentas of male fetuses showed that approximately 30% of macrophages expressed XX (maternal) chromosomes and 70% expressed XY (fetal) chromosomes. These findings showed that placental $CD14^+$ macrophages from healthy donors were a mixed population of Hofbauer cells and decidual macrophages.

TABLE 1 | List of genes associated with macrophage polarization.

Gene symbol	Forward primer (5′-3′)	Reverse primer (5′-3′)
β-actin	GGAAATCGTGCGTGACATTA	AGGAGGAAGGCTGGAAGAG
M1 genes		
CCL2	GCTGGAGAGCTACAAGAGGATCA	TCTCTCTTGAGCTTGGTGACAAAA
NOS2	TTGCAAGCTGATGGTCAAGATC	CAACCCGAGCTCCTGGAA
EDN1	CCTCCATCCCCCATACTAAATC	GTCTCCAAAAATCAAGGACAGG
HESX1	GCTCGGGGAAAACAAACC	TTCTTCTGGCATTGGGTGA
IDO1	TCATCTCACAGACCACAAGTCA	CAAAATAGGAGGCAGTTCCAGT
TNFSF10	GAAAATAATCCCCACACGCTAC	GTCACTCTCTCCACCCTCACA
IL15RA	ATCTTCCGTCCCTCATCCTAAC	CTCAGCATCTCTCCCACCTTT
CXCL9	ACACTTGCGGATATTCTGGACT	GGGAGATGGTGTGTAATTGAT
IL2RA	GTTGAAGAGGAAGGGCAAAAC	ACTGGGAAGTTGGAATGAGATG
TNF	CATCTATCTGGGAGGGGTCTTC	AGGAGGGGGTAATAAAGGGATT
M2 genes		
ALOX15	AACTTCCACCAGGCTTCTCTC	GGGGGCTGAAATAACCAAAG
CCL13	GAGCAGAGAGGCAAAGAAACA	ATGTGAAGCAGCAAGTAGATGG
CCL23	CATCTCCTACACCCCACGAA	CATTCTCACGCAAACCTGAACT
CLEC4F	GGCATTTCTGGTAGAGTTCACA	ATACTTTCTGAGTGGGCAGGA
CHN2	AGAACTGTGGCTGGAAAATGAG	GTGGTTGTCGTTGTGTGTGAG
CTSC	GAGGTTGTGTCTTGTAGCCAGT	CCCCTTTTTGTAGTGGAGGAAG
FN1	ACACCTGGAGCAAGAAGGATAA	CCACAGAGTAGACCACACCACT
HRH1	ACTTGGAGGTGGTATGTGCTG	CTCAGGGCTTGCTTCTTGTAGT
SLC4A7	CCCTCAAAACAGTCCTCCTTCT	TTTCCTCATTCTTCTGCTCCTC
CD209	GGATACAAGAGCTTAGCAGGGTG	GCGTGAAGGAGAGGAGTTGC

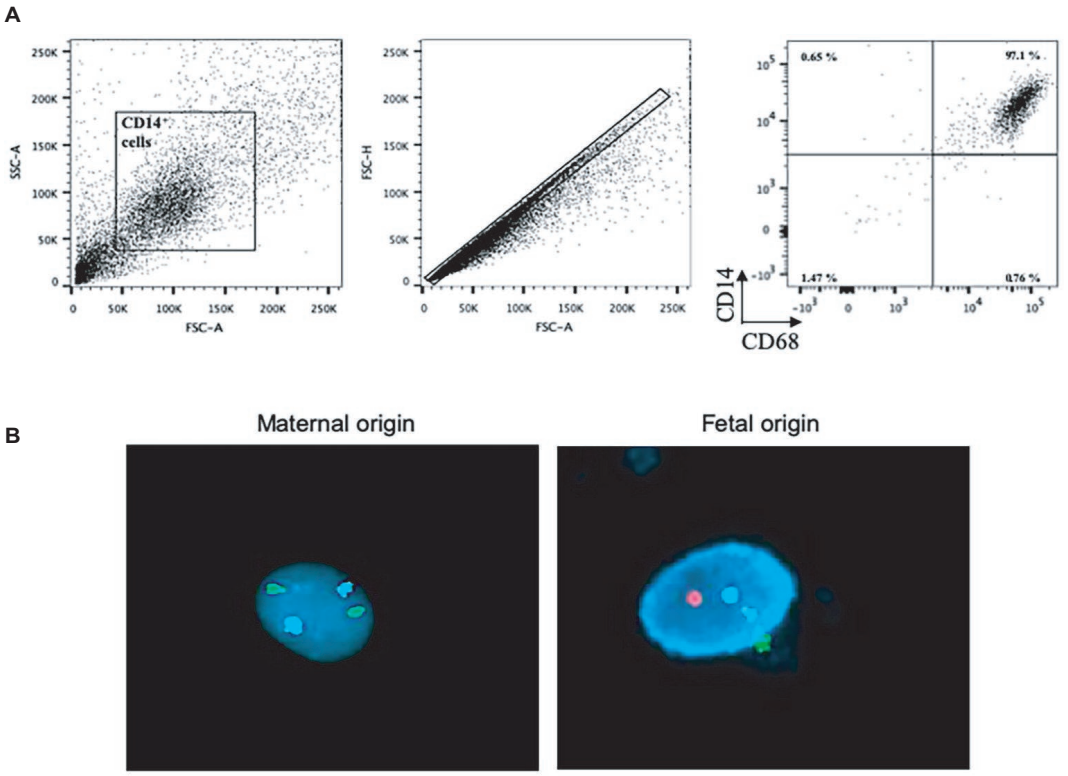

FIGURE 1 | Characterization of placental macrophages. **(A)** Placental CD14⁺ cells from 20 different placentas were analyzed for the expression of CD14 and CD68 by flow cytometry. Representative dot-blots are shown. **(B)** Placental macrophages from the placentas of male fetuses were analyzed for the expression of X, Y, and 18 chromosomes. Representative pictures show the X chromosome (in green), the Y chromosome (in red), and the chromosome 18 (nucleus in blue, used as control).

C. burnetii Infection of Placental Macrophages

Isolated CD14⁺ placental macrophages were infected or not with *C. burnetii* (bacterium-to-cell ratio of 50:1) for 4 h (**Figure 2A**) and cells were washed to remove unbound bacteria (this time was defined as day 0). Then, every 3 days of infection, the lactate dehydrogenase (LDH) release was quantified. No significant differences were observed between infected and resting placental macrophages (**Figure 2B**). The uptake, replication, or elimination of organisms by macrophages was observed (**Figure 2C**), and quantified by evaluation of the number of bacterial DNA copies using qPCR targeting the *com1* gene (**Figure 2D**). As a single *com1* gene is found per bacterium, it was easy to deduce that about 30 bacteria infected placental macrophages at day 0. A significant and steady decrease in the number of bacterial DNA copies was observed. At day 3, the number of bacterial DNA copies ($1.6 \pm 0.9 \times 10^7$) significantly ($p = 0.0049$) decreased (75%) to reach 8.11% of initial value at day 6. At day 9, the number of bacterial copies represented only 1.22% of the number found at day 0 (**Figure 2D**) with as many live bacteria as dead (**Figure 2E**). Taken together, these results provide evidence that placental macrophages were able to internalize *C. burnetii* organisms and that they had a microbicidal effect on these bacteria.

C. burnetii Infection and Multinuclear Giant Cell Formation

Because placental macrophages spontaneously form MGCs after culture, we wondered if *C. burnetii* interfered with MGC formation. Placental macrophages were infected with *C. burnetii* (bacterium-to-cell ratio of 50:1) for 4 h (day 0), washed to discard unbound bacteria and the formation of MGCs was quantified from day 0 to day 9. MGC formation was similar in uninfected and *C. burnetii*-infected macrophages (**Figure 3A**), demonstrating that *C. burnetii* did not prevent MGC formation. We then assessed the ability of these MGCs to ingest *C. burnetii*. MGCs formed after 3 days of culture were able to ingest bacteria as demonstrated by confocal imaging (**Figure 3B**). Interestingly, the formation of MGCs and *C. burnetii* DNA copies were significantly correlated ($R^2 = -0.88$, $p = 0.043$) (**Figure 3C**), suggesting that MGCs derived from placental macrophages were involved in *C. burnetii* elimination.

Molecular Mechanism of *C. burnetii* Elimination

To understand how bacteria were eliminated by placental macrophages, we investigated their transcriptional response to *C. burnetii*, and LPS as control, after 6 h of incubation. First, the expression of 8 M1-related genes including *IL-15RA*, *CXCL9*,

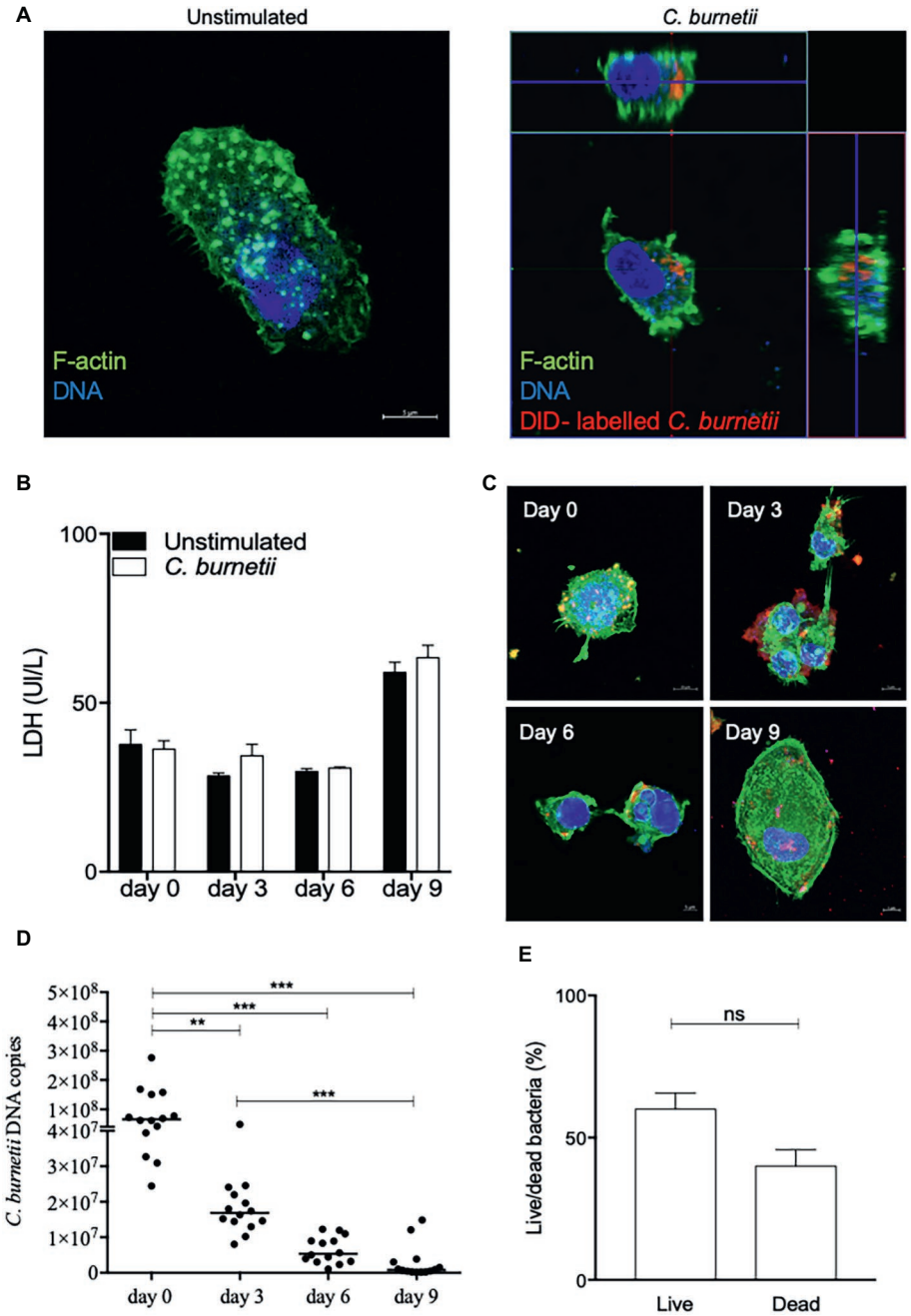

FIGURE 2 | Microbicidal activity of placental macrophages. Placental CD14⁺ macrophages (2 × 10^6 cells per assay) were infected or not by *C. burnetii* (bacterium-to-cell ratio of 50:1) for 4 h. **(A)** Confocal pictures show placental macrophage infected of not by DID-labeled bacteria. Infected macrophages were illustrated by the sections at the top and the right of the image was bacteria indicated in red, F-actin labeled with phalloidin-488 in green, and nucleus stained with Hoechst 3342 in blue. **(B)** Placental macrophages were incubated with *C. burnetii* for 4 h (day 0), then washed to eliminate free bacteria and incubated for 9 days. Lactate dehydrogenase (LDH) quantification was performed at each time of kinetic time of infection (n = 4 placentas). **(C)** Pictures of placental macrophages infected by *C. burnetii* at 0-, 3-, 6- and 9-day post-infection. Bacteria are indicated in red, F-actin labeled with phalloidin-488 in green and nucleus stained with Hoechst 3342 in blue. **(D)** Every 3 days, the number of bacterial DNA copies was evaluated by qPCR (n = 14 placentas). $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$. **(E)** Percentage of live/dead bacteria number was evaluated per macrophages at each time (ns = not significant).

EDN1, *IDO1*, *TNF*, *TNFSF10*, *IL-2RA*, and *HESXI* and 10 M2-associated genes including *CLEC4F*, *CCL13*, *SCL4A7*, *CTSC*, *HRH1*, *CHN2*, *CD209*, *ALOX15*, *FN1,* and *CCL23* was evaluated by qRT-PCR. Using a principal component analysis approach of M1 genes, we found that unstimulated and LPS-stimulated macrophages were associated whereas *C. burnetii*-stimulated

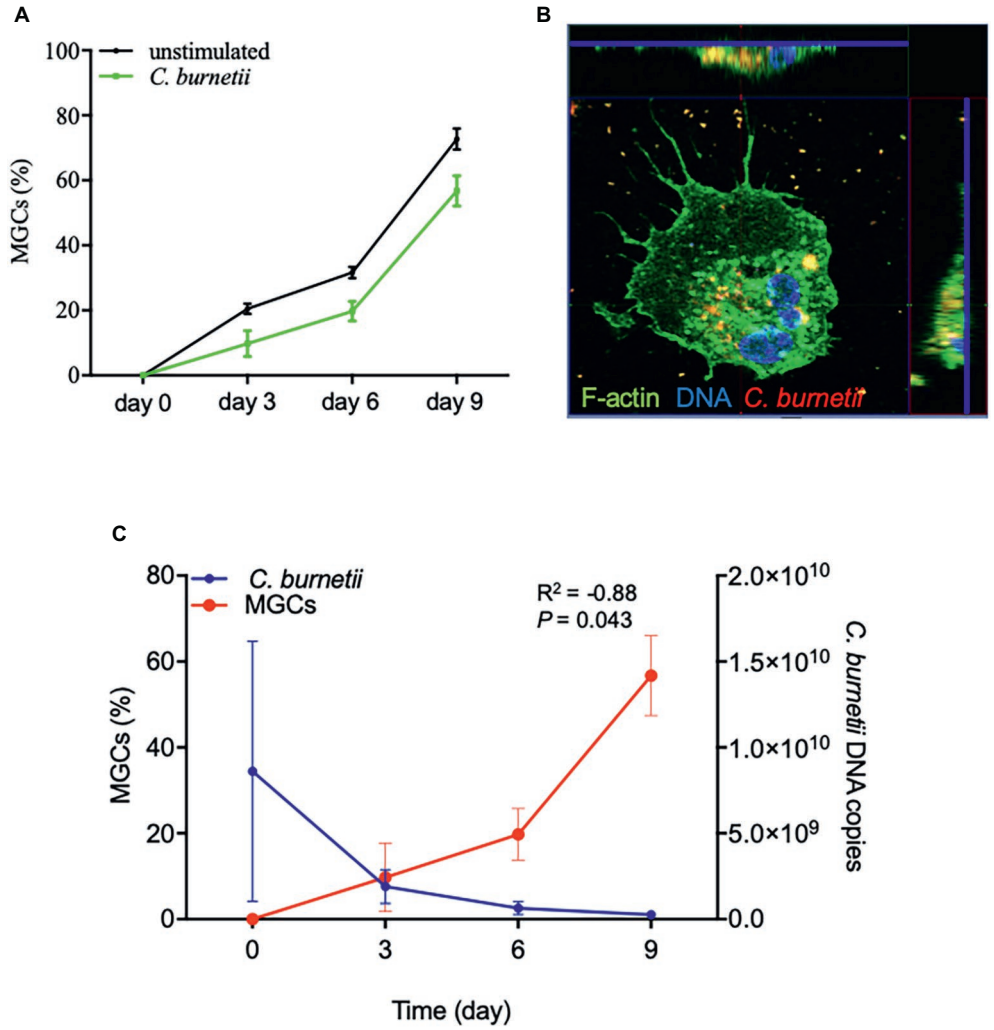

FIGURE 3 | MGC formation and *C. burnetii* infection. Isolated placental macrophages (2×10^5 cells per assay) from eight different placentas were stimulated by *C. burnetii* (bacterium-to-cell ratio of 50:1) for 4 h, then washed to eliminate unbound bacteria and cultivated for 9 days. **(A)** The time course of the number of MGCs was determined by optical determination and the results expressed in percentage of cells presenting at least two nuclei. **(B)** A representative confocal picture is shown after 3 days with F-actin labeled with phalloidin-488 in green, nucleus stained with Hoechst 3342 in blue, and bacteria in red. Intracellular bacteria were observed in the sections found at the top and the right of the image. **(C)** The analyses of MGC formation and *C. burnetii* survival performed using four placentas are correlated ($R^2 = -0.88$, $p = 0.043$).

macrophages were in an eccentric position. In contrast, the expression of M2 genes was strongly interrelated in unstimulated, LPS- and *C. burnetii*-stimulated cells (**Figure 4A**). A hierarchical clustering approach showed that modulated genes of placental macrophages stimulated by *C. burnetii* formed a cluster distinct from those of unstimulated and LPS-stimulated macrophages (**Figure 4B**). M1 genes are found in a different cluster than that of M2 genes, even if they included the *CLEC4F*, *CCL13*, and *SCLC4A7* M2-related genes. Interestingly, *C. burnetii* induced the up-modulation of all M1 genes compared to unstimulated and LPS-stimulated cells. Contrary to *C. burnetii*, LPS stimulation of placental macrophages led to the up-modulation of M2 genes. Taken together, these results demonstrated that *C. burnetii*, and not LPS, induced a M1-type transcriptional profile.

