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Translational virology in pregnancy

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Translational virology in pregnancy

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EDITORIAL
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Editorial: Translational Virology in Pregnancy

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Editorial on the Research Topic

Translational Virology in Pregnancy

Viral pandemics, like the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), are becoming more common and disproportionately impact the health of pregnant individuals and their fetuses. Recently, the probability of an extreme epidemic similar in intensity to the 1918 "Spanish influenza" with roughly 32 million deaths was estimated at 1.9% annually (1). For an individual born in 2000, this estimate translates to a 38% lifetime probability of experiencing an extreme epidemic by 2020. This probability estimate was expected to increase up to three-fold due to human-environment interactions, disease emergence from zoonotic reservoirs, and climate change (1). Depending on the virus, an infection during pregnancy may impart risk for more severe maternal disease, preterm birth, preeclampsia, stillbirth, fetal congenital anomalies and/or a long-term risk for neuropsychiatric disease in the children (2–9). This Research Topic includes scientific manuscripts broadly covering viral infections in pregnancy and their impact on pregnancy outcome and the maternal-placental-fetal immune response. Here, we summarize the articles included in this Research Topic and their importance to the field of translational virology in pregnancy.

Understanding the impact of viral infections in pregnancy on the fetus typically begins with *in vitro* and *in vivo* models with a focus on the placental response to infection. Sheridan et al. reviewed the wide range of current human placental models that include primary trophoblast cell and explant cultures, trophoblast cell lines, and trophoblast stem cells and organoids. This review also discusses how these models may be used to learn about viral trafficking and placental immune defense as well as their strengths and limitations. Since viral infection of the placenta is thought to play a pivotal role in adverse pregnancy outcomes, an important research area is the investigation of placental immunopathology and factors permitting or restricting viral replication at the maternal-fetal interface. Devi Negi et al. explored the role of interleukin-10 (IL-10) in viral clearance from uterine and placental tissues in a murine model of lymphocytic choriomeningitis virus (Armstrong; LCMV-Arm). High IL-10 at the maternal-fetal interface impaired viral clearance suggesting that modulation of IL-10 expression may be beneficial in preventing adverse pregnancy outcomes. In a study of human placentas from women testing positive for COVID-19, Sharps et al. evaluated the distribution of immune cells and found a significant increase in the number of $CD163^{+}$ placental macrophages (Hofbauer cells) and vascularity in the COVID-19 group. The assortment of available *in vitro* and *in vivo* systems for modeling placental viral infections are essential for understanding the role of immune responses in controlling viruses at the maternal-fetal interface.

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The Zika virus (ZIKV) pandemic in 2015-2016 was linked to thousands of cases of congenital microcephaly and a wide spectrum of central nervous system anomalies in newborns (10). A series of articles in this Research topic addresses the impact of ZIKV infection on placental cellular processes, immune defenses, and vertical transmission. Guzeloglu-Kayisli et al. reviewed the molecular mechanisms underlying ZIKV infection of placental cells and highlight the role that decidual cells play in the propagation of ZIKV in extravillous cytotrophoblast cells and subsequent transplacental infection. Using a rhesus macaque model and single cell RNA sequencing, Haese et al. found that ZIKV infection induced the stable expression of antiviral genes within $CD14^+$ cells of the placenta, which may act to limit ZIKV from propagating in the placenta and causing further tissue injury. McKinney et al. demonstrated that downregulation of autophagy genes and proteins occurs later in ZIKV infection by triangulating data from primary human trophoblast cells, human placentas, and a marmoset model. ZIKV continues to pose a viral threat globally as it is endemic in large geographic regions with sporadic outbreaks and the potential to collapse health care sectors (11). Investigating the molecular and cellular consequences of ZIKV infection during pregnancy and potential pharmaceutical targets to prevent vertical transmission remains a high priority.