Second, we investigated IFN-γ release by placental macrophages stimulated by *C. burnetii*. Placental macrophages stimulated by *C. burnetii* induced a significant increase of IFN-γ release compared to unstimulated cells ($p = 0.038$, One-way ANOVA) (**Figure 5A**). Then, every 3 days of infection we showed a gradual increase of IFN-γ release over time ($p = 0.0041$, ANOVA Kruskal-Wallis test) (**Figure 5B**), suggesting a key role for this cytokine in cell response to bacterial infection. Finally, while investigating a relationship between IFN-γ release and *C. burnetii* survival, we showed a significant correlation between these two data ($R^2 = -1$, $p = 0.0417$, non-parametric Spearman test) (**Figure 5C**). Collectively, these results suggest that IFN-γ production was correlated with *C. burnetii* elimination.

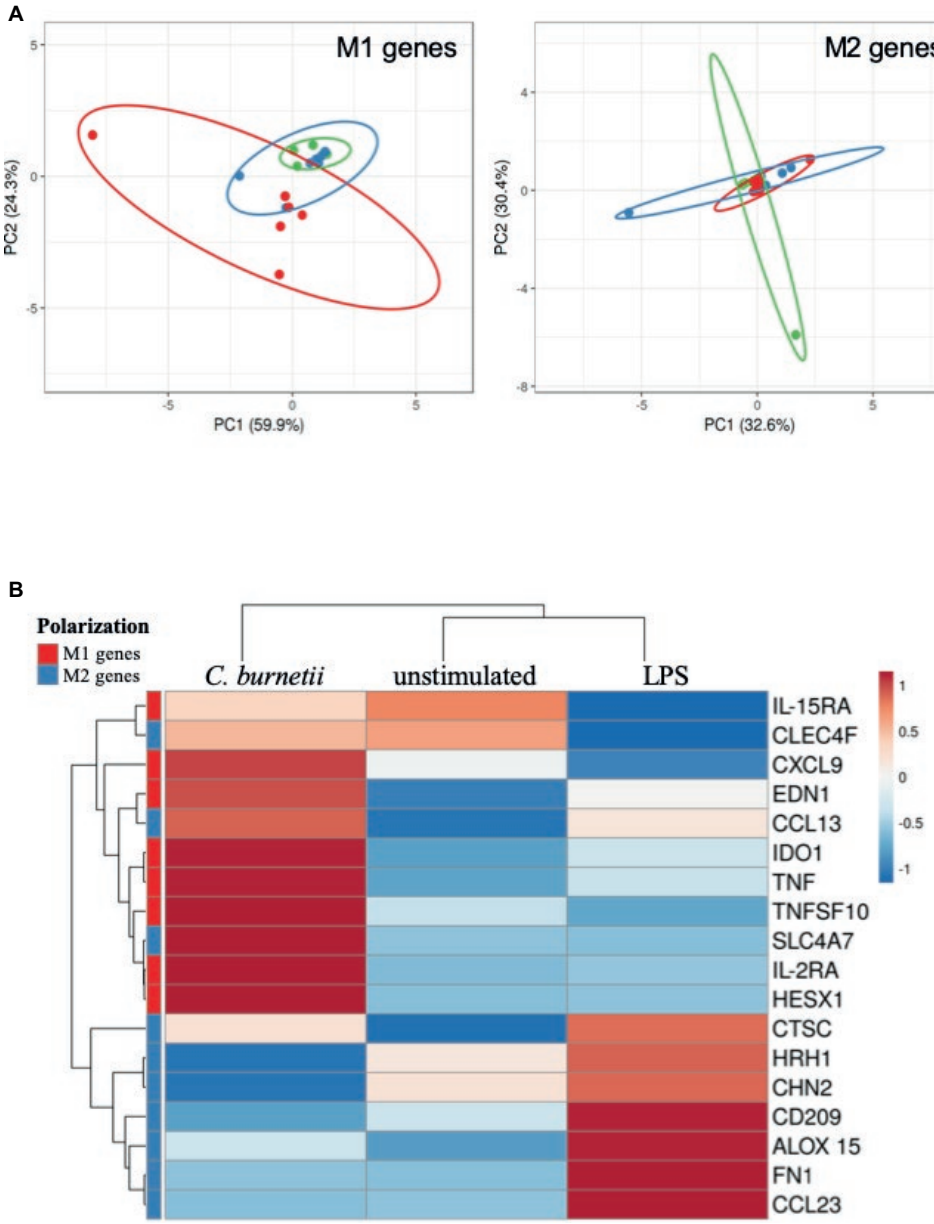

FIGURE 4 | Polarization profile of placental macrophages stimulated by *C. burnetii.* Placental macrophages (1×10^6 cells per assay) were incubated with *C. burnetii* (bacterium-to-cell ratio of 50:1) or 1 μg/ml LPS for 6 h, and their M1/M2 transcriptional response was analyzed by qRT-PCR. **(A)** A principal component analysis showed the repartition of unstimulated placental macrophages (in green, six placentas), *C. burnetii*-stimulated (in red, seven placentas), and LPS-stimulated macrophages (in blue, seven placentas) according to the expression of M1 genes (left panel) and M2 genes (right panel). **(B)** A heat-map analysis showed the modulation of the relative quantity expression of M1 genes (in red) and M2 genes (in blue) when placental macrophages were unstimulated or stimulated by *C. burnetii* or LPS.

DISCUSSION

In the present study, we first characterized $CD14^+$ cells isolated from full-term placentas. We found that more than 97% of $CD14^+$ placental cells expressed CD68, a marker associated with macrophage differentiation confirming that $CD14^+$ placental cells were macrophages, not circulating monocytes (Pinhal-Enfield et al., 2012). Next, we quantified fetal and maternal cells in placental macrophages by genotyping. We found a mixed cell population with maternal and fetal origins, even if the proportion of fetal macrophages seemed to be higher than that of maternal cells. In previous studies, the origin of placental macrophages (Hofbauer cells *versus* decidual macrophages) was based on the location of these cells within the placenta tissue (Reyes and Golos, 2018). These findings highlight the phenotype and origin of placental macrophage populations.

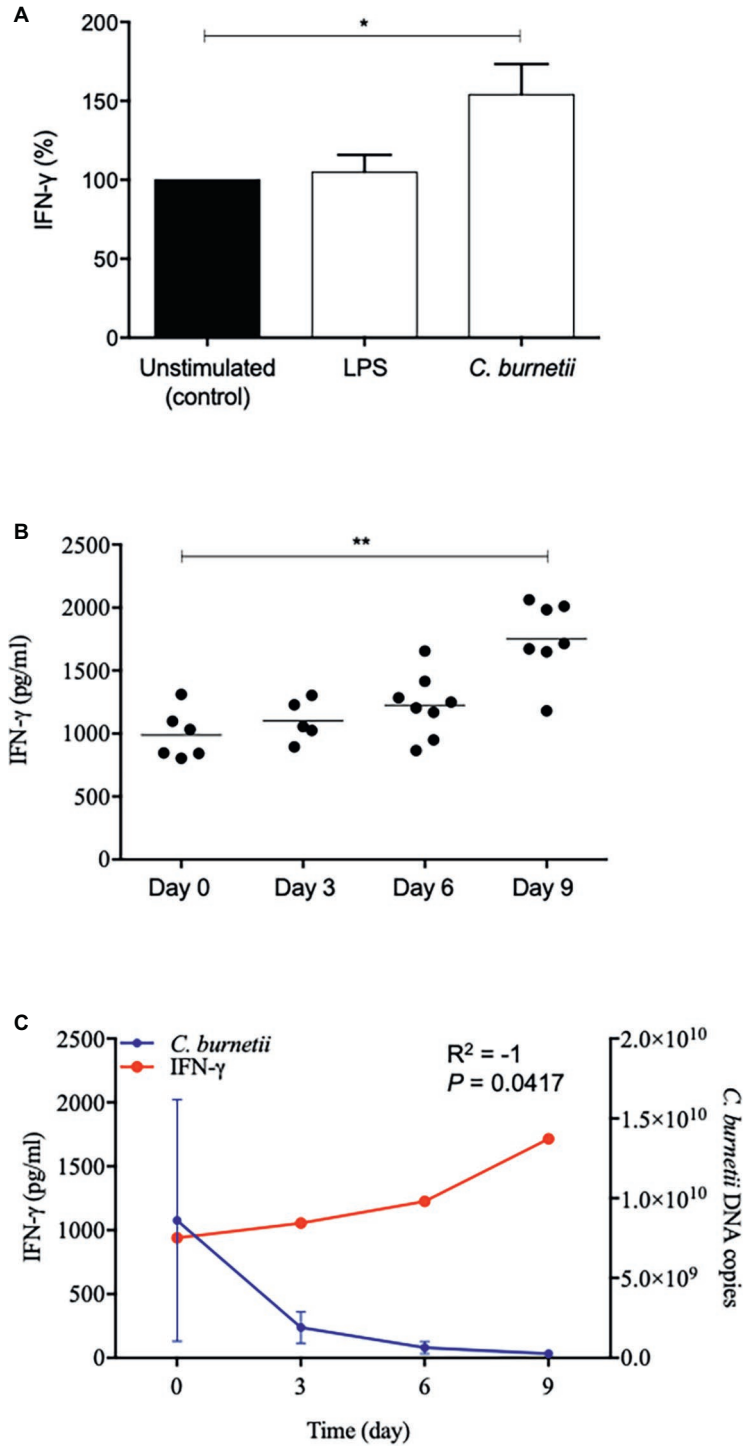

FIGURE 5 | IFN-γ release and *C. burnetii* infection. **(A** and **B)** Placental macrophages (1 × 10^6 cells per assay) were incubated or not with *C. burnetii* (bacterium-to-cell ratio of 50:1) or 1 μg/ml LPS for 4 h (day 0) and then washed to eliminate free bacteria. The release of IFN-γ was quantified at 0-, 3-, 6-, and 9-day post-infection or unstimulated cells as control when six placentas were used. **(C)** The release of IFN-γ by placental macrophages was correlated with *C. burnetii* survival ($R^2 = -1$, $p = 0.0417$) when four placentas were used. *$p \leq 0.05$ and **$p \leq 0.01$.

Here, we provided evidence that placental macrophages belong to the target cells for *C. burnetii,* as determined by the efficiency of bacterial uptake. This finding amplifies our previous observations that BeWo trophoblast cell line (Ben Amara et al., 2010) and decidual dendritic cells (Gorvel et al., 2014) are able to internalize *C. burnetii* whereas placental mast

cells exhibit a powerful extracellular antimicrobial mechanism against the bacterium (Mezouar et al., 2019c,d). In contrast to BeWo trophoblast cell line, *C. burnetii* was eliminated by macrophages from full-term placentas. The use of macrophages from full-term placentas is consistent with the observation that the risk of fetal infection by *C. burnetii* is higher during the first and second trimesters than during the third trimester (Eldin et al., 2017).

C. burnetii did not prevent the spontaneous formation of MGCs, a peculiar property of placental macrophages (Ben Amara et al., 2013; Belhareth et al., 2018). This is distinct from infectious placental pathologies such as chorioamnionitis in which MGC formation is prevented (Ben Amara et al., 2013). Additionally, MGC formation and *C. burnetii* elimination were correlated, suggesting a microbicidal role of these cells derived from placental macrophages. This hypothesis is supported by the observation that MGCs from other origins can play an anti-microbial role (Enelow et al., 1992; Hernandez-Pando et al., 2000).

It is well known that the functional properties of macrophages including microbicidal activity are controlled by their microenvironment. We found that *C. burnetii* induced a transcriptional program consisting of M1-related genes. Surprisingly, LPS, a powerful M1 agonist of tissue macrophages, rather induced an M2 program, demonstrating that the placental microenvironment governs the inflammatory or the immunoregulatory activity of placental macrophages. This is distinct from our previous results with monocyte-derived macrophages that express an atypical M2 program in response to *C. burnetii* associated with bacterial persistence (Benoit et al., 2008a). We suggest that *C. burnetii* induces a M1 program in placental macrophages that, in turn, plays a role in the elimination of the bacteria.

Interestingly, we showed that two members of the TNF family, including TNF and TNFSF10, were up-modulated by *C. burnetii* infection compared to LPS stimulation. A large variety of anti-microbial responses have been attributed to genes of the TNF family such as direct killing of infected cells, inhibition of intracellular pathogen or induction of apoptosis (Rahman and McFadden, 2006). Here, we found an absence of cell apoptosis and a strong anti-microbial response. The anti-microbial role of TNF has been previously reported using mice model. Indeed, during *C. burnetii* infection TNF deficient mice presented a defect of infection control as for other pathogens such as *Listeria monocytogenes* or *Mycobacterium tuberculosis* (Rahman and McFadden, 2006; Andoh et al., 2007). Interestingly, during pregnancy an excess of TNF production by inflammatory immune cells leads to the abrogation of fetus tolerance and its rejection as observed in pregnant women with *C. burnetii* infection (Chabtini et al., 2013; Eldin et al., 2017). In contrast, other inflammatory cytokines such as IL-1β are not specifically modulated by *C. burnetii* (data not shown). All together these findings suggested that further investigations are needed to clarify the role of genes for TNF family in the placental response during *C. burnetii* infection.

We also found that *C. burnetii* stimulated the release of large amounts of IFN-γ by placental macrophages. IFN-γ is known to be produced by innate lymphoid cells, NK and Th1 cells but tissue macrophages are generally considered as poor producers of IFN-γ. The placental context plays likely an important role in the ability of placental cells to produce IFN-γ. Indeed, trophoblasts produce high levels of type III interferons (Aboagye-Mathiesen et al., 1994; Lee et al., 2001) that are likely to be involved in pregnancy. The role of the IFN-γ produced by placental macrophages remains elusive. Interestingly, the release of IFN-γ by placental macrophages and their microbicidal activity were significantly correlated, suggesting that an autocrine loop could lead to their microbicidal activity. Additionally, IFN-γ was found involved in the increased expression of IDO1 leading to the deprivation of tryptophan, as essential amino acid for bacteria (Nelp et al., 2018). Interestingly, we found an up modulation of IDO1 in *C. burnetii* infected placental macrophages. Indirectly it has been previously reported that this process lead the inhibition of bacterial growth (Pantoja et al., 2000). This result is reminiscent of the observations that the co-culture of decidual macrophages and NK cells increases the production of IFN-γ, which contributes to control the infection of macrophages with HIV (Quillay et al., 2016).

In conclusion, our results showed that macrophages from full-term placentas were resistant to *C. burnetii* infection; such resistance may be related to an autocrine effect of IFN-γ. This study provides new insight into the pathophysiology of Q fever disease at the placenta level and suggests the microbicidal role of MGCs.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the manuscript/supplementary files.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by "Comité d'Ethique d'Aix-Marseille Université" (number 08-012). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SM and J-LM conceived and designed the experiments. SM, IB, AB, and AD performed experiments. CC, JB, and CB performed flow cytometry, genotype, and LDH assay experiments, respectively. SM, IB, AB, and AD analyzed the data. SM, PH, MK, and J-LM supervised the study. SM and J-LM wrote the paper.

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frontiers in Microbiology

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Yellow Fever Vaccination in a Mouse Model Is Associated With Uninterrupted Pregnancies and Viable Neonates Except When Administered at Implantation Period

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The potential risk of yellow fever (YF) infection in unvaccinated pregnant women has aroused serious concerns. In this study, we evaluated the effect of the YF vaccine during gestation using a mouse model, analyzing placental structure, immunolocalization of the virus antigen, and viral activity at the maternal-fetal barrier and in the maternal liver and fetus. The YF vaccine (17DD) was administered subcutaneously at a dose of 2.0 $\log_{10}$ PFU to CD-1 mice on gestational days (gd) 0.5, 5.5, and 11.5 (n = 5–10/group). The control group received sterile saline (n = 5–10/group). Maternal liver, implantation sites with fetus, and placentas were collected on gd18.5. The numbers of implantation sites, reabsorbed embryos, and stillborn fetuses were counted, and placentas and live fetuses were weighed. Tissues (placenta, fetuses, and liver) of vaccinated pregnant mice on gd5.5 (n = 15) were paraffin-embedded in 10% buffered-formalin and collected in TRIzol for immunolocalization of YF vaccine virus and PCR, respectively. PCR products were also subjected to automated sequence analysis. Fetal growth restriction ($p < 0.0001$) and a significant decrease in fetal viability ($p < 0.0001$) occurred only when the vaccine was administered on gd5.5. In stillbirths, the viral antigen was consistently immunolocalized at the maternal-fetal barrier and in fetal organs, suggesting a transplacental transfer. In stillbirths, RNA of the vaccine virus was also detected by reverse transcriptase-PCR indicating viral activity in the maternal liver and fetal tissues. In conclusion, the findings of this study in the mouse suggest that vaccination did not cause adverse outcomes with respect to fetal development except when administered during the early gestational stage, indicating the implantation period as a susceptible period in which the YF vaccine virus might interfere with pregnancy.

Keywords: yellow fever (YF) vaccine, pregnancy, congenital transmission, placenta, fetal losses

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INTRODUCTION

Yellow fever (YF) is an acute viral infection associated with hepatitis, jaundice, hemorrhage, and renal failure, and it may progress to death (Monath, 2008; Staples and Monath, 2011). Acute febrile syndrome and hemorrhagic phenomena are the most prominent manifestations of this disease. Vaccination and strategic programs for controlling the main vectors—hematophagous mosquitoes

of the genera *Aedes, Haemogogos,* and *Sabethes*—have been successfully implemented in many countries, even though the number of infected persons has increased worldwide over the last two decades (Monath, 2013; Monath and Vasconcelos, 2015). Large outbreaks have been reported in Africa as well as South and Central America, mainly in forested areas infested by the transmission vectors (Staples and Monath, 2011).

In Brazil, no urban outbreak of YF has occurred since the 1940s, when the urban cycle of transmission was eradicated. However, in 1997, YF viral infections were detected in North and Central Brazil, and a new outbreak was registered in 2008 in South and Southeast regions of the country (Monath and Vasconcelos, 2015; Waggoner et al., 2018). In 2017, YF virus (YFV) was detected in non-human primates, and human cases were reported in places where vaccination coverage was low (Leal et al., 2016; Fernandes et al., 2017), a situation that may have been aggravated by public hesitancy toward vaccines.

YF virus are single-stranded RNA viruses of the Flaviviridae family. The direct cytopathic effect of the virus and a potent host immune response are associated with the secretion of TGF-b, TNF-a, and IFN-g, which are believed to play roles in the manifestations and severity of the disease (Quaresma et al., 2006a,b, 2013; Woodson et al., 2011; Engelmann et al., 2014). Transcriptomic analyses of viscerotropic YF in a rhesus macaque model also showed that YF infection correlates with changes in cytokine gene expression before the emergence of clinical symptoms, suggesting these immune factors (cytokines) may influence the disease outcome (Engelmann et al., 2014). The results of *in vitro* studies have suggested that effects of infection on endothelial cells may also contribute to YF pathogenesis (Khaiboullina et al., 2005; Quaresma et al., 2006a). Inoculation of flaviviruses in monkeys indicates that the initial site of replication is the inoculation site in the skin followed by the lymph nodes, where additional replication occurs (Marchette et al., 1973; Monath and Barrett, 2003; Dean et al., 2005). The virus reaches the bloodstream via the lymphatic system and spreads to prevalent organs, such as the liver (Monath and Vasconcelos, 2015). In the liver, the virus infects the Kupffer cells and the hepatocytes, causing severe necrosis (Monath and Barrett, 2003).

The YF vaccine virus is a live attenuated virus, which is prepared and obtained by culturing the 17D strain virus in living chick embryos, and it can induce neutralizing antibodies and T lymphocyte responses. The vaccine differs from YFV by the loss of viscerotropism, despite its replicative activity in cell culture, and by 20 amino acid changes in the envelope protein (Lee and Lobigs, 2008; Monath et al., 2013). In monkeys, the YF 17D vaccine causes a transient low viremia (Monath and Barrett, 2003) accompanied by innate immune responses with detectable levels of cytokines and toll-like receptor-mediated signaling (Monath and Vasconcelos, 2015). Neutralizing antibodies are the principal mediators of protective immunity against flaviviruses (Monath et al., 2013).

After a single dose of the YF 17D vaccine, 80–90% of human subjects become seropositive by day 10 (Monath and Barrett, 2003). A small number of adverse events have been associated with vaccination in humans and monkeys, ranging from severe encephalitis and hepatic failure to neurological symptoms of benign prognosis (Monath and Barrett, 2003; Martins et al., 2015).

In pregnant women, Nishioka et al. (1998) found a relative risk of 2.29 for spontaneous abortions after vaccination. Another study performed on women who inadvertently received YF vaccine during pregnancy reported abortion, stillbirth, and malformation rates similar to those found in the general population (Nasidi et al., 1993; Robert et al., 1999; Suzano et al., 2006; D'Acremont et al., 2008). Nonetheless, due to the theoretical risk of maternal-fetal transmission associated with fetal hepatic and neuronal susceptibility to the YF 17D virus, there is a general recommendation to avoid vaccine administration during pregnancy except when epidemiologically justified (Hagmann et al., 2017). However, the absence of vaccination during pregnancy is a risk to the mother and fetus, thereby increasing the risk of infection to local mosquitoes. Therefore, it is imperative to disseminate knowledge and awareness on gestational vaccination.

A small number of viruses are transmitted from mother to fetus, showing the effectiveness of the hemochorial barrier against these infections (Marinho et al., 2017). However, the mechanisms by which the viruses overcome the placental barrier is still uncertain. Recent studies suggest that maternal immunity, time of gestation, coinfections, and many other factors may be associated with this effectiveness (Marinho et al., 2017).

In this study, we used a mouse model to analyze the effect of YF vaccination during gestation and to identify potential susceptible phases that might compromise embryo/fetal health. This study addressed the birth/mortality rates, as well as the morphology and localization of YF 17D virus in fetuses, placentas, and maternal tissues after vaccination through immunohistochemical reactions and polymerase chain reaction (PCR).

MATERIALS AND METHODS

Animals

Female adult mice (CD-1 mice, 3 months old) were caged overnight with males (1:1) and successful mating was verified the following morning. The presence of a vaginal plug indicated day 0.5 of gestation (gd). All animal care and experimental procedures were carried out according to the Brazilian Society of Science in Laboratory Animals (COBEA) and was approved by the Ethics Committee for Animal Research (CEEA) of Biomedical Sciences Institute of the University of São Paulo, Brazil.

Vaccination

The vaccination protocol was carried out at the Department of Cellular and Developmental Biology in the Biomedical Sciences Institute of the University of São Paulo, under the supervision of the Department of Immunization Center for Epidemiological Surveillance of the State of São Paulo. YF vaccine (17DD, parts 00PVFA028Z; 066VFA061Z; and 082VFB006Z) was obtained from the Oswaldo Cruz Foundation (Bio-Manguinhos, Rio de Janeiro, Brazil). The vaccine was reconstituted with 5 mL of saline

and administered subcutaneously at a dose of 2.0 $\log_{10}$ PFU in a final volume of 0.1 mL. In the control group, the vaccine was replaced by sterile saline (PBS, Gibco BRL, Grand Island, NY, United States).

Experimental Design

The experiments were divided into two phases. In the first phase, vaccination was carried out at different stages of pregnancy (0.5, 5.5, and 11.5 gestation days [gd]) for gestational parameter evaluation (n = 5–10 pregnant females/experimental or control group). Day 0.5 of gestation is a period when the zygote is in the lumen of the uterine tube; day 5.5 of gestation represent the onset of implantation, in which the trophoblast giant cells surrounding the blastocyst come into direct contact with the maternal blood; and day 11.5 of pregnancy is the usual time for placenta maturation (Adamson et al., 2002; Cross, 2005; Watson and Cross, 2005; Hu and Cross, 2009; Croy et al., 2014).

In the second phase, immunolocalization of the viral antigen and signals of viral activity were analyzed at periods in which the vaccination had caused relevant changes (gd5.5, n = 15 pregnant females/experimental groups and n = 10/control groups).

Sample Collection for Gestational Performance

Vaccinated and control animals were anesthetized with hydrochloride xylazine (Rompun 2%®, Bayer, São Paulo, Brazil) and ketamine (1:1, v/v, Ketalar, Bayer, São Paulo, Brazil) on gd18.5. Uterine horns were dissected under a stereoscopic microscope, and implantation sites with fetuses and their placentas were exposed. The fetuses were euthanized in a CO_2 chamber. Placentas, resorptions, and living and dead fetuses were counted and weighed. The total number of implantation sites and early reabsorbed embryos was evaluated by incubating the uterine horns in 10% ammonium sulfide for 10 min after the removal of the fetuses, placentas, and late resorptions (Salewsky, 1964).

Birth index (BI) and mortality rate (MR) were respectively calculated as follows:

$$\mathrm{BI} = \frac{\text{Total number of implantation sites} - \text{number of stillbirths and resorptions}}{\text{Total number of implantation sites}}$$

$$\mathrm{MR} = \frac{\text{Total number of implantation sites} - \text{number of live fetuses}}{\text{Total number of implantation sites}} \times 100$$

Results were expressed as the mean value ± SD, and Student's t-test was used to determine significant differences in comparison with age-control groups. A probability level of less than 5% was considered significant. Statistical analysis was performed using the program Statistical Package for Social Science for Windows.

Collection and Processing of Samples for Morphological Analysis

Under deep anesthesia, fragments of the maternal liver and the uterine horns were obtained. Placentas, fetuses (live fetuses were euthanized by CO_2 inhalation) and material resulting from resorptions were then dissected. Tissues were fixed in 10% buffered formalin and routinely processed for embedding in Histosec® (Merck KGaA, Darmstadt, Germany). Sections were either stained with hematoxylin and eosin or processed for immunoreactions and light microscopy analysis.

Sections of the maternal liver, placenta, and fetus of control females (n = 5) and vaccinated mothers with living (n = 5) and dead (stillbirth, n = 5) fetuses obtained from different mothers were assessed with immunohistochemical (IH) assays. At least three sections from each placenta (three placentas per animal) and of maternal liver and fetus were obtained from each experimental animal for analysis. Deparaffinized and hydrated sections were incubated in the citric acid solution (10 mM, pH 6.0) for 3 min at 60°C and thereafter blocked for 15 min in 3% hydrogen peroxide in distilled water. Sections were incubated for 1 h in M.O.M. mouse IgG blocking reagent (Vector Lab, Burlingame, CA, United States). Next, the samples were immunostained using the polyclonal mouse anti-YF virus antibody (Division of Medical Biology, Department of Virology, Adolfo Lutz Institute, São Paulo, Brazil) diluted at 1:2,000 in TBS containing 1% bovine serum albumin, for 30 min at 37°C, followed by 18 h at 4°C. Labeled polymer-HRP anti-mouse (EnVision + System HRP [DAB], Dako Cytomation) was used as a secondary antibody for 1 h at room temperature. Color development was obtained by incubating with DAB substrate-chromogen solution (0.05% 3,3′- diaminobenzidine in hydrogen peroxide, Sigma-Aldrich, St. Louis, MO, United Ststes) for 5 min. Sections were counterstained with Mayer's hematoxylin and examined using an Axioskop 2 light microscope (Carl Zeiss, Oberkochen, Germany). The images were captured with Axio Vision 4.7 software (Carl Zeiss, Oberkochen, Germany). Negative control was performed by omission of the primary antibody and/or by replacing this antibody with non-immune serum. The sensitivity of the reaction was tested by using immunoreactive liver samples of a patient diagnosed with YF who had a known expression of the viral antigen (positive control).

Detection of Viral RNA by RT-PCR

YF virus envelope protein gene fragments were detected through the PCR assay in maternal liver, placenta, fetal brain, and liver of live fetuses and stillbirths of vaccinated mothers on gd5.5 and in the placenta and liver of mothers vaccinated on gd0.5 and 11.5. Positive control reactions were performed using samples of the YF vaccine. Total RNA was isolated with TRIzol (Invitrogen™, Carlsbad, CA, United States) and suspended again in sterile distilled water according to Chomizynski and Sacchi (1987). All reagents were purchased from Sigma Aldrich (St. Louis, MO, United States), unless otherwise stated. RNA concentration and purity were determined by spectrophotometric measurement of absorbance at 260 nm, and the purity was determined at A260/A280 ratio. The RNA integrity was checked by using

1% agarose gel electrophoresis with 0.4 mol/L Tris-acetate and 0.001 mol/L EDTA buffer. Viral RNA was converted to cDNA using 5.0 μg of RNA, 5.0 μL of specific antisense primer (5′-GCT TTT CCA TAC CCA ATG AA-3′ (MG922934.1), 2.0 μL dNTPs Mix, 0.75 μL M-MLV reverse transcriptase (Invitrogen™), 6.0 μL reaction buffer 5 × and 3.0 μL DTT 0.1 M. The mixture was incubated at 37°C for 90 min and at 95°C for 5 min to inactivate the reverse transcriptase. The viral cDNA (6.0 μL) was amplified by PCR using a 2.5 μL 10 × PCR buffer (Biotools B&M Labs S.A., Madrid, Spain), 2.0 μL $MgCl_2$ (50 mM), 1.0 μL of dNTPs Mix, 0.5 μL DMSO 4%, 0.75 μL DNA polymerase (1 U/μL, Biotools B&M Labs S.A., Madrid, Spain), and 5.0 μL (10 pmol/μL) each of the forward and reverse primers (**1**: 5′-TAC CCT GGA GCA AGA CAA GT-3′; **2**: 5′-GCT TTT CCA TAC CCA ATG AA-3′). The PCR was performed in a Bio-Rad Gene Cycler™ (Bio-Rad Laboratories, Portland, ME, United States). Cycling conditions included denaturation at 94°C for 5 min, 35 PCR cycles of 94°C for 1 min, 58°C for 2 min, 72°C for 3 min and the last step for a final extension at 72°C for 10 min in a thermocycler. PCR was performed using reverse transcripted products from the vaccine's RNA as a template. PCR products were analyzed on 1% agarose gel electrophoresis using a molecular weight marker DNA (100 base pairs, DNA Ladder, Ludwig Biotec, Nova Alvorada, Brazil) as reference. The gel was exposed to a Molecular Imaging screen (G: Box Chemil-R, Syngene, Frederick, MD, United States) for computerized gel documentation (Scion image program, Scion Corp., Frederick, MD, United States). The identity of the 482 bp-amplified products was confirmed by sequence analysis (automated sequence analysis, MegaBACE 1000, GE Healthcare, Buckinghamshire, United Kingdom) at the Center for Human Genome Studies at the University of São Paulo (Brazil). Fluorograms were analyzed using the Cimarron 3.12 base-caller software. A sequence database search was performed using the BLAST network service of the National Center for Biotechnology Information[1].

RESULTS

Vaccination on gd0.5 and 11.5 did not affect the average number of implantation sites and fetal resorption per pregnant female in comparison to controls (**Table 1**). In the group that received vaccination on gd5.5, the mean number of degenerated fetuses and stillbirths and the number of embryo/fetal losses significantly increased (from 0.13 in control group to 1.37 in vaccinated group, $p = 0.01$; and from 0.38 in controls to 3.5 in vaccinated animals, $p = 0.002$, respectively), resulting in an increased mortality rate (from 5.1% in control group to 33.1% in vaccinated group, $p = 0.001$) in comparison to sham controls, as shown in **Table 1**.