A major knowledge gap is the impact of maternal viral infections on placental and fetal development. Many viruses are known to cross the placenta and infect the fetus, such as ZIKV and the 'TORCH' infections [varicella-zoster virus, parvovirus B19, rubella virus, cytomegalovirus, herpes simplex virus]. Creisher et al. used an immunocompetent mouse model of ZIKV infection to demonstrate a broad downregulation of transcriptional activity of genes in the placenta that drive tissue morphology, neurological development, cell signaling and inflammation. Maternal infections and immune activation have also been associated with an increased long-term risk in the child for neuropsychiatric disease including autism spectrum disorders (ASD) (3, 12). Sharma and Jash discussed the link between viral infection in pregnancy, uterine immune activation and regulatory T cells in the development of ASD-like fetal brain pathology. This highlights a potential transcriptional mechanism for ZIKV-mediated placental and fetal pathology. Finally, an intriguing article by Goetzl et al. proposed a novel non-invasive method for diagnosing fetal viral infection, and thus viral infection-associated fetal brain injury, through the quantitation of fetal central nervous system extracellular vesicles from maternal serum or plasma. For nearly all viruses with pandemic potential, our knowledge of how infection impacts fetal neurodevelopment is either incomplete or completely unknown.

In addition to highly pathogenic viruses known to adversely impact pregnancy and fetal outcomes, there are also commensal viruses colonizing humans with an unknown impact on pregnancy outcomes. Anelloviruses (family Anelloviridae) are non-enveloped circular single-stranded DNA viruses that are a nearly ubiquitous colonizer of humans and can be found in maternal blood, amniotic fluid, cervical and vaginal fluid, and breast milk. Kyathanahalli et al. presented an intriguing possible link between anellovirus colonization and birth timing through an analysis of what is known regarding the interactions of anelloviruses with other microbes and impact on maternal host defense. The understanding of the vaginal microbiota and innate/adaptive immune crosstalk that contributes to the risk for spontaneous preterm birth continues to evolve (13), which leaves the possibility that immunomodulation by anelloviruses, or other unknown microbes, may change the vaginal immune milieu and increase preterm birth risk.

There is a great need for therapeutic development to mitigate the maternal-placental-fetal injury posed by a viral infection in pregnancy. Viral infections may be associated with an increased risk for preeclampsia, a hypertensive disorder in pregnancy that can be life-threatening for the mother. Indeed, recent data indicates that SARS-CoV-2 infection is strongly associated with the development of preeclampsia (14). Modeling this *in vivo*, preeclamptic-like symptoms have been linked to the activation of the Toll-like receptor 3 (TLR3) pathway by double-stranded RNA (dsRNA), which is produced by positive-strand RNA viruses, like SARS-CoV-2 (15, 16). In this mouse model, Balasubbramanian et al. found that high-dose vardenafil blunted the hypertensive effects of TLR3 activation by the viral dsRNA mimic, Poly(I:C). Drug repurposing combined with new approaches for drug validation in animal models can significantly accelerate the approximate 10-20 year timeline for moving a drug from bench-to-bedside (17). Evaluating potential therapeutics for efficacy in pregnancy is not a challenge embraced by the pharmaceutical industry and one that the reproductive scientific community bears disproportionately (18).

Pandemic preparedness requires an understanding of the impact of a wide array of viruses on maternal-fetal immunity, maternal health, pregnancy outcomes and fetal development. The risk for new viral epidemics and pandemics is increasing with climate change and human-environment interactions. Investigation of the molecular and cellular processes contributing to placental immunopathology and immune defense is the foundation for understanding the effect of viruses on the maternal-fetal dyad, which is essential for the development of clinical approaches to improve maternal and fetal outcomes.

AUTHOR CONTRIBUTIONS

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

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Placental Autophagy and Viral Replication Co-localize in Human and Non-human Primate Placentae Following Zika Virus Infection: Implications for Therapeutic Interventions

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Background: Multiple studies have shown both induction and inhibition of autophagy during Zika virus (ZIKV) infection. While some have proposed mechanisms by which autophagic dysregulation might facilitate ZIKV vertical transmission, there is a lack of *in situ* data in human and non-human primate models. This is an especially pertinent question as autophagy-inhibitors, such as hydroxychloroquine, have been proposed as potential therapeutic agents aimed at preventing vertical transmission of ZIKV and other RNA viruses.

Objectives: Given the paucity of pre-clinical data in support of either autophagic enhancement or inhibition of placental ZIKV viral infection, we sought to assess cellular, spatial, and temporal associations between placental ZIKV infection and measures of autophagy in human primary cell culture and congenital infection cases, as well as an experimental non-human primate (marmoset, *Callithrix jacchus*) model.