Fetal weight gain was significantly lower only when the vaccine was administered in pregnant animals on gd5.5 ($p = 0.004$; **Table 2**). Placental weight was not altered significantly in any vaccinated group compared to the control group. These data indicate the susceptible period to YF vaccination during pregnancy, justifying the subsequent experimental procedures on only day 5.5 of gestation.

Fetuses were macroscopically divided into live fetuses, stillbirths (absence of heartbeat, but no visible degeneration and therefore, considered as late-dead fetuses), early-dead fetuses (with apparent developmental delay and degenerative signals), and post-implantation resorptions (implantation site with no recognizable fetal structures) (**Figure 1**). The subsequent analyses were performed on live fetuses and stillbirths.

In the maternal liver as well as in the liver of live fetuses of the vaccinated females on gd5.5, the viral antigen was rarely immunolocalized (**Figures 2A,B,a,b**). For IH reactions, no immunolabeling was detected in the non-vaccinated group (not shown) in the negative control, which was performed by omitting the primary antibody (**Figures 2G–I**). Biopsies of human liver diagnosed with YF were used as positive control for the IH reactions (**Figure 2J**).

[1]http://www.ncbi.nlm.nih.gov/BLAST/

TABLE 2 | Effect of anti-yellow fever vaccination administered on gestation days 0.5, 5.5, or 11.5 on placental and fetal weights.

	n	Fetal weight (g)	Placental weight (g)
Control gd 0.5	53	0.89 ± 0.17	0.15 ± 0.03
Vaccinated gd 0.5	50	0.87 ± 0.09	0.14 ± 0.02
Control gd 5.5	180	0.95 ± 0.10	0.14 ± 0.04
Vaccinated gd 5.5	158	0.82[a] ± 0.12	0.13 ± 0.01
Control gd 11.5	83	0.91 ± 0.11	0.13 ± 0.02
Vaccinated gd 11.5	57	0.88 ± 0.06	0.13 ± 0.02

Values correspond to mean ± SD; values indicated by letters highlight statistical differences in comparison to the respective age-control. Student's t-test ([a]p = 0.004).

TABLE 1 | Effect of anti-yellow fever vaccination administered on gestation days 0.5, 5.5, and 11.5 on the gestational parameters.

	n	Implantation sites	Number of fetuses	Degenerated fetuses/stillbirths	Embryo/fetal losses	Mortality rate (%)
Control gd 0.5	56	11.2 ± 1.64	10.6 ± 1.95	0.4 ± 0.55	1.0 ± 1.2	9.2 ± 10.3
Vaccinated gd 0.5	53	10.6 ± 3.71	10.0 ± 4.06	0.4 ± 0.89	1.0 ± 1.0	11.6 ± 11.1
Control gd 5.5	96	12.0 ± 2.33	11.7 ± 2.25	0.13 ± 0.35	0.38 ± 0.52	5.1 ± 7.3
Vaccinated gd 5.5	97	12.1 ± 1.89	9.5 ± 1.77	1.37[a] ± 1.19	3.5[b] ± 1.69	33.1[c] ± 17.1
Control gd 11.5	83	10.3 ± 2.38	9.8 ± 2.23	0.25 ± 0.46	0.75 ± 1.16	6.7 ± 9.9
Vaccinated gd11.5	54	10.8 ± 2.17	10.0 ± 2.65	0.4 ± 0.55	1.2 ± 1.10	7.6 ± 9.0

n, Total number of implantation sites. Values correspond to mean number per pregnant female ± SD; [a−c]highlights statistical differences in comparison to the respective age-control. [a]p = 0.01; [b]p = 0.002; [c]p = 0.001 (Student's t-test).

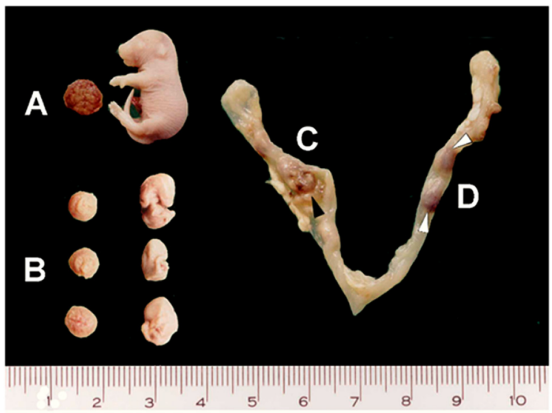

FIGURE 1 | Macroscopic fetal features on gd18.5. Samples from vaccinated pregnant mice present different fetal development, as shown in **(A)** apparently normal (living and stillborn) fetuses, **(B)** early-dead fetuses and **(C,D)** resorptions (black and white arrowheads).

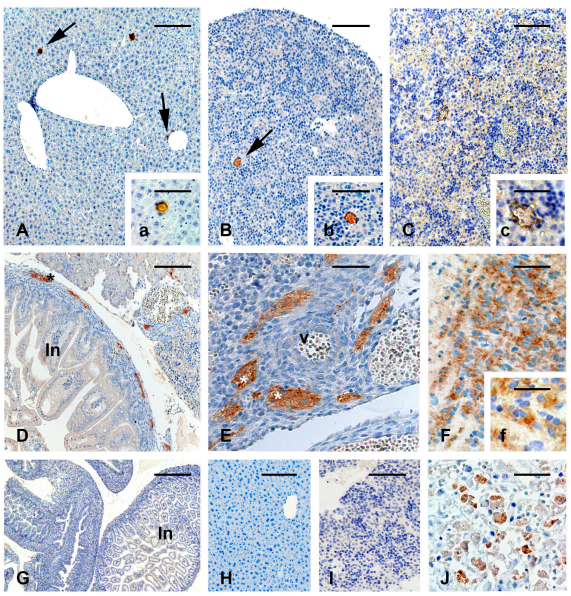

FIGURE 2 | Immunolocalization of the yellow fever (YF) antigen after vaccination on gd 5.5. **(A–C)** In maternal and fetal liver, immunoreactive areas are rare **(A,a)**. Liver of one vaccinated pregnant female **(B,b)**. Liver of a live fetus **(C,c)**. Liver of a stillborn. Reactivity is characterized by a brown colored (arrows) **(D–F)**. Immunolocalization of YF antigen in stillbirths shows reactivity for the virus vaccine in nervous cells in the peripheral area of the developing digestive tract **(D)** and in nerves **(E)** surrounding one arterial vessel (v). Strong reactivity can also be seen in the brain **(F,f)**. Figures **(G–I)** are negative controls of the reaction, in which the primary antibody was replaced by non-immune serum. Figure **(J)** shows the positive control of the IH reaction (liver of a patient with YF). Bars in **(A)** = 150 μm, in **(a,b**,**E)** = 100 μm, in **(B,C,H,I)** = 200 μm, in **(c,F)** = 60 μm, in **(D) =** 350 μm, in **(f)** = 50 μm, in **(G)** = 600 μm, in **(J)** = 75 μm.

Unlike live fetuses, reactivity to YFV in the stillbirths was intense and distributed in the cells of several organs (**Figures 2C–F**). Reactivity was seen in the liver (**Figures 2C,c**), nervous tissue in the developing intestine (**Figure 2D**), cells surrounding arterial vessels (**Figure 2E**), and in the fetal brain (**Figures 2F,f**).

Histological analysis of placentas from control and viable fetuses of vaccinated animals showed typical morphological features. In live fetuses, immunoreactions revealed the presence of the viral antigen in the trophoblast cells of the junctional zone (trophoblast giant cells, **Figure 3A**) and spongiotrophoblast cells (**Figure 3C**), and only occasionally in the cells of the labyrinthine layers (**Figure 3D**).

In general, placentas from stillbirths showed common characteristics in relation to age-control placentas from vaccinated and control living fetuses. Occasional morphological changes found in this group included the scattering of glycogen cell clusters toward the labyrinthine zone. Viral antigen was seen in the trophoblast giant cells (**Figure 3E**), the spongiotrophoblast cells (**Figure 3F**), and the glycogen cells

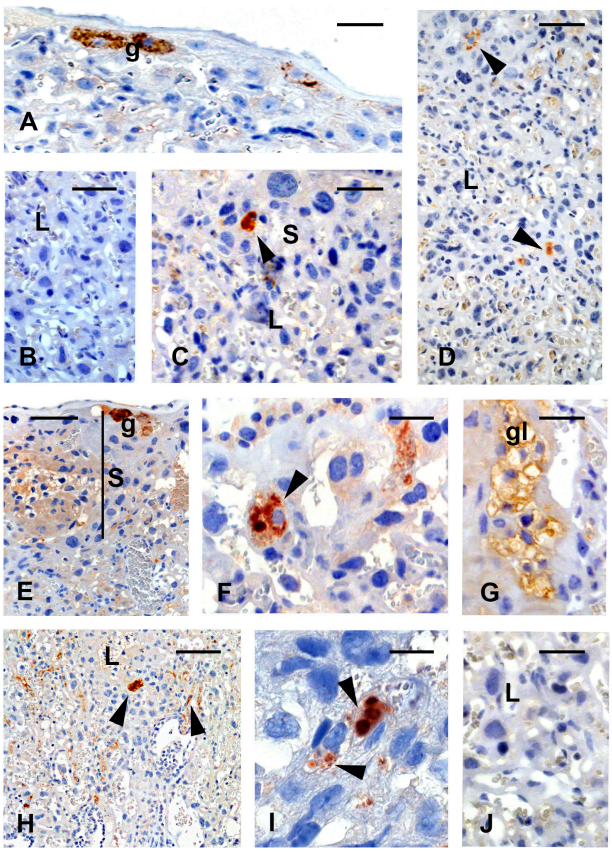

FIGURE 3 | Immunolocalization of the YF antigen after vaccination on gd5.5. Placentas of live fetuses **(A–D)** and stillbirths **(E–J)**. Reactions are characterized by brownish color. The viral antigen is seen at the junctional zone in trophoblast giant cells **(A, g)** and **(C**, arrowhead) in cells of the spongiotrophoblast (S) area. Few cells **(D**, arrowheads) reacted with the antibody against YF virus (YFV) in the labyrinthine region. In stillbirths, the immunoreaction in spongiotrophoblast (S) is intense, in giant cells **(E, g)**, spongiotrophoblast cells **(F**, arrowhead) and in glycogen cells **(G, gl) (H,I)**. Reactivity is also seen in the labyrinth (L, arrowheads). Figures **(B,J)** are negative controls of the reaction. Bars in **(A,D,E)** = 120 μm, in **(B)** = 100 μm, in **(C,G,J)** = 80 μm, in **(F)** = 60 μm, in **(H)** = 240 μm, in **(I)** = 25 μm.

(**Figure 3G**). Reactivity was stronger in the labyrinthine area (**Figures 3H,I**) in different cell types. The negative IH control did not show any reaction in these tissues (**Figures 3B,J**).

The YF vaccine was also detected by RT-PCR using the YFV consensus primer pair. RT-PCR analysis of YFV produced amplicons, as shown in **Figures 4A,B**, in maternal liver, placenta, fetal brain, and liver in stillbirths. In live fetuses, only traces of the amplicons were found in part of the samples (in tissues of 2 fetuses from 7 analyzed). DNA amplification was not observed in the negative control nor in samples from vaccination on gd0.5 and 11.5 (not shown). YFV identity was confirmed by sequencing PCR products obtained from the brain samples of stillbirths ($n = 3$). The sequence was aligned to the YFV strain 17DD-Brazil, and 100% identity was observed (**Figure 4C**).

DISCUSSION

We examined the effect of vaccination against YFV at three different gestational periods in mice and found clear evidence of changes in pregnancy outcomes and fetal infection only when the vaccine was administered during the embryo implantation period (gd 5.5). Vaccination on this particular day of gestation led to increased embryo/fetal losses and growth restriction in live fetuses. The vaccine virus was only occasionally observed in the liver of the live fetuses but consistently found in stillbirths' tissues. Immunolocalization of the vaccine virus was detected in the placenta of both live fetuses and stillbirths, but was more intense in samples of the stillborn fetuses. The presence of the virus in the placenta and fetal organs in the stillbirths indicated that a vertical passage of the vaccine virus may have occurred. In living fetuses, by contrast, the presence of the virus only in the placenta suggests the maternal-fetal interface may assume a protective role. The differences in the fate of individuals belonging to the same litter, however, are not clearly understood.

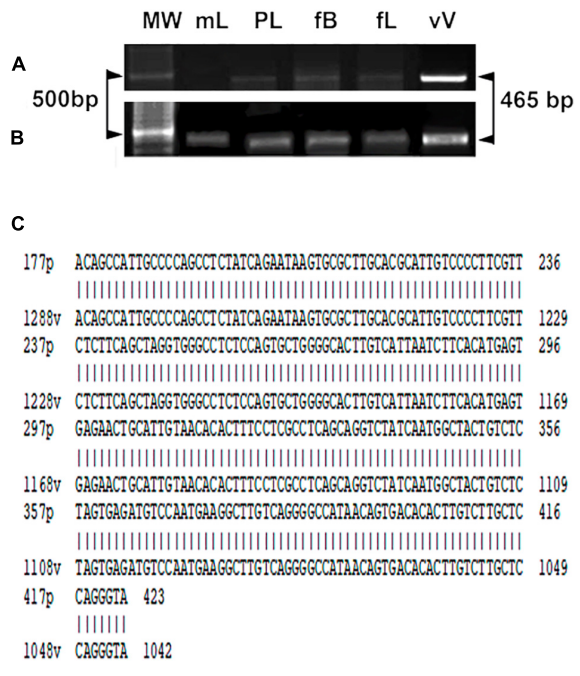

FIGURE 4 | Gene expression of the YF viral capsid protein (465 bp) in maternal liver and tissues of viable fetuses **(A)** and stillbirths **(B)**. Maternal liver (mL), placenta (Pl), fetal brain (fB), liver (fL), (Vv) vaccine YF virus (positive control). The first band of each gel corresponds to the DNA weight in which 500 base pairs are highlighted (arrowheads). **(C)** Sequencing of the experimental PCR product obtained from brain of stillbirths. Note the alignment and 100% identity with the yellow fever virus strain 17DD-Brazil.

Genetic background of each individual may be a factor to be considered, as the strain used here is an outbred mouse. In humans, it has been suggested that modulation of susceptibility to the virus may occur as a result of genetic variation, particularly in loci encoding innate immune mediators (Blake and Garcia-Blanco, 2014). In addition, mice generally have an innate resistance to flavivirus-induced mortality/morbidity due to the autosomal dominant *Flvr* allele. When infected, mice exhibit low levels of viral titers in their tissues, which is associated with a low mortality rate (Nathanson and Brinton, 2007). While that might be one contributing factor, there is also evidence suggesting that the transplacental transfer of a flavivirus is a stochastic process (Yuan and Allen, 2011; Björnberg et al., 2014). Stochastic models have been developed to explain the dynamics of viral infection/transmission, which includes random transitions between infection, latent infection, or non-infection in cells, tissues, and organisms (Tuckwell and Le Corfec, 1998; Yuan and Allen, 2011; Björnberg et al., 2014).

A key question in this study is why changes occurred only following the administration of the vaccine on gd5.5. A plausible explanation may be the immaturity of the cells that comprise the maternal-fetal barrier at this stage of gestation. Day 5.5 of gestation marks the beginning of the implantation process. At this time, the trophoblast giant cells assume an invasive phenotype, thereby opening subluminal endometrial capillaries and establishing the first contact with maternal blood (Bevilacqua and Abrahamsohn, 1988, 1989). This process lasts for a few more days until the embryo is fully lodged in the uterine tissue. The placenta then starts the maturation process, which is characterized by the differentiation of the trophoblast cells to assume defensive, endocrine, immune regulatory, and nutritional properties (Adamson et al., 2002; Cross, 2005; Hu and Cross, 2009).

Evidence from a previous study in humans showed that vaccination results in viremia from the second to the sixth day after administration (Reinhardt et al., 1998). In this context, it is possible that in our experiments, maternal viremia had occurred at the phase of trophoblast immaturity soon after implantation. This might foster a condition of viral access to the embryonic tissues, boosting reactions not found when the vaccine is administered in later stages of pregnancy. In summary, on gd5.5 and the subsequent few days, the trophoblast giant cells may not be mature or differentiated enough to act as a barrier to viral passage. Based on this, vaccination in the later stages of gestation (gd11.5) might be related to full placental differentiation and ability for efficient activation of antiviral mechanisms. In contrast, the lack of contact between maternal

blood and the embryo during the early stages of development, when the mothers were inoculated on gd0.5 may be the critical factor that prevented adverse fetal outcomes in this group. In both cases, our results over these periods are consistent with the findings of vaccination studies in humans.

The disparity with human vaccination data, however, occurred when vaccine inoculation was performed specifically during embryo implantation (day 5.5 of gestation), which resulted in early and late losses (stillbirths).

Studies assessing YF vaccination during early stages of pregnancy also reported a trend toward increased odds of several adverse events (miscarriages, premature births, and low birth weight) when women inadvertently received the vaccine through mass vaccination programs (Tsai, 1993, 2006; Nishioka et al., 1998). The deleterious effect on gestation outcome and fetal development, however, was considered within the expected population indices (Nasidi et al., 1993; Robert et al., 1999; Suzano et al., 2006; D'Acremont et al., 2008). As mentioned earlier, one possible explanation for this disparity may be the organization of the maternal-fetal barrier in its early stages, as having species-specific functional and structural aspects (Georgiades et al., 2002) may determine the passage of the virus to the fetal organism in rodents, but not in humans. In addition, the discrepancy may in part be due to the impossibility of determining in which gestational phase the vaccine has been administered. Peri-implantation losses in humans are hardly detectable, and hence, they are usually not reported and considered for statistical analysis reported in the literature.

The incidence of growth restriction and stillbirths in mothers vaccinated on gd5.5 may be based on several mechanisms, alone or in association. Viral access and lodgment in the developing mouse placenta may be a major factor. Although, morphologically, we did not see any placental damage or placental maturation defects, the immunoreactivity to the vaccine virus at the spongiotrophoblast and labyrinthine zones (also detected by PCR) may represent early access (gd5.5) and further colonization leading to different degrees of placental function impairment.

Virus immunolocalization graded from placental and fetal tissues with occasional antibody reactions (in the live fetuses) to areas of extensive antibody reactivity (in stillbirths), suggesting that the degree of placental/fetal infection might be related to fetal death.

Another possibility is the commitment of fetal metabolism when the placental barrier has not been able to prevent the passage of the virus. YF infection is characterized by a viral viscerotropism in which the liver and nervous system can be aggressively infected, as reported in humans who have succumbed to the infection post-vaccination (Monath, 2008; Martins et al., 2015; Monath and Vasconcelos, 2015). Our immunolocalization and PCR results also detected the presence of the vaccine virus and activity in the nervous tissues of stillborn fetuses, which may have contributed to the impairment of this system and fetal death as a direct cytopathic effect.

The fetal and maternal immune environment may also be an important factor in determining fetal infection when vaccination occurred on gd5.5. There is a well-orchestrated pro- and anti-inflammatory cytokine network locally produced to modulate the complex process of implantation (Dey et al., 2004; Chaouat et al., 2007). On the other hand, YF vaccination also induces a proinflammatory response, where TNF-α, among other cytokines seems to be a key factor (Monath and Vasconcelos, 2015). This may lead to an immune overreaction with the release of proinflammatory cytokines and other mediators of the innate immune system into the fetal circulation, resulting in outcomes such as early embryo deaths and low birth weight in the surviving fetuses (Chaouat et al., 2007; Chattopadhyay et al., 2010; Kurtis et al., 2011). Recent evidence has also shown that pregnant rat females with Zika virus infection exhibited a robust inflammatory response, including critical cytokines and chemokines, regardless of the mother's response to the virus (Khaiboullina et al., 2019). A similar response is also observed in Zika virus infection in pregnant Rhesus monkeys (Hirsch et al., 2018). From this perspective, the plethora of immune factors that are triggered by viral infections may also have been the cause of the gestational outcomes obtained in this study, a question that deserves further research effort.

This study has no exact answers as to why this does not occur at the same frequency in humans. The greater fragility of the trophoblastic barrier in the early stages of gestation in mice may also be an essential factor. Protective neutralizing antibodies were found 14 days after vaccination in humans (Kohler et al., 2012), which were transferred to the embryo/fetus throughout the term of the human pregnancy, limiting viral growth and its deleterious effects. However, in this study, the short gestational period associated with the interval between vaccination and sacrifice of the mice (gd5.5 to gd18.5) might not be sufficient to transfer appropriate protective IgG to the fetal organism.

In summary, our results showed that mouse vaccination does not change gestational parameters when administered in early or mid-gestation. Adverse outcomes such as fetal growth restriction and increased rate of mortality could be observed only when vaccination occurred during the embryo implantation period. The localization of virus particles in the placenta and fetus indicates that YF vaccine virus may have crossed the placental barrier in a stochastic process. In living fetuses, the presence of the virus was limited or absent, whereas in stillbirths, the immunoreactivity and the viral load were high in the placenta and fetal organs. The heterogeneity of responses suggests that the stage of embryo implantation represents a window of susceptibility in which vaccination and associated immune response may interfere with the course of gestation.

CONCLUSION

The yellow fever vaccine virus passed the placental barrier only when administered during embryo implantation, inducing fetal growth restriction and increased fetal mortality rate.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethics Committee for Animal Research (CEEA) of Biomedical Sciences Institute of the University of São Paulo, Brazil (no. 126/37 book2) – Institute of Biomedical Sciences – USP.

AUTHOR CONTRIBUTIONS

FS and EB wrote the draft of the manuscript. EB and HS designed the study. All authors except HS participated in data collection. Data analysis was conducted by FS and FM, who vouch for the findings.

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Association of Maternal Factors and HIV Infection With Innate Cytokine Responses of Delivering Mothers and Newborns in Mozambique

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Maternal factors and exposure to pathogens have an impact on infant health. For instance, HIV exposed but uninfected infants have higher morbidity and mortality than HIV unexposed infants. Innate responses are the first line of defense and orchestrate the subsequent adaptive immune response and are especially relevant in newborns. To determine the association of maternal HIV infection with maternal and newborn innate immunity we analyzed the cytokine responses upon pattern recognition receptor (PRR) stimulations in the triad of maternal peripheral and placental blood as well as in cord blood in a cohort of mother-infant pairs from southern Mozambique. A total of 48 women (35 HIV-uninfected and 13 HIV-infected) were included. Women and infant innate responses positively correlated with each other. Age, gravidity and sex of the fetus had some associations with spontaneous production of cytokines in the maternal peripheral blood. HIV-infected women not receiving antiretroviral therapy (ART) before pregnancy showed decreased IL-8 and IL-6 PRR responses in peripheral blood compared to those HIV-uninfected, and PRR hyporesponsiveness for IL-8 was also found in the corresponding infant's cord blood. HIV infection had a greater impact on placental blood responses, with significantly increased pro-inflammatory, T_H1 and T_H17 PRR responses in HIV-infected women not receiving ART before pregnancy compared to HIV-uninfected women. In conclusion, innate response of the mother and her newborn was altered by HIV infection in the women who did not receive ART before pregnancy. As these responses could be related to birth outcomes, targeted innate immune modulation could improve maternal and newborn health.

Keywords: cytokines, pattern recognition receptors, innate immunity, cord, pregnant women, anemia, HIV, HIV exposed uninfected

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INTRODUCTION

Globally, there were 2.5 million estimated deaths in children within the first month of life in 2017 (WHO, 2017), mostly in low-income countries. Infectious diseases are one of the main causes of mortality in children under the age of 5 (WHO, 2017). It is well known that newborns and infants are more susceptible to infections and severity of infectious diseases than adults (Brook et al., 2017). Infants' vulnerability reflects differences in the immune system in early life compared to adults, which may result in a slower immune response defending against infecting pathogens yet increased immunopathology upon infection. The underlying specific causes are yet to be elucidated (Kollmann et al., 2017).

The immune system of newborns and infants is adapted to the peculiarities of this age period. Newborns come from a tolerogenic fetal environment and suddenly they are exposed to the maternal and environmental microbiome, which requires extensive efforts to maintain homeostasis. Studies of immune ontogeny have demonstrated that immune development undergoes intense changes during the early period of life, yet follows a stable developmental trajectory (Olin et al., 2018; Lee et al., 2019). The changes during this developmental phase, which are key for health and disease, are probably driven by a range of environmental exposures. Maternal factors and exposure to pathogens during pregnancy have a clear impact on the health outcomes of infants. A clear example is malaria during pregnancy. Infants exposed to *Plasmodium in utero* have an increased risk for malaria (Schwarz et al., 2008) as well as non-malaria infections (Rachas et al., 2012); this can also affect vaccine responses (Malhotra et al., 2015). Similarly, children born to HIV-infected mothers, but not themselves infected, suffer higher morbidity and mortality (Slogrove et al., 2012, 2017; Moraleda et al., 2014; Rupérez et al., 2017; Goetghebuer et al., 2018). This is a high concern for public health since the successful implementation of measures to prevent mother-to-child transmission of HIV has resulted in a reduced incidence of pediatric HIV, but an increase in the number of HIV-exposed uninfected (HEU) infants, particularly in sub-Saharan Africa. In some of the sub-Saharan countries, up to 30% of pregnant women are HIV-infected (González et al., 2012). Biological causes behind the increased morbidity and mortality in HEU include, lower transfer of maternal antibodies to newborns, alterations in the immune system of infants due to exposure to antiretroviral drugs, the immune activation in the mother driven by the infection and also HIV exposure *in utero* (Ruck et al., 2016). Adverse birth outcomes in HEU such as prematurity (Et et al., 2004; Chen et al., 2012) and fetal anemia (González et al., 2017) may also negatively affect the newborn's health outcomes.

The immunology behind the reduced HEU health outcomes is not clear, but studies comparing HEU with unexposed (HUU) children have shown an altered immune response (Abu-Raya et al., 2016; Evans et al., 2016). Innate immune responses are an essential defense against infectious agents in newborns and direct and shape the adaptive immune response. Importantly, innate responses can also contribute to immunopathology. The innate immune system senses microbial pathogens through pattern recognition receptors (PRR), such as toll-like receptors (TLR) and nucleotide-binding oligomerization domain (NOD)-like receptors, which recognize conserved pathogen-associated molecular patterns. Innate immunity can present immunological memory, i.e., initial stimulation can lead to enhanced (trained immunity) or diminished innate responses (tolerance) to the same or different stimulus (Netea et al., 2011). Previous studies have shown that PRR-mediated innate immune responses differ between neonates, infants and adults (Kollmann et al., 2012; Georgountzou and Papadopoulos, 2017). Most studies show that upon *in vitro* stimulation of cord blood with TLR ligands compared to adult peripheral blood, lower levels of pro-inflammatory cytokines such as TNF and IL-1β and higher levels of the anti-inflammatory cytokine IL-10 are induced (Kollmann et al., 2012; Georgountzou and Papadopoulos, 2017). Also, responses of IFN and T_H1-supporting cytokines such as IL-12p70 are weaker in cord than adults, whereas responses of the T_H17-promoting cytokines IL-6 and IL-23 are enhanced.

Differences in the innate system of HEU vs. HUU infants have been found. Overall, a pro-inflammatory and activated immune profile has been described in HEU infants (Lohman-Payne et al., 2018; Dirajlal-Fargo et al., 2019) with changes in the proportion and profile of innate cell subsets (European Collaborative Study, 2004; Bunders et al., 2005; Velilla et al., 2008). To our knowledge, very few studies have assessed innate responses in HEU infants. We previously found higher TNF, IL-6, and IL-12 concentrations in myeloid dendritic cells (DC) and monocytes in response to bacterial PRR ligands in South African children, particularly at 2 and 6 weeks of age, but no differences in plasmacytoid DC (Reikie et al., 2013). However, other studies reported a reduced IL-12 production by cord monocytes in HEU infants (Chougnet et al., 2000) and no IL-6 responses upon TLR4 stimulation in some HEU infants (Maloupazoa Siawaya et al., 2018). Another study showed no differences upon TLR9 stimulation on DC (Velilla et al., 2008).

Despite evidence of the impact of maternal condition on the infant's health, there is limited data on the relationship between the innate responses in the pregnant woman and their infants. Specifically, maternal cytokines may represent a stronger determinant of child immune responses than genetic factors (Djuardi et al., 2016). The extent to which innate immune responses to PRR differ or correlate between mothers and their newborns and how innate responses are altered by HIV exposure and other maternal biological factors such as anemia has not been well characterized. Here we aimed to (i) compare TLR- and NOD-mediated innate cytokine responses in HIV infected and uninfected women (peripheral and placental blood) and their infants (cord blood), (ii) estimate the association of HIV infection, maternal factors (age, gravidity, and anemia), and fetal sex with the innate cytokine profile, and (iii) explore the association of innate responses with birth outcomes.

MATERIALS AND METHODS

Study Design

This is an observational study that included 48 women-cord pairs at the time of delivery. Study women were randomly selected among those recruited at the antenatal care clinic (ANC) in the Manhiça District Hospital (Mozambique) for an immunology study ancillary to two clinical trials comparing: (a) two-dose Intermittent Preventive Treatment in pregnancy (IPTp) with mefloquine (MQ) vs. two-dose IPTp-sulphadoxine-pyrimethamine (SP) in HIV-uninfected women (González et al., 2014b) and (b) three-dose IPTp-MQ and daily cotrimoxazole (CTX) prophylaxis vs. three-dose ITPp-placebo and daily CTX in HIV-infected women (González et al., 2014a; **Supplementary Figure S1**). The intensity of malaria transmission at the time of the study (2011–2013) was low/moderate (Mayor et al., 2015). All study women received bed nets treated with long-lasting insecticide, folic acid and ferrous sulfate. At the time of the study, antiretroviral therapy (ART) was recommended when CD4 + T cell count was below <350 cells/μL and/or when the woman was in 3 or 4 HIV/AIDS WHO clinical stage (WHO, 2005). ART was delivered to pregnant women at the monthly ANC visits. Prevention of mother-to-child transmission of HIV was based on antenatal administration of daily monotherapy with zidovudine (AZT) to the mother from 14 weeks of gestation, and combined antiretrovirals (single-dose nevirapine [NVP] and daily AZT plus lamivudine [3TC]) during labor and up to 1 week postpartum.

Participant characteristics and maternal and birth outcomes were recorded during the two clinical trials as described previously (González et al., 2014a,b). At delivery, blood samples (peripheral, placental, and cord blood) were collected into 10 mL Sodium Heparin tubes (BD Vacutainer, Cat No. 368480). Cord blood was collected from the umbilical arteries and placental blood was obtained from small incisions (1–1.5 cm-deep and long incisions) on the maternal-facing side of the placenta. Thick and thin blood films, as well as placental-biopsy samples (stored in 10% buffered formalin) were assessed for detection of *Plasmodium* species according to standard procedures (González et al., 2014a,b). Tissue samples from the maternal side of the placenta, as well as 50 μl of maternal peripheral, placental, and cord blood samples on filter papers, were collected for detection of *Plasmodium falciparum* in duplicate by means of a real-time quantitative polymerase-chain-reaction (qPCR) assay targeting 18S ribosomal RNA (Mayor et al., 2009). A capillary blood sample was collected from the infant at 6 weeks of age onto filter paper for HIV PCR analysis, following national guidelines for prevention of mother-to-child transmission of HIV. All newborns from whose blood we analyzed in the study were HIV-uninfected.

Pattern Recognition Receptor Stimulations

Blood samples were processed in less than 4 h from collection. Whole blood was mixed 1:1 with sterile pre-warmed (37°C) RPMI 1640 medium. Two hundred microliters were added to each well of pre-made 96-well round-bottom polystyrene plates containing 22 μl of specific TLR and NOD ligands: PAM3CSK4 (PAM, TLR2/1); polyinosinic-polycytidylic acid (poly I:C, TLR3); lipopolysaccharide (LPS, TLR4); resiquimod (R848, TLR7/8); peptidoglycan (PGN, NOD1/2) and muramyl dipeptide (MDP, NOD2); and media alone. All ligands were diluted in RPMI medium to obtain the desired concentration: PAM (InvivoGen, San Diego, CA, United States) at 1 μg/mL; Poly I:C (GE Healthcare, Fairfield, CT, United States) at 100 μg/mL; LPS (InvivoGen) at 10 ng/mL; R848 (InvivoGen) at 10 μM; PGN (InvivoGen) at 10 μg/mL; MDP (InvivoGen) at 0.1 μg/mL. To standardize the assays, the pre-made plates were prepared, sealed with aluminum plate sealer and stored at −80°C until use.

The diluted whole blood was incubated for 24 h at 37°C in 5% CO_2. After 24 h in culture, plates were centrifuged, 100 μL of supernatant were taken and stored at −80°C. The samples were shipped on dry ice via World Courier to Vancouver (Canada) where they were stored at −80°C until Luminex and ELISA-based measurements.