Study Design: Primary trophoblast cells were isolated from human placentae ($n = 10$) and infected *in vitro* with ZIKV. Autophagy-associated gene expression (*ULK-1, BECN1, ATG5, ATG7, ATG12, ATG16L1, MAP1LC3A, MAP1LC3B, p62/SQSTM1*) was then determined by TaqMan qPCR to determine fold-change with ZIKV-infection. In *in vivo* validation experiments, autophagy genes *LC3B* and *p62/SQSTM1* were probed using *in situ* hybridization (ISH) in the placentae of human Congenital Zika Syndrome (CZS) cases ($n = 3$) and ZIKV-infected marmoset placenta ($n = 1$) and fetal tissue ($n = 1$). Infected and uninfected villi were compared for mean density and co-localization of autophagic protein markers.

Results: Studies of primary cultured human trophoblasts revealed **decreased** expression of autophagy genes *ATG5* and *p62/SQSTM1* in ZIKV-infected trophoblasts [*ATG5* fold change (±SD) 0.734-fold (±0.722), $p = 0.036$; p62/SQSTM1 0.661-fold (±0.666), $p = 0.029$]. Histologic examination by ISH and immunohistochemistry confirmed spatial association of autophagy and ZIKV infection in human congenital infection cases, as well as marmoset placental and fetal tissue samples. When quantified by densitometric data, autophagic protein LC3B, and p62/SQSTM1 expression in marmoset placenta were significantly *decreased* in *in situ* ZIKV-infected villi compared to less-infected areas [LC3B mean 0.951 (95% CI, 0.930–0.971), $p = 0.018$; p62/SQSTM1 mean 0.863 (95% CI, 0.810–0.916), $p = 0.024$].

Conclusion: In the current study, we observed that in the non-transformed human and non-human primate placenta, disruption (specifically down-regulation) of autophagy accompanies later ZIKV replication *in vitro, in vivo,* and *in situ*. The findings collectively suggest that dysregulated autophagy spatially and temporally accompanies placental ZIKV replication, providing the first *in situ* evidence in relevant primate pre-clinical and clinical models for the importance of timing of human therapeutic strategies aimed at agonizing/antagonizing autophagy. These studies have likely further implications for other congenitally transmitted viruses, particularly the RNA viruses, given the ubiquitous nature of autophagic disruption and dysregulation in host responses to viral infection during pregnancy.

Keywords: autophagy, congenital Zika syndrome, placental viral infection, autophagy and viral infection, viral transmission, Zika virus

HIGHLIGHTS

- Given the contradictory nature of pre-clinical data supporting both autophagic enhancement and inhibition of placental ZIKV viral infection, we sought to assess cellular, spatial, and temporal associations between human and non-human primate placental ZIKV replication and measures of autophagy.
- Dysregulated autophagy spatially and temporally co-localizes with placental ZIKV replication in human placentae and non-human primate placental and fetal tissue from congenital infection cases.
- Demonstrating the relationship between dysregulated autophagy and placental ZIKV infection provides the first *in situ* evidence in relevant pre-clinical models for human therapeutic strategies aimed at agonizing/antagonizing autophagy, potentially suggesting a means for mediating vertical transmission of ZIKV.

INTRODUCTION

Autophagy is a critical cytoplasmic process of degradation, which is felt to be crucial in the maintenance of cellular and tissue integrity. It occurs at a basal level in eukaryotic cells, in which cytoplasmic contents are partitioned into a double membrane-enclosed autophagosome (AP). This AP complex can then fuse with lysosomes to participate in cellular degradation and recycling. Every step of the process (initiation, vesicle elongation, maturation, docking and fusion, vesicle degradation) is controlled by a complex set of regulatory proteins (1–3). Over the past decade, interest in the role of autophagy in many areas of the pathophysiology of human disease has increased, including cellular response to infection.