Cytokine Quantification

The Luminex assay was performed in 26 Luminex plates and in two different phases some weeks apart. Supernatants from the 24 h culture plates were diluted 1:2 and 1:150 with RPMI and assessed in single replicates. Cytokines were assessed using the 13-plex Millipore Milliplex Map Kit (MPXHCTYO-60K): IFN-α2, IFN-γ, IL-1β, IL-6, IL-8, IL-10, IL-12p40, IL-12p70, IP10, MDC, MIP-1α, MIP-1β, and TNF-α. Manufacturer's instructions were followed and the controls included in the kit were used. Data were analyzed in MiraiBio Masterplex QT. A 5-parameter logistic plot was used to calculate the standard curve and the sample concentrations. The lower and upper limits of detection were set as the lowest and highest concentration of the standard curve, respectively. The sample dilution factors were accounted for in setting the upper limit of detection. Samples with values below the detection limit for a specific cytokine were assigned a value half of the detection limit. When pooling the data of different plates, the higher lower limit of detection was applied for all plates. For a given sample and analyte, if readings were less than 50 beads, values were discarded; also, samples that had all analytes for all stimulations below the lower limit of detection were excluded from the analysis as this indicated failure of the biological assay. Twenty-five microliters of supernatants were used to also measure IL-23 in single replicates using the eBioscience Human IL-23 ELISA Ready-SET-Go kit (88-7237-86). Limits of detection were set as above. We selected these 14 cytokines because they cover key functional categories: cytokines supporting T_H1-responses (IFN-α, IFN-γ, IP10, IL-12p70), cytokines supporting T_H17 cytokines (IL-12p40, IL-6, and IL-23), pro-inflammatory cytokines (TNF and IL-1β) and chemokines (IL8, MIP-1α, MIP-1β, MDC) and the anti-inflammatory cytokine IL-10.

Statistical Analysis

Descriptive analysis comparing characteristics HIV-uninfected and HIV-infected groups was performed using the compareGroups R package (Subirana et al., 2014). The

compareGroups function performs a Shapiro–Wilk test for normality to decide if the variables are normal or non-normal distributed performing subsequently parametric or non-parametric tests, respectively.

Unsupervised analysis to visualize factors affecting variability in the cytokine concentration was performed using principal component analysis (PCA) with the FactoMineR package (Lê et al., 2008). Crude cytokine concentration values were used for the PCA and the three first components that explained most of the data variability were selected. A technical batch effect (effect of the phase in which Luminex plates were assayed) was detected and all analyses were performed by multivariable linear and logistic regressions adjusting by assay phase (**Supplementary Figure S2C**). Age was detected as one of the main factors influencing cytokine responses by univariable analyses and therefore was considered a confounding factor and was also used to adjust all analyses in multivariable models. Multivariable models were not adjusted for additional variables due to the limited sample size.

The analysis of cytokine responses to the PRR (agonist-specific cytokine responses) was performed with ratios of the concentrations of the stimulations divided by the unstimulated control (background). Concentration values from the unstimulated controls, considered spontaneous cytokine expression, were also analyzed. Ratios and concentration values were $\log_{10}$-transformed. Differences of crude cytokine concentrations between agonists and the unstimulated control were assessed by one-sided Wilcoxon signed-rank tests and differences between the three compartments (placental, cord, and peripheral) were assessed by Friedman tests.

Fold-change differences in cytokine responses in pairwise comparisons between compartments were assessed by multivariable linear regression analysis with cytokine ratios or concentrations as the dependent variable. Correlations of cytokine concentrations and ratios between different compartments were performed by Spearman. The association of gravidity (primigravidae [first pregnancy] vs. multigravidae [≥2 pregnancies], sex, maternal anemia at delivery [hemoglobin < 11 g/dL], HIV infection and being on ART at study baseline with cytokine responses was assessed separately by different multivariable linear regression models, with each factor as independent variable, age as a co-variable and cytokine ratio or concentration as the dependent variable. The relationship of cytokine responses with pregnancy outcomes (birth weight, low birth weight [<2500 gr], fetal hemoglobin, fetal anemia [<12.5 g/dL in cord blood], gestational age measured by Ballard score (Ballard et al., 1991) and prematurity [<37 weeks of gestational age] was assessed through multivariable logistic regressions (one for each factor separately), with cytokine response as independent variable. These multivariable models were also adjusted by age and batch effect.

P-values were adjusted for multiple testing by Benjamini–Hochberg (False Discovery Rate, FDR); the number of tests performed for each outcome was stated in the respective tables' footnote. Adjustments of *p*-values were performed separately for each maternal variable and birth outcome to allow the assessment of each variable independently. Due to the exploratory nature of this study we chose to consider adjusted *p*-values ≤ 0.3 (FDR 30%) as significant. All analyses were performed in R software version 3.5 and 3.6.0 (R Core Team, 2019). Additional packages used for data management were reshape2, tidyverse and dplyr (Wickham, 2007, 2016b; Wickham et al., 2015). The package ggplot2 (Wickham, 2016a) was used for all boxplots, heatmaps, and forestplots.

RESULTS

Baseline Characteristics of Study Participants

A total of 48 women (35 HIV-uninfected and 13 HIV-infected) with peripheral, placental and corresponding cord blood samples available were included in the study. All HIV-uninfected women participated in a clinical trial comparing MQ to SP as IPTp (González et al., 2014b). Most of them (N = 33) received MQ whereas only 3 received SP (**Table 1** and **Supplementary Figure S1**). All HIV-infected women participated in the trial comparing MQ plus daily CTX vs. only CTX (González et al., 2014a) and only 3 received MQ (**Table 1** and **Supplementary Figure S1**). Therefore, the associations with the different IPTp treatments were not assessed. The median age of the women was 22 years, 33.3% of them were primigravidae, 33.3% had anemia and 43.8% gave birth to a female baby (**Table 1**). Based on the middle-upper arm circumference (MUAC), only one HIV-uninfected and one infected woman had malnutrition (MUAC < 22 cm). There were five cesarean deliveries. Regarding birth outcomes, 6.3% of the newborns had fetal anemia, 8.3% had low birth weight, and 16.7% were premature. There were no significant differences between HIV-uninfected and HIV-infected women with the exception of fetal hemoglobin levels in the cord, which were significantly lower in those born to HIV-infected women. HIV PCR of all study infants was negative at 6 weeks of age.

Six out of 13 HIV-infected women received ART before pregnancy. None of the study women had placental malaria and all had negative *P. falciparum* blood smears during ANC visits; parasites were detected by qPCR in 5 out of 43 study women at recruitment. No submicroscopic data was available for the other two visits and therefore associations with malaria were not assessed in this study.

Innate Cytokine Profile

We initially explored all cytokine data in an unsupervised approach using PCA that allows the reduction of the dimensionality of data into a new set of uncorrelated variables (principal components). **Supplementary Figure S2A** shows the PCA scores of all maternal peripheral, placenta and cord blood samples by the 6 PRR agonists using the first three dimensions. Only pI:C and R848 had a clear differential profile from the unstimulated control. Despite no clear clustering in the PCA, cytokine levels still differed significantly from the unstimulated control for all PRR agonists for most cytokines (**Supplementary Figure S3**). R848 showed the highest responses

TABLE 1 | Summary descriptive of study population by groups of HIV.

	[All] *N* = 48	HIV-uninfected *N* = 35	HIV-infected *N* = 13	*P*-value
Age, median[Q1;Q3]	22.0 [18.8;27.0]	21.0 [17.5;26.5]	25.0 [21.0;27.0]	0.153[a]
Gravidity, N(%)**:**				0.170[b]
MG	32 (66.7%)	21 (60.0%)	11 (84.6%)	
PG	16 (33.3%)	14 (40.0%)	2 (15.4%)	
Fetal sex, N(%)**:**				0.437[b]
female	21 (43.8%)	17 (48.6%)	4 (30.8%)	
male	27 (56.2%)	18 (51.4%)	9 (69.2%)	
Maternal hemoglobin at delivery, mean (SD)	11.2 (1.52)	11.3 (1.69)	11.0 (0.93)	0.436[c]
Maternal anemia at delivery (hemoglobin < 11 g/dL), N(%)**:**				0.735[b]
no	32 (66.7%)	24 (68.6%)	8 (61.5%)	
yes	16 (33.3%)	11 (31.4%)	5 (38.5%)	
Fetal hemoglobin, mean (SD)	14.2 (1.24)	14.4 (1.23)	13.5 (1.00)	0.011[c]
Fetal anemia (<12 g/dL in cord blood), N(%)**:**				0.174[b]
no	45 (93.8%)	34 (97.1%)	11 (84.6%)	
yes	3 (6.3%)	1 (2.7%)	2 (15.4%)	
Birth weight, median[Q1;Q3]	3050 [2900;3300]	3100 [2900;3250]	3000 [2900;3300]	0.692[a]
Low birth weight (<2500 gr), N(%)**:**				0.294[a]
no	44 (91.7%)	33 (94.3%)	11 (84.6%)	
yes	4 (8.3%)	2 (5.7%)	2 (15.4%)	
Gestational age, median[Q1;Q3]	38.0 [37.0;39.0]	38.0 [37.0;39.0]	38.0 [37.0;39.2]	0.601[a]
N missings	4	3	1	
Prematurity (<37 weeks), N(%)**:**				1.000[b]
no	40 (83.3%)	29 (82.9%)	11 (84.6%)	
yes	8 (16.7%)	6 (17.1%)	2 (15.4%)	
MUAC (cm) at recruitment, mean (SD)	25.6 (2.16)	25.7 (2.16)	25.3 (2.21)	0.531[c]
N missings	1	–	1	
Mode of delivery, N(%)**:**				1.000[b]
Normal vaginal	42 (89.4%)	31 (88.6%)	11 (91.7%)	
Cesarean section	5 (10.6%)	4 (11.4%)	1 (8.3%)	
N missings	1	–	1	
CD4+ T cell counts at delivery, mean (SD)	–	–	837 (580)	na
HIV viral load at delivery, median[Q1;Q3]	–	–	1116 [1.00;20457]	na
IPTp, N(%):				na
2-dose MQ	–	32 (91.4%)	–	
2-dose SP	–	3 (8.6%)	–	
3-dose MQ + daily CTX	–	–	3 (23.1%)	
Placebo + daily CTX		–	10 (76.9%)	

[a]Non-parametric test. [b]Chi-square. [c]Parametric test. CTX, cotrimoxazole; IPTp, Intermittent Preventive Treatment in pregnancy; MG, multigravidae; MQ, mefloquine; MUAC, middle upper arm circumference; na, not applicable; PG, primigravidae; Q, quartile; SD, standard deviation; SP, sulphadoxine-pyrimethamine.

while MDP was the weakest stimulant with no statistically significant differences in IL-23, IL-12p70, IP10, and MDC concentrations compared to the unstimulated control. Therefore, we used the cytokine concentrations in the unstimulated control sample and all the agonists-specific cytokine responses for the analysis. The agonist-specific responses were analyzed for each cytokine using ratios between the cytokine concentration in the agonist stimulation and the cytokine concentration in the unstimulated control (**Supplementary Figure S4**).

No clustering of data was observed for blood compartment (periphery, cord, placenta, **Supplementary Figure S2B**) or any other factor in the PCA analysis, reflecting that samples did not differ substantially in their cytokine profile despite being from different compartments and coming from mothers with diverse characteristics. The Luminex assays were performed in two phases which we detected to affect the cytokine data (**Supplementary Figure S2C**), therefore the phase in which the assays were performed was taken into account in the subsequent analyses adjusting the models by phase.

Comparison of Cytokine Levels Between Compartments

Overall cytokine production was positively correlated between the different compartments, especially between maternal peripheral blood and cord blood (**Figure 1A**). In particular, spontaneous production of TNF and MIP-1β, IL-12p40, IL-12p70, and IFN-α was highly correlated between those two compartments (rho = 0.58–0.73). Regarding the cytokine responses to PRR agonists (**Figure 1B**), MIP1-α, MIP-1β, and IP10 were strongly correlated in peripheral and cord blood for all agonists. Some other high positive cytokine correlations were found but varied between agonists. In general, spontaneous production of cytokines and agonist-specific responses in cord blood were also positively correlated with the ones in the placental blood (**Figure 1A**), similarly to correlations between cord and periphery, but weaker.

Cytokine concentrations in the unstimulated control and in the PRR stimulations differed among periphery, placenta and cord, particularly for the pro-inflammatory cytokines and chemokines, the anti-inflammatory cytokine IL-10 and the T_H17 cytokines IL-6 and IL-12p40 (**Supplementary Figure S5**). **Figures 2**, **3** show the fold-change difference between compartments in the crude cytokine concentrations (**Figure 2**) and the agonist-specific cytokine responses (**Figure 3**), calculated using adjusted models. Overall, cytokine concentrations were higher in cord than in maternal peripheral blood. Specifically, spontaneous production of the pro-inflammatory MDC and IL-8 chemokines was 2.4 and 3.5 times higher, respectively, in cord than in peripheral maternal blood (**Figure 2**). Also, IL-12p40 production for all PRR with the exception of MDP, IL-1β for NOD1/2, IL-12p70 for TLR3 and IFN-γ for TLR7/8 were higher in cord (**Figure 3**), but lower for MDC in response to TLR7/8 and for IFN-α in response to TLR1/2 stimulation (**Figure 3**). Spontaneous production of cytokines in placenta was higher than in periphery and cord for the pro-inflammatory cytokines IL-1β, TNF, MIP-1α, and IL-8, the anti-inflammatory IL-10, the T_H17 cytokine IL-6 and the T_H1 cytokine IP10 (**Figure 2**). However, there were diminished PRR agonist-specific responses in placental blood compared to the other two compartments (**Figure 3**). Therefore, despite a general positive correlation of innate cytokine responses between compartments, some differences were found in cord compared to peripheral maternal blood.

Association of HIV-Infection and Other Maternal Factors With Cytokine Responses

HIV-exposure was not associated with spontaneous production of cytokines (**Table 2**). For some PRR stimulations, particularly TLR3, HIV-infected compared to HIV-uninfected mothers, produced less IL-6 and IL-8 in peripheral blood, yet much higher IL-1β, MIP-1β, IL-10, IL-12p40, and IL-6, but less IFN-α (for TLR7/8) in placental and lower IL-8 and IP10 in cord blood (**Figure 4**). However, these associations were only statistically significant after adjusting for multiple testing when analyzing HIV-infected women who did not receive ART prior to pregnancy (**Figure 4**).

In analysis including HIV-uninfected and -infected women, higher maternal age was associated with lower spontaneous production of pro-inflammatory cytokines as well as the IL-10 anti-inflammatory cytokine in women's peripheral blood (**Supplementary Table S1**). Age also was associated with higher cytokine production following PRR stimulation in peripheral blood when not adjusting for multiple comparisons (**Supplementary Table S2**). As age was the non-clinical variable with the largest association with cytokine responses, we adjusted all analyses, including the above, by age.

Gravidity was also associated with spontaneous production of cytokines in maternal peripheral blood: Primigravidae had lower spontaneous production of MIP-1α, MIP-1β, and IL-23 (**Table 2**). Upon stimulation though, primigravidae produced more IL-10, IL-8, TNF, and IL-23 in maternal peripheral blood, but less IL-1β in placental and less IFN-γ in cord blood (**Supplementary Figure S6**). However, these later results were not statistically significant after adjusting for multiple testing.

Fetal sex had a remarkable impact on cytokine responses. Mothers who delivered a male vs. female newborn exhibited lower spontaneous production of IL-1β, IL-8, IL-6, and IFN-α in their peripheral blood (**Table 2**), although associations of the first two were not significant after adjusting for multiple testing. It is worth mentioning that male vs. female newborns had lower IP10 responses for all PRR stimulations despite lack of statistical significance after adjusting for multiple testing (**Supplementary Figure S6**).

When considering raw p-values < 0.05, maternal anemia was associated with higher spontaneous production of IL-8 and IL-6 in peripheral blood and lower production in cord blood of IFN-γ (**Table 2**). In contrast, upon PRR stimulation (**Figure 4**), anemic women had lower IL-8 and IL-6 responses. Maternal anemia was also associated with cytokine responses in cord (particularly for MDP and LPS), with higher IL-10, IL-1β, MDC, TNF, IL-12p70, IL-12p40 responses following PRR stimulation.

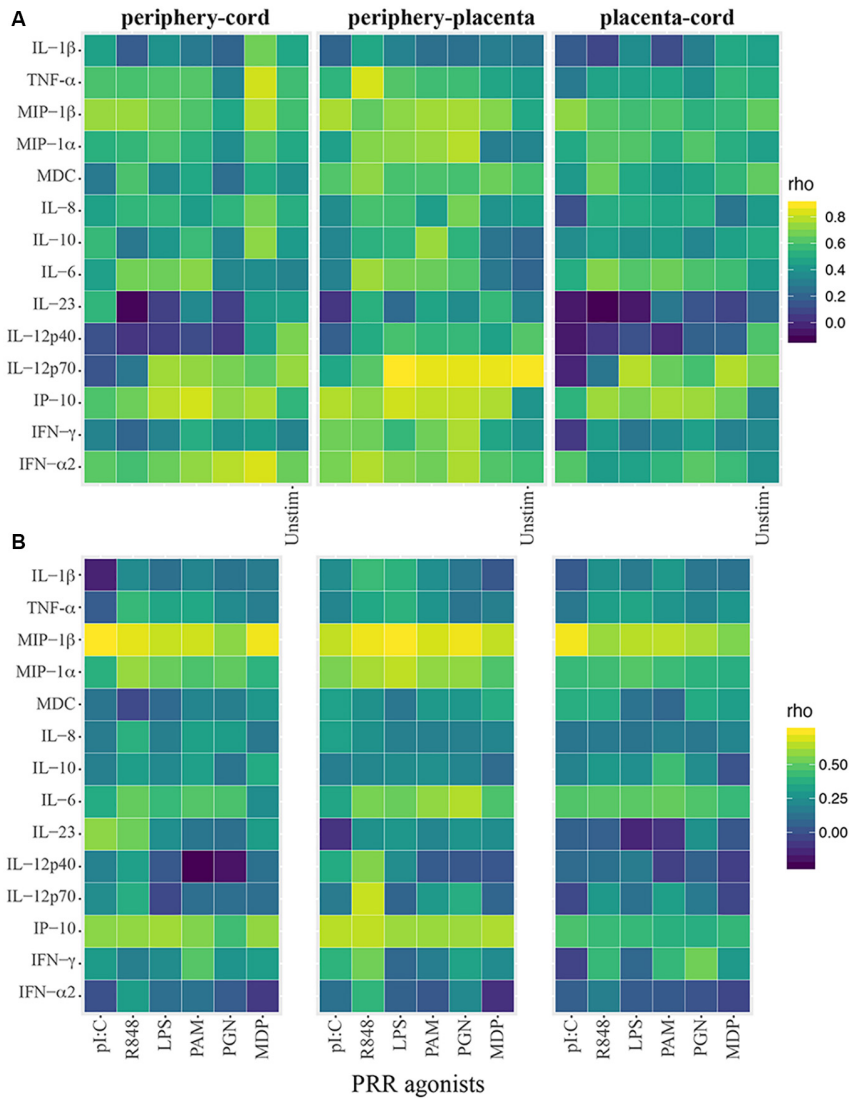

FIGURE 1 | Heatmaps showing innate cytokine correlations between cord and periphery and placenta. **(A)** Spearman correlation coefficients (rho) using crude cytokine concentrations upon innate stimulations or spontaneous production of cytokines (background control = Unstim). **(B)** Spearman correlations using agonist-specific cytokine responses expressed as ratios of cytokine production of innate stimulations over background control.

In summary, HIV infection when ART was not administered before pregnancy, affected mainly placental blood responses, with little to no impact on maternal peripheral or cord blood responses. On the other hand, age and gravidity (independently from age) affected some spontaneous maternal peripheral responses, while maternal anemia associations did not reach statistical significance after adjusting for multiple testing. Curiously, fetal sex was associated with particular spontaneous peripheral maternal responses.

Association of Innate Cytokines With Birth Outcomes

We did not find any statistically significant association of spontaneous production of cytokines with birth outcomes when adjusting for multiple testing (**Supplementary Table S3**). However, higher spontaneous production of IL-8 and IL-6 in maternal peripheral blood could be associated with lower fetal hemoglobin (raw p-value < 0.05). This is consistent with the trends found for maternal anemia and higher IL-8 and IL-6, considering that maternal anemia can be associated with fetal anemia. On the other hand, maternal peripheral blood PRR responses of IFN-γ were significantly associated with lower fetal hemoglobin, whereas IL-6 and IL-8 were associated with considerable increases (**Table 3**).

Regarding newborn's gestational age and prematurity, there were no statistically significant associations with cytokine responses (**Table 3** and **Supplementary Tables S3–S5**). Nevertheless, consistent associations when considering raw p-values were detected: Ten-fold changes in spontaneous

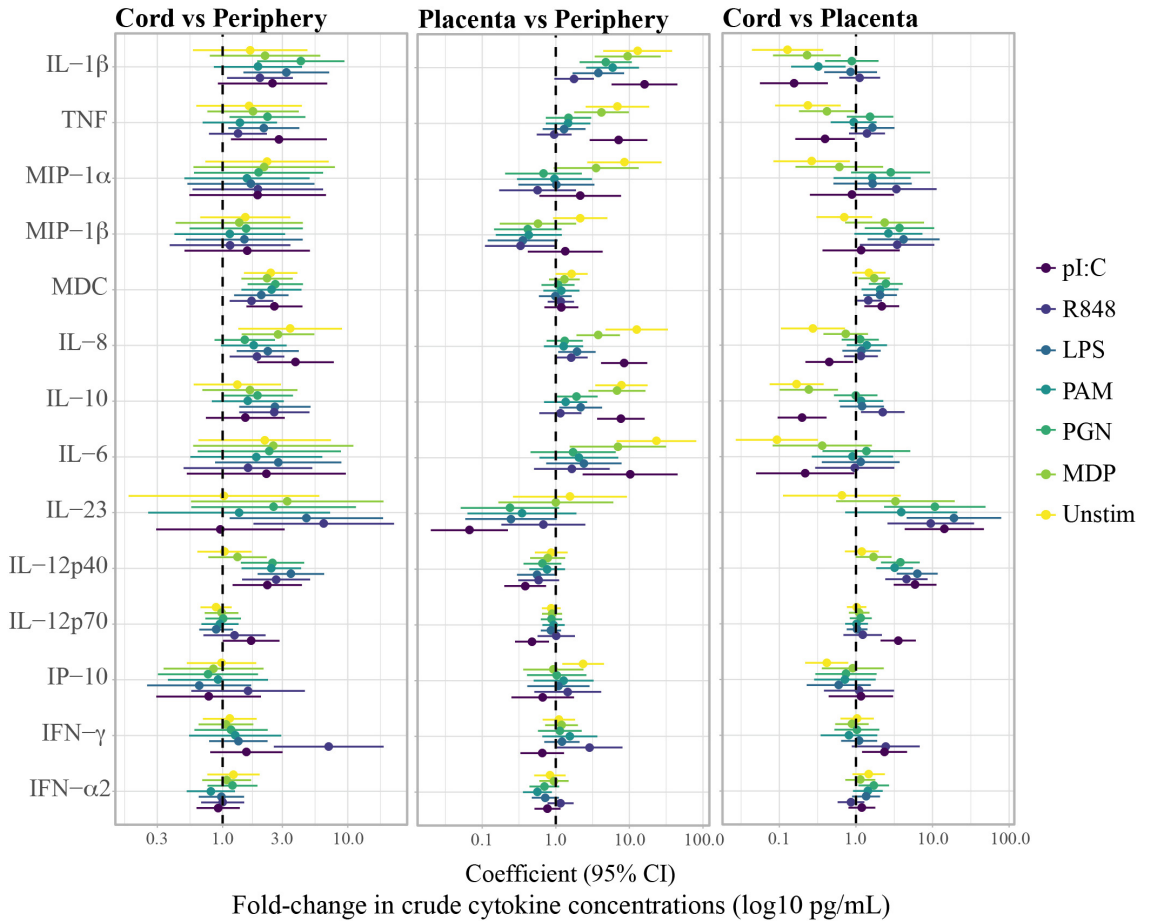

FIGURE 2 | Fold-change differences between compartments in crude cytokine concentrations upon whole blood stimulations with agonists of pattern recognition receptors. Forest plots showing the effect (coefficients and 95% confidence intervals) of cord vs. periphery, cord vs. placenta and placenta vs. periphery, on cytokine concentrations. Coefficients were calculated as 10^beta and beta was obtained by multivariable regressions adjusted by age (log_{10}-transformed) and technical batch, with cytokine concentrations (log10-transformed) as outcome.

production of IL-8 in cord blood were associated with 0.337 odds of being premature (**Supplementary Table S4**), whereas IL-8 responses in cord blood were associated with lower gestational age (**Table 3**); and 10-fold increases in IL-8 responses to all PRR stimulations, with the exception of TLR1/2, were associated with 3.5–5 increased odds of prematurity (**Supplementary Table S5**).

DISCUSSION

Our study contrasting innate immunity across the three compartments of maternal peripheral blood, placental blood and newborn cord blood provides evidence in support of the notion that maternal HIV infection impacts innate responses in both the woman and the HIV-exposed but uninfected child. Given the unique access to the triad of maternal peripheral and placental blood as well newborn cord blood, we were able to identify that the relationship of maternal HIV-infection with cytokine responses was predominantly evident in the placental blood compartment. Furthermore, our study design also allowed us to identify that this impact of HIV on maternal and newborn innate immunity was restricted to women who had not received ART before pregnancy. This has substantial public health implications, as this change in immune status is presumed to be related to adverse birth outcomes such as low birth weight and prematurity, and thus all HIV-infected women should be on ART. Our results further reveal that other maternal factors could further influence innate responses, such as maternal age, gravidity and infant's sex.

HIV-infected women who did not receive ART at baseline had decreased IL-6 and IL-8 responses to PRR stimulations in peripheral blood. HIV itself may activate TLR7/8 (Meier et al., 2007) and other PRR (Mogensen et al., 2010) contributing to the HIV chronic immune activation. In addition, systemic bacterial translocation caused by HIV infection (Brenchley et al., 2006) may also be activating TLR and promoting further immune activation. According to our results, higher baseline immune activation and TLR stimulation could be causing hyporesponsiveness or tolerance upon further stimulation, which we speculate could lead to increased susceptibility to infections.

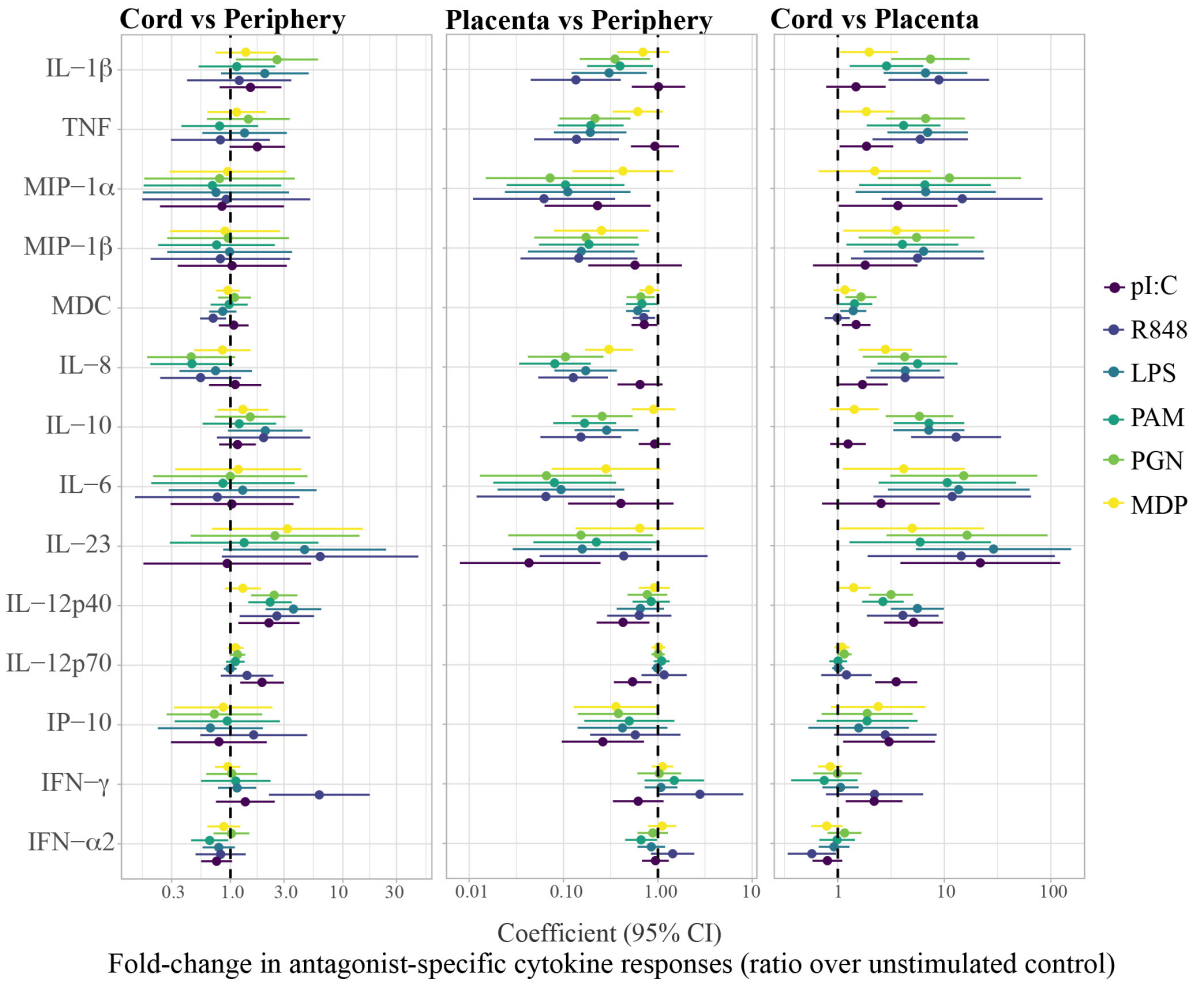

FIGURE 3 | Fold-change differences between compartments in agonist-specific cytokine responses (ratios). Agonist-specific cytokine responses are expressed as ratios of cytokine concentrations upon stimulation over background control (unstim). Forest plots showing the effect (coefficients and 95% confidence intervals) of cord vs. periphery, cord vs. placenta and placenta vs. periphery, on cytokine responses. Coefficients were calculated as 10^beta and beta was obtained by multivariable regressions adjusted by age (log_{10}-transformed) and technical batch, with cytokine ratios (log10-transformed) as outcome.

Our findings are consistent with previous studies showing a decreased response to TLR in HIV-infected individuals, which inversely correlated with viral load (Scully et al., 2016). However, other studies showed that responses depended on the cell subset and the HIV infection stage (Chang et al., 2012) and increased TLR expression and responsiveness with HIV infection have also been observed (Lester et al., 2008; Hernández et al., 2012). Of note, in these studies the response to TLR was measured in PBMC instead of whole blood and in different conditions than ours. In addition, all of the other studies were performed in non-pregnant adults, and sex (Meier et al., 2009) and pregnancy (Ziegler et al., 2018), which is characterized by immunoregulatory mechanisms to maintain the semi-allogeneic fetus, affect innate responses. To our knowledge, only one study assessed innate responses to TLR ligands in HIV-infected pregnant women and in their newborns (Cardoso et al., 2013). In that study and similarly to our results, a compromised cytokine (TNF, IFN-α, and IL-10) response to TLR was detected in both maternal and cord blood with the exception of TLR7/8 response in myeloid DC.

The associations of HIV infection when not receiving ART before pregnancy with placental PRR responses, suggest that HIV may be particularly affecting the placenta. We found that women who had not received ART before pregnancy had higher pro-inflammatory and anti-inflammatory IL-10 (which usually go hand in hand with pro-inflammatory cytokines), and T_H1 and T_H17 PRR responses in placental blood than HIV-uninfected women. The only exception worth noting was IFN-α in response to R848 stimulation, which was lower in HIV-infected than uninfected women. It would be of interest to further investigate how these responses may affect the fetus and if they could be associated with the adverse birth outcomes described in HIV-infected women (Chen et al., 2012). The increased impact of HIV-infection when not receiving ART before pregnancy is in line with recent results showing that ART initiation during pregnancy, instead of before, was associated with the

TABLE 2 | Association of HIV infection, gravidity, infant's sex and maternal anemia with spontaneous cytokine production.

	Compartment	Cytokine	Cytokine group	Coefficient[a]	95% CI	*P*-value[b]	BH *P*-value[c]
HIV infection (ref: HIV-uninfected)							
	Cord	IL-12p70	TH1	0.648	0.429; 0.979	0.04	0.993
Gravidity (ref: multigravidae)							
	Periphery	MIP-1α	Pro-inflammatory	0.068	0.01; 0.471	0.008	0.28
	Periphery	MIP-1β	Pro-inflammatory	0.158	0.034; 0.729	0.019	0.28
	Periphery	IL-23	TH17	0.018	0.001; 0.509	0.02	0.28
	Cord	IP10	TH1	0.312	0.103; 0.947	0.04	0.993
Sex (ref: females)							
	Periphery	IL-1β	Pro-inflammatory	0.243	0.065; 0.914	0.037	0.389
	Periphery	IL-8	Pro-inflammatory	0.211	0.05; 0.895	0.035	0.389
	Periphery	IFN-α2	TH1	0.35	0.163; 0.752	0.008	0.21
	Periphery	IL-6	TH17	0.127	0.027; 0.601	0.01	0.21
Maternal anemia (ref: no anemia)							
	Periphery	IL-8	Pro-inflammatory	5.984	1.315; 27.234	0.022	0.651
	Periphery	IL-6	TH17	6.433	1.193; 34.679	0.031	0.651
	Cord	IFN-γ	TH1	0.432	0.188; 0.993	0.048	0.672

Only results with raw p-values ≤ 0.05 are shown. [a]Coefficients are 10^beta and beta was obtained in multivariable regression models adjusted by age (log_{10}-transformed) and technical batch with cytokine concentrations (log_{10}-transformed) as outcome. [b]Raw p-value. [c]Significance was established at a Benjamini–Hochberg (BH) adjusted p-value ≤ 0.3. P-values were adjusted by a total of 42 tests performed for each independent variable.

activation of newborn monocytes, a reduced placental transfer of maternal antibodies and a higher risk of hospitalization of infants (Goetghebuer et al., 2018). Our findings support that initiation of ART before conception could benefit pregnancy and newborns, beyond prevention of perinatal transmission.