There are several examples of co-evolution of viral mechanisms that take advantage of (or co-opt) host clearance mechanisms to accentuate their virulence. For example, recognition of viral antigens and the subsequent triggering of interferons and neutralizing antibodies limit viral replication, and thus viruses have co-opted key aspects of immune recognition and interferon production to instead facilitate their replication with enhanced cell entry and suppressed immunoreactivity (1, 4). Autophagic mechanisms that clear viruses, as obligate intracellular organisms, are no exception. Japanese encephalitis (5), HIV (6), Dengue (7, 8), Ebola (9), Parvo, Polio, Coxsackie, Hepatitis B, and Hepatitis C (10) viruses are all examples of viruses that exploit autophagy to enhance their replication and trigger host pathogenesis in the process (11).

Zika virus (ZIKV), a positive-strand RNA arbovirus in the *Flaviviridae* family initially identified in Uganda in 1947, evolved an Asian clade that then was identified as causing birth anomalies after emerging in the Caribbean and South America in late 2014 and early 2015 (12, 13). The latest pathological Asian strain has since become endemic, not only

regionally, but has re-emerged in Africa and Asia in a largely immuno-naïve population (14). Recognition of congenital Zika syndrome (CZS), a phenotype that includes severe fetal neural anomalies (12, 15) during the last epidemic in South America, spurred a wave of research into mechanism and route of ZIKV infection and perinatal transmission (13, 16–23). Through this research, autophagy was identified as a potential key interrupted process rendering CZS. This was not entirely surprising, as dysregulation of autophagy has been previously linked to neural pathogenesis in neurodegenerative conditions (24), is known to be important for normal neurogenesis in human fetal neural stem cells (25, 26), and has been shown to affect central nervous system consequences of ZIKV infection in a pregnant mouse model (27). Moreover, *in vitro* infection models demonstrate that some degree of dysregulation of autophagy accompanied ZIKV infection of cell lines, including human umbilical vein endothelial cells (28), fetal neural stem cells (25, 29), and human skin fibroblasts (30). Several potential genes and pathways involved in the process have been identified in non-human or primate models (29, 31–35). However, the exact manner of and mechanisms by which autophagy dysregulation might facilitate ZIKV vertical transmission is still under investigation, especially regarding avoidance of clearance through the normal anti-viral autophagic processes after initial induction. This is crucial, as pharmacologic autophagy inhibitors are both available and have historical use with safety data for other indications in pregnancy (36–38). Specifically, anti-autophagic drugs such as mefloquine (37) or chloroquine (38, 39) have been proposed as potentially effective treatment to prevent maternal-fetal transmission in ZIKV-exposed pregnancies.

Autophagic processes have previously been shown to affect resistance to infection in human placentae [for example Cao et al. (40)]. Biologically plausible candidates for autophagy dysregulation facilitating vertical transmission include several of the cells of the human placenta. Indeed, we and others previously demonstrated *in vivo*, *in vitro,* and *in situ* replication of ZIKV in primary human and primate placental cells (cyto- and syncytiotrophoblasts, Hofbauer cells, endothelial cells, fibroblasts) (13, 19), as well as cells in the chorioamniotic membrane (epithelial cells and trophoblast progenitors) (41). We demonstrated previously that even small focal infections of the villous tissue are sufficient for vertical transmission of the virus, even with asymptomatic maternal infection (16). Experimental infections in animals (primates and manipulated murine models) demonstrated that placental trophoblast replication preceded fetal infection (18, 42). Collectively, these data document varying degrees of placental cellular infection in both the absence and presence of fetal infection and pathogenesis, leading to the conclusion that the placenta potentially seeds and serves as a reservoir for subsequent diffuse fetal infection.

These observations inspired our current study. Because the placenta is a highly active in autophagic flux throughout pregnancy (43), we and a few others have hypothesized that the interaction of autophagy and ZIKV infection in the placenta may contribute to pathogenesis. For example, impairment of autophagy through use of knock out mouse models reduces vertical transmission and fetal and placental damage following ZIKV infection (40). Results were echoed in studies of human neuronal precursors and glial cells in culture in which ZIKV seems to induce autophagy early on during infection, but then downregulates autophagy through induction of mTOR in order to facilitate ZIKV replication (44).

Given the paucity of human clinical (observational cases) or pre-clinical experimental animal data in support of either autophagic enhancement or inhibition of placental viral infection, we sought to leverage our previous human translation and non-human experimental infection research to assess cellular, spatial, and temporal associations between placental ZIKV replication and measures of macroautophagy. The objectives were therefore to first directly assess autophagic gene expression changes in primary syncytiotrophoblast cell culture infected *in vitro*. We then aimed to provide detailed histopathology *in vivo* employing human placental tissue and our non-human primate model (*Callithrix jacchus,* common marmoset) that, unlike other mammalian models, is naturally susceptible to infection and vertical viral transmission.

MATERIALS AND METHODS

ZIKV-Associated Autophagy Gene Expression Changes in Primary Human Trophoblasts by qPCR

Placental donors were comprised of primarily white Hispanic women with uncomplicated term deliveries, without major maternal or fetal comorbidities or anomalies, and high integrity placental tissue samples. Subjects and their samples were recruited via methods described previously in the index study for the current report (13). The clinical characteristics of the donor pregnancies are summarized in **Supplementary Table 1**. Briefly, placental tissue was dissected, then enzymatically digested, then Percoll separated to isolate cytotrophoblasts, which then syncytialized *in vitro*. After 2–5 days, syncytiotrophoblasts were infected with 1×10^5 RNA copies of first passage clinically isolated ZIKV FLR [10 Tissue Culture Infectious Dose (TCID) or mock]. Active replication was confirmed as described in our previously published work (13). After 4–5 days cells were scraped and flash-frozen in RNA*later* (ThermoFisher Scientific). Exosomal RNA was isolated from ZIKV-infected trophoblast cultures using TRIzol LS (Invitrogen) purification as previously described (13). Levels of specific mRNAs for autophagy-associated genes (*ULK1, BECN1, ATG5, ATG7, ATG12, ATG16L1, MAP1LC3A, MAP1LC3B, p62/SQSTM1*) were assessed against GAPDH as the endogenous control, using TaqMan qPCR assays (ThermoFisher Scientific) (assay IDs are listed in **Supplementary Table 2**). Template cDNA was generated using High Capacity cDNA reverse transcription Kit (Applied Biosystems) per manufacturer's instructions. Triplicate reactions were then prepared using 1 ug template cDNA per well. TaqMan assays were performed with a StepOnePlus platform (Applied Biosystems) according to standard conditions of 60°C annealing temperature for 40 cycles. Delta C_t data was filtered for outliers with a $Q = 0.01$. Differences were determined using paired *t*-tests within donors compared to mock. Fold change was

calculated by delta delta C_t method. All statistics were performed with Prism software (GraphPad, v8.4.3, La Jolla, CA).

Co-localization of ZIKV Replication and Autophagy Activity in Human and Marmoset Placentae via *In situ* Hybridization and Immunohistochemistry

No new samples were obtained for this current work. Histological sections from human case reports as well as experimental non-human primate infections were obtained from two separate studies that have been previously published (16, 19) [human studies were approved by Baylor College of Medicine IRB H-25735; non-human primate studies were performed at Southwest National Primate Research Center and approved by local Institutional Animal Care and Use Committee (IACUC) and Biohazard Committee]. For the human study, subjects with risk for, or suspected, CZS were recruited and consented as described in the previous publication (16). A clinical description of the cases is summarized in **Supplementary Table 3**. Immediately following delivery, a full cross section of placental tissue was removed 4 cm from the cord insertion site for three suspected CZS cases, and one healthy neonate. Tissue was then further dissected and fixed in formalin for ∼8 h then processed into paraffin blocks.

Under IACUC approval, two pregnant common marmosets were experimentally infected with an injection of 2.5×10^5 plaque forming units (PFU) of the first passage contemporaneous Brazil ZIKV strain SPH2015 (GenBank accession number KU321639) twice 4 days apart, on estimated gestational days 79/83 and 68/72, respectively, as previously published (19). After 16 days (dam 1) and 18 days (dam 2) of asymptomatic infection, both pregnancies spontaneously aborted, from which tissue was obtained and formalin fixed prior to processing. Because of tissue destruction prior to initial collection, sections of collected tissue from only dam 2 were paraffin-embedded into blocks and used for the current work: marmoset fetus [full sagittal ($n = 1$) and frontal/coronal ($n = 1$)] and placenta ($n = 1$). Detailed examination of the tissues as previously described revealed focal infection and viral replication in diverse placental and fetal tissues.