HIV exposed uninfected newborns had lower pro-inflammatory (IL-8) responses in cord blood compared to HUU newborns, although these differences were only statistically significant after adjusting for multiple testing when analyzing mothers not receiving ART before pregnancy. In our previous study, we had found higher IL-6, TNF, IL-12 responses in innate cells of HEU compared to HUU, but other cytokines besides IFN-α were not analyzed (Reikie et al., 2014). Moreover, in that study we did not assess concentrations in supernatants nor responses in cord. The diminished IL-8 PRR responses in cord in HEU could be of particular interest because of the consistent associations of IL-8 with gestational age and prematurity, although they were not statistically significant when adjusting for multiple testing.

Anemia during pregnancy is frequent, particularly in low-income countries, and HIV infection further increases the risk of anemia in pregnancy (González et al., 2017). There are multifactorial causes for the anemia, being malaria an important factor. However, none of the women of this study had microscopic or placental malaria. Our results were not statistically significant after adjusting for multiple testing, but were biologically plausible and consistent with the literature. Anemic mothers showed higher spontaneous production of pro-inflammatory IL-8 and the T_H17 cytokine IL-6. Chronic inflammation is another of the factors causing anemia and IL-6 contributes to the inflammation and development of anemia through the iron regulatory hormone hepcidin, and the iron exporter ferroportin (Fraenkel, 2015). Furthermore, activation of TLR results in iron sequestration (Abreu et al., 2018). Although not significant after adjusting for multiple testing, we also found that anemia could be associated with lower responsiveness to PRR stimulations, similarly to what has been described in anemic children (Liao et al., 2018). While higher spontaneous production of IL-8 and IL-6 may reflect the pro-inflammatory status of the mothers, particularly the HIV-infected ones, and may be mediators of the development of anemia, the response to PRR suggest an impact of anemia on the immune response. Diminished IL-6 and IL-8 PRR responses observed in HIV-infected mothers who did not receive ART before pregnancy were associated with decreased fetal hemoglobin. Unfortunately, the interaction of HIV, maternal anemia and fetal anemia with the innate immune responses cannot be disentangled in our study and it is impossible to infer causality.

Other factors had some associations with the cytokine profile. Age is usually associated with cytokine responses and our study seems not to be an exception. Gravidity, which is related to age, was independently associated with lower spontaneous production of three cytokines on maternal peripheral blood. Curiously, sex of the newborn could be associated with the mother's spontaneous cytokine production. Women carrying male fetuses had lower spontaneous production of T_H1 and T_H17 cytokines. Sexual dimorphism has been observed in maternal inflammation and cytokine profile in other studies (Stevenson et al., 2000; Enninga et al., 2015). Carrying a female fetus has been associated with more severe asthma in the mothers (Clifton, 2010) and fetal sex affects birth outcomes such as low fetal growth restriction, prematurity, and preeclampsia (Edwards et al., 2000). Sexual dimorphisms in the placenta have also been described (Clifton, 2010), but we did not find differences in placental blood in our study.

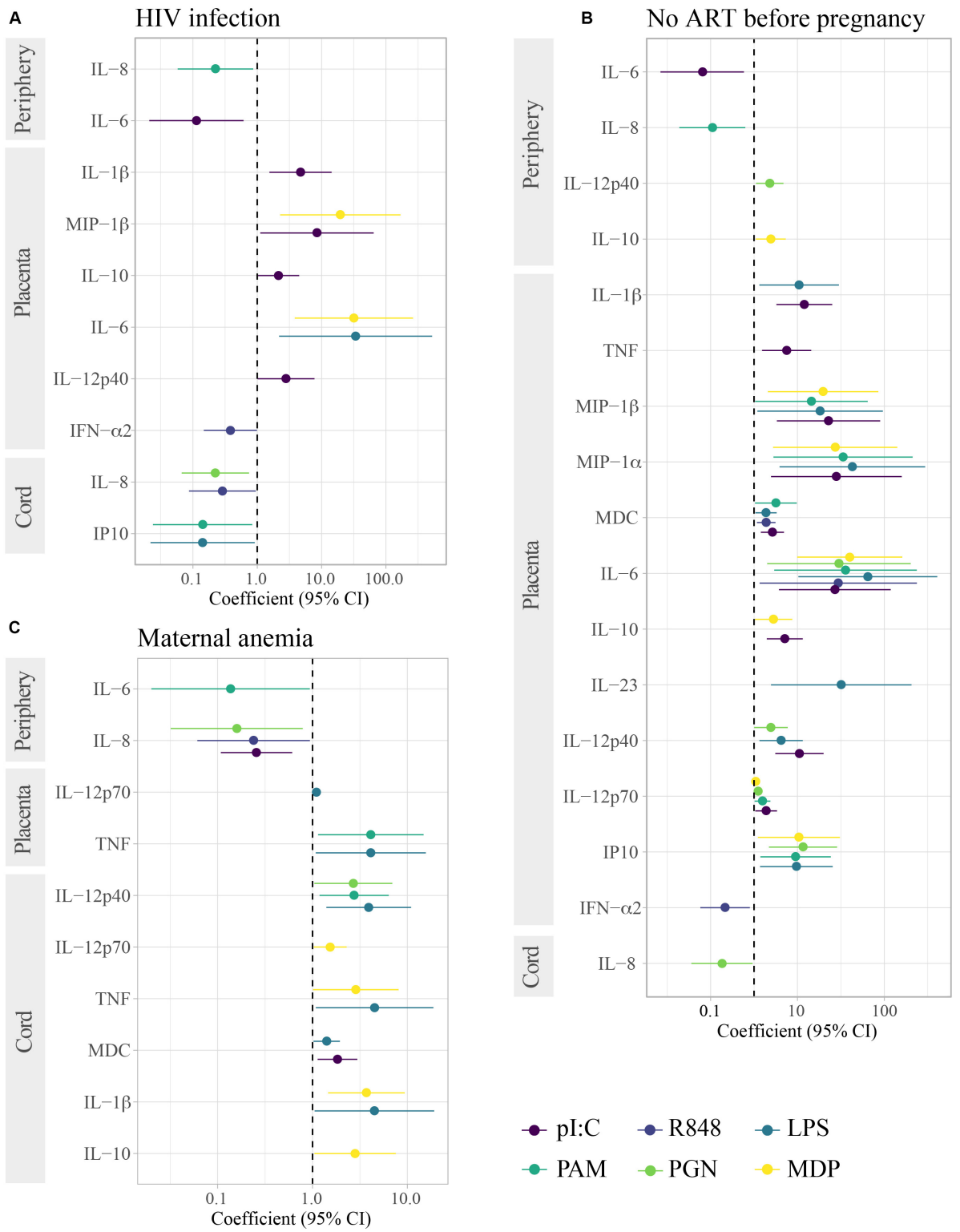

FIGURE 4 | Association of HIV infection, lack of ART before pregnancy and maternal anemia with cytokine responses induced by innate stimulations. Forest plots show the effect of **(A)** HIV-infected in reference to HIV-uninfected women, **(B)** lack of ART before pregnancy in HIV-infected women in reference to HIV-uninfected women, and **(C)** maternal anemia in reference to no anemia in HIV-infected and uninfected women. Only results with raw p-values < 0.05 are shown. All results in **(C)** were statistically significant after adjusting for multiple testing by Benjamini–Hochberg. The coefficient was calculated as 10^beta and beta was obtained in multivariable regression models adjusted by age (log_{10}-transformed) and technical batch with cytokine ratios (log_{10}-transformed) as outcome.

TABLE 3 | Association of cytokines induced by innate stimulations with birth outcomes.

Compartment	Agonist	Receptor	Receptor localization	Cytokine ratio	Cytokine group	Coefficient (%)[a]	95% CI	*P*-value[b]	BH *P*-value[c]
Birth weight									
Periphery	R848	TLR7/8	Endosomal	IL-12p70	TH1	0.599	0.019; 1.181	0.043	0.983
Placenta	R848	TLR7/8	Endosomal	IL-12p40	TH17	0.366	0.036; 0.697	0.031	0.983
Fetal hemoglobin									
Periphery	MDP	NOD2	Cytosolic	IFN-γ	TH1	−32.054	−47.201; −12.561	0.004	0.294
Periphery	PAM	TLR1/2	Surface	IL-6	TH17	3.625	1.049; 6.267	0.007	0.294
Periphery	LPS	TLR4	Surface	IL-6	TH17	3.499	0.975; 6.086	0.007	0.294
Periphery	R848	TLR7/8	Endosomal	IL-6	TH17	2.594	0.307; 4.932	0.027	0.601
Periphery	R848	TLR7/8	Endosomal	IL-8	Pro-inflammatory	5.578	1.963; 9.321	0.003	0.294
Periphery	pI:C	TLR3	Endosomal	IL-8	Pro-inflammatory	8.297	2.803; 14.084	0.004	0.294
Periphery	PAM	TLR1/2	Surface	IL-8	Pro-inflammatory	5.675	1.731; 9.773	0.006	0.294
Periphery	PGN	NOD1/2	Cytosolic	IL-8	Pro-inflammatory	4.243	1.068; 7.517	0.01	0.360
Periphery	LPS	TLR4	Surface	IL-8	Pro-inflammatory	5.271	1.141; 9.569	0.013	0.410
Periphery	MDP	NOD2	Cytosolic	IL-8	Pro-inflammatory	6.625	1.011; 12.551	0.021	0.588
Periphery	PAM	TLR1/2	Surface	IP10	TH1	2.85	0.031; 5.749	0.048	0.657
Periphery	LPS	TLR4	Surface	MIP-1β	Pro-inflammatory	2.891	0.109; 5.75	0.042	0.657
Periphery	PAM	TLR1/2	Surface	MIP-1β	Pro-inflammatory	2.904	0.094; 5.794	0.043	0.657
Cord	PGN	NOD1/2	Cytosolic	IL-12p40	TH17	−5.078	−9.712; −0.207	0.042	0.657
Cord	PGN	NOD1/2	Cytosolic	IL-12p70	TH1	−13.054	−23.336; −1.393	0.03	0.601
Cord	pI:C	TLR3	Endosomal	IL-1β	Pro-inflammatory	−4.931	−9.182; −0.48	0.031	0.601
Cord	pI:C	TLR3	Endosomal	MDC	Pro-inflammatory	−10.339	−18.504; −1.356	0.026	0.601
Gestational age									
Placenta	R848	TLR7/8	Endosomal	IL-12p40	TH17	0.092	0.003; 0.182	0.044	0.837
Placenta	LPS	TLR4	Surface	IP10	TH1	−0.104	−0.179; −0.028	0.008	0.837
Placenta	MDP	NOD2	Cytosolic	IP10	TH1	−0.089	−0.159; −0.02	0.013	0.837
Placenta	PAM	TLR1/2	Surface	IP10	TH1	−0.094	−0.174; −0.014	0.023	0.837
Placenta	MDP	NOD2	Cytosolic	TNF	Pro-inflammatory	0.13	0.024; 0.236	0.018	0.837
Placenta	PAM	TLR1/2	Surface	TNF	Pro-inflammatory	0.094	0.006; 0.182	0.038	0.837
Cord	MDP	NOD2	Cytosolic	IL-8	Pro-inflammatory	−0.123	−0.225; −0.02	0.021	0.837

Only results with raw p-values ≤ 0.05 are shown. [a]Coefficients show the difference in percentage in the outcome with 10% increases in cytokine ratios. It was calculated as (1.10^beta-1) × 100 and beta was obtained in multivariable regression models adjusted by age (log_{10}-transformed) and technical batch with cytokine ratios (log_{10}-transformed) as predictor variable and birth outcomes as dependent variable. Birth weight and gestational age were log_{10}-transformed. [b]Raw p-value. [c]Significance was established at a Benjamini–Hochberg (BH) adjusted p-value ≤ 0.3. P-values were adjusted by a total of 252 tests performed for each independent variable.

Instead, in cord, IP10 responses in male infants for all PRR were lower than females, although these differences were not statistically significant after adjusting for multiple testing. Sex has a clear effect on immune response through life and particularly during infancy has been associated with different susceptibility to diseases and vaccine responses (Muenchhoff and Goulder, 2014; Fischinger et al., 2019). Future studies should assess if differences in IP10, a key chemokine in protective immunity and vaccine responses, are maintained through infancy and related to sex differences in clinical and vaccine outcomes.

It is not clear how maternal peripheral blood differs from placental or newborn cord blood, although cytokine responses in the mother seem to have a strong influence on cytokine responses in their infants (Djuardi et al., 2016). While overall cytokine concentrations and PRR responses were positively correlated between the mother peripheral and the placental blood and the cord blood, cytokine levels tended to be higher in cord blood stimulations than in peripheral blood, with some specific PRR responses higher in cord than in maternal blood. This is, apparently, in contradiction to previous reports showing a diminished T_H1 and other responses in cord blood than adults (Kollmann et al., 2012; Georgountzou and Papadopoulos, 2017). We found only lower responses to IFN-α upon PAM in cord. However, here we compared cord blood responses to blood responses from their mothers at delivery instead of non-related non-pregnant adults. Of note, TLR7/8 responses were the most robust response as previously described (Kollmann et al., 2012; Georgountzou and Papadopoulos, 2017). Despite the coordinated innate response of the mothers with their children found here or in other studies (Djuardi et al., 2016), we did not find a clear association of peripheral blood cytokine responses with birth weight or gestational age and prematurity, only with fetal hemoglobin levels. Placental blood responses were less correlated with peripheral and cord responses and probably influenced by the delivery process which is known to be a pro-inflammatory placental process. In fact, we found higher spontaneous production of pro-inflammatory cytokines in the placenta. Due to the exposure of the placenta to the mothers' microbiome during delivery and the procedure of sample collection, there is the potential risk of bacterial contamination in the placental blood. While that could be a problem because bacteria would stimulate PRR, we had unstimulated controls in the assay and our interpretation is in comparison of stimulated to those unstimulated samples. The finding that HIV may be associated specifically with placental responses and the association of some of the placental blood responses with gestational age and prematurity calls for further research on the immunopathology of this organ. The role of trophoblasts may be relevant, since these cells induce innate responses upon sensing of pathogens and orchestrate the recruitment and activation of innate cells at the maternal-fetal interface (Guleria and Pollard, 2000).

Despite some associations and other interesting findings of biological plausibility, the low sample size and the multiple tests performed demand interpreting the results with prudence, particularly because the potential for false positives is high. Findings should be confirmed with other techniques such ELISAs and additional cohorts. We measured secreted cytokines in whole blood, but particular cell subsets may be differently producing the measured cytokines upon PRR stimulation and therefore, further studies should include single-cell analyses. Also, it would be of interest to assess the expression of PRR in the cells of each compartment. Some findings may be specific from the study area and innate responses may vary in different geographic areas as has been shown in children from different continents (Smolen et al., 2014). Furthermore, inclusion of women participating in clinical trials may have posed some bias due to different treatments and additional medical attention that they may have received compared to other women in the area. We cannot discard either that the IPTp treatment received or the CTX administered to all HIV-infected women may have had an effect on the innate responses. Nevertheless, our data provide evidence of the association of HIV infection with innate responses to PRR in the mother, particularly obvious in the placenta. Results also suggest a complex relationship between HIV, maternal and fetal anemia and innate responses that may provide clues on anemia development. In addition, we report important data on specific innate responses correlating with birth outcomes that warrant follow up studies. Modulation of the innate response could be a powerful strategy to improve maternal and neonatal outcomes, but the causal association of innate responses with birth outcomes and infant's health requires further detailed investigation.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

This study was carried out in accordance with ICH Good Clinical Practice guidelines and the Declaration of Helsinki. The study protocols and informed consent forms were reviewed and approved by the Comité Ètic d'Investigació Clínica (CEIC, Hospital Clínic, UB), Spain, and the Comité Nacional de Bioética (CNBS), Mozambique. Written informed consent was obtained from all participants.

AUTHOR CONTRIBUTIONS

GM, AM, CD, and TK wrote the first draft of the manuscript. TK, AM, CD, and GM conceived the study and the experimental design and interpreted the data. GM performed the statistical analysis. MM, RB, and CJ collected the samples and performed the stimulation experiments. KS performed the cytokine

Luminex assay. RG, MR, JA, EM, and CM designed and enrolled participants in the clinical trials. JA was the clinical trial statistician. CD, RG, KS, CM, TK, and AM contributed to the write up of the manuscript. All authors reviewed and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.01452/full#supplementary-material

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