In the current study, for all tissues, serial sections were meticulously prepared in parallel to allow for cross-comparison using different stains to label viral replication and markers of autophagy. To assess for viral infection, *in situ* hybridization (ISH) was carried out against the ZIKV positive strand viral genome using a ZIKV-specific probe set largely according to manufacturer's instructions. An additional pretreatment with Protease Plus for 15 min and Target Retrieval Reagent for 15 min (RNAscope, ACD Biosciences) was performed for optimal retrieval. To assess for the levels of autophagy-associated proteins LC3B and p62/SQSTM1, immunohistochemistry was employed. Serial sections of the human placental blocks were deparaffinized and rehydrated, then probed with antibodies against LC3B (#3868, Cell Signaling Technology, Danvers, MA, USA) or p62/SQSTM1 (#H00008878-M01, Abnova, Walnut, CA, USA). It was determined that these were non-cross reacting antibodies with testing, and therefore LC3B (NB100-2220) and p62/SQSTM1(NBP1-49956) (Novus, Centennial, CO, USA) were alternatively used for non-human primate tissue sections.

Bright field slides were examined under low (4x), medium (20x), medium-high (40x), and high power (60x) using a Nikon Eclipse 90i microscope (Nikon Instruments, Melville, NY, USA). As a first pass, areas of active ZIKV infection were identified and marked. The same villi and areas were then identified on each serial immunohistochemistry section by comparing those markings and confirmed by comparing landmarks within the microanatomy of those areas. In this way, precise co-localization could be confidently determined. Images were captured using NIS Elements 4.20 (Nikon). Staining within areas of interest was assessed visually and using metrics within the software as described below. Minor adjustment for contrast and background levels were made using NIS Elements 4.20 for publication (Nikon).

Quantitative Measures of Autophagy Activity in Marmoset Placentae

Sections were inspected for focal areas of infected villi. Four such areas per slide in the marmoset placental tissue were selected, where heavily ZIKV-infected villi were adjacent to apparently un-stained, and uninfected villi. Densitometry was then performed within these villi to establish and compare the levels of autophagic marker proteins. Standard colorimetric thresholds were established for each of the three stains (ZIKV, LC3B, and p62 antibodies) using NIS Elements 4.20 (Nikon). To do this, the most densely stained area of the highest power image was selected, and serial areas of staining were selected until all apparent staining was captured as assessed visually. Villi of interest were then manually traced using the inside of the syncytiotrophoblast as the perimeter. From the ZIKV stained slides, densitometry was used to systematically establish the placental villi within the captured fields with the most ($n = 2$) and least ($n = 2$) ZIKV infection. The most and least infected villi were then set as our regions of interest in the serially stained autophagy marker protein slides.

Areas were then traced to the same villi in the probed serial sections. The established thresholds for each antibody were then applied to each region of interest across all the slides, and densitometric measurements were made. Mean area density of the stain (as defined by previously-set colorimetric thresholds) was calculated for each region of interest and used as the basis of comparison. Differences between the most and least infected areas on all marmoset placental slides were determined using paired *t*-tests. All statistics were performed with Prism software (GraphPad, v8.4.3, La Jolla, CA).

RESULTS

Autophagy-Related Gene Expression Changes in ZIKV-Infected Primary Human Trophoblasts

To determine whether transcription of autophagic genes were altered (either up or down regulated, shown as fraction of mock-infected same subject controls) with cellular ZIKV infection, we analyzed *in vitro* infection of primary human trophoblasts.

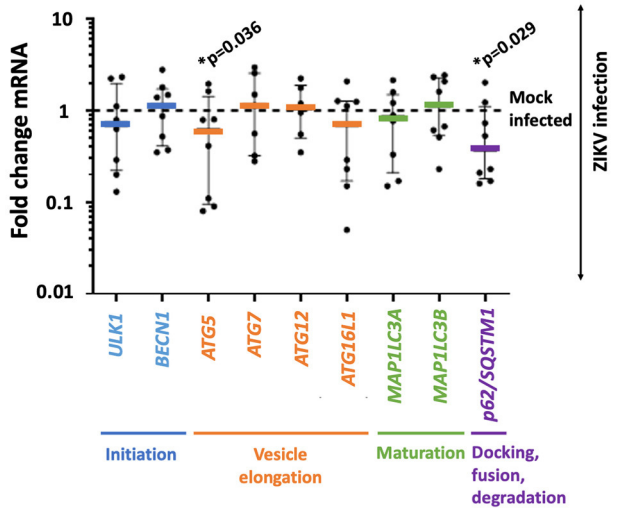

FIGURE 1 | ZIKV infection is accompanied by decreased gene expression in autophagy-related genes *ATG5* and *p62/SQSTM1* in primary human trophoblasts. Primary human trophoblasts were infected with 10^5 RNA copies of first passage clinically isolated ZIKV FLR or mock, and autophagic gene expression was assessed. Genes were selected based on involvement in multiple phases of autophagy (stage noted in colored text underlying the *x* axis). Expression of *ATG5* and *p62/SQSTM1* were significantly decreased (*ATG5* mean fold change expression 0.734 ± 0.722 SD; *p*-value 0.036, *p62/SQSTM1* mean fold change expression 0.661 ± 0.666 SD, *p*-value 0.029) following infection 2–5 days post-isolation. Fold change in expression was calculated by delta delta Ct method, normalizing first to GAPDH and then mean delta Ct of mock-infected controls. Data was filtered for outliers with $Q = 0.01$. Significance was determined using *t*-tests of *Ct*-values with a $p < 0.05$ denoting significance (*).

Placentas from normal, healthy term pregnancies were collected, dissected, and cytotrophoblasts isolated. *In vitro* syncytialization was monitored by assessing the βhCG levels in the conditioned media, and cell purity was assessed on a subset by subjecting the cells to flow cytometry as we have previously published (13). The syncytiotrophoblasts were then infected with first passage ZIKV FLR or a mock. The infection was confirmed by daily monitoring of the conditioned media for ZIKV RNA. After 3–5 days cells were then collected, and RNA purified to measure for gene changes of interest.

Autophagy-related genes *ULK1, BECN1, ATG5, ATG7, ATG12, ATG16L1, MAP1LC3A, MAP1LC3B*, and *p62/SQSTM1* were assessed by TaqMan qPCR. Two significant changes in gene expression were observed by *t*-test. The expression of *ATG5* was decreased by 27% (mean fold change expression 0.734 ± 0.722; *p*-value 0.036), and *p62/SQSTM1* was decreased by 33% (mean fold change expression 0.661 ± 0.666, *p*-value 0.029) compared to mock infected placental trophoblasts (**Figure 1**).

Spatial Association of ZIKV Replication and Autophagy in Human Placental Tissue

Given the observed changes to key autophagy genes with *in vitro* ZIKV infection, we set out to examine known ZIKV-infected human placentae (with CZS-affected fetuses) for similar changes in protein *in situ*. Specifically, we chose LC3B as the primary marker of autophagy, as it directly interacts with our gene products of interest, as well as p62 protein. However, we first assessed the placenta for ZIKV presence via +strand RNA detection, so as to identify areas of interest with autophagic activity for examination.

Histologic examination of cases of CZS alongside uninfected, unaffected controls demonstrated the presence of replicating ZIKV in cases but not controls (brown staining, black arrowheads, **Figure 2A**). The virus was noted in multiple areas of placental villi including multinucleated syncytiotrophoblasts at the maternal–fetal interface. Histologic examination of serial placental sections stained with autophagy markers LC3B and p62/SQSTM1 was performed with careful identification of exact areas of ZIKV. Co-localization of autophagy and ZIKV infection were noted in all three cases of CZS. **Figure 2B** demonstrates colocalization of LC3B with red-brown staining, while **Figure 2C** demonstrates colocalization of p62/SQSTM1 with bright red staining. Black arrowheads identify areas of ZIKV infection noted in prior sections. The small amount of visible ZIKV infection at time of tissue harvesting is likely due to the inclusion criteria of the initial study which was for infants suspected with CZS, allowing for maternal serum IgM seronegativity for ZIKV at the time of delivery.

ZIKV and Autophagy Co-localize in Marmoset Placental Tissue as Well as Marmoset Fetal Neuro-Ophthalmic Structures

Unsurprisingly, examination of placental tissue from timed experimental infections in non-human primates showed a very high degree of ZIKV infection compared to the human cases. Dense dark brown staining was most apparent in the parenchyma of the tissue, while some staining was also apparent in the syncytiotrophoblasts. The darkest staining is most likely attributed to ZIKV infection of Hofbauer cells. Histologic examination of the placental tissue demonstrated focal areas of infection, with highly infected villi adjacent to villi without evidence of apparent ZIKV (**Figure 3A**, brown labeling). By comparing serial sections for morphological markers within the villous shapes, autophagy markers could be compared between infected and non-infected areas. Again, autophagy-related proteins LC3B and p62/SQSTM1 were selected as the markers of autophagy for these studies. At first glance, the staining appears universal for the constitutively expressed proteins, however co-localization within the same areas as ZIKV revealed spatial distinction (**Figure 3B**: LC3B, red/brown labeling; **Figure 3C**: p62/SQSTM1, red/brown labeling).

Examination was also made of marmoset fetal tissue from a second pregnancy with experimental infection that was spontaneously aborted. A frontal cross-section immediately posterior to the fetal eye was examined. ZIKV infection was noted within the neural tissue of the developing visual cortex, within the cortical plate of the developing frontal brain, and within the periorbital musculature. Co-localization of autophagic activity via autophagy markers LC3B (red/brown labeling) and

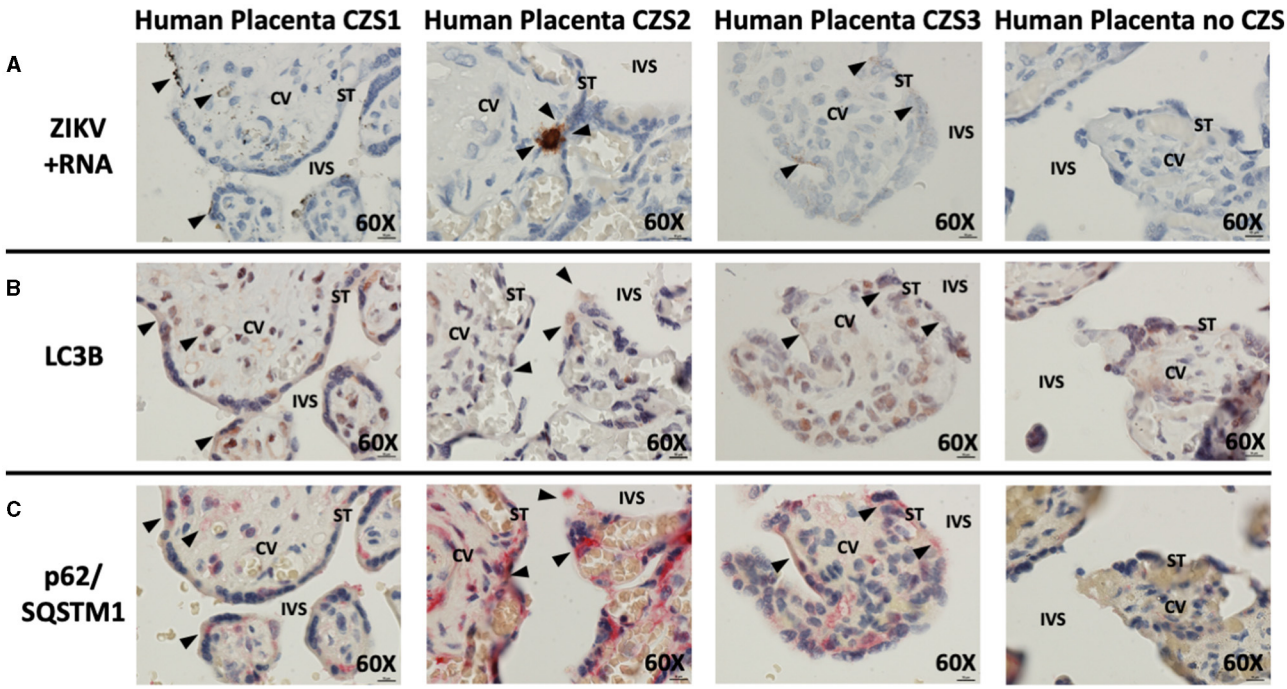

FIGURE 2 | *In situ* hybridization and immunohistochemistry staining demonstrate autophagy and ZIKV replication co-localize in human placentae of neonates affected by CZS. *In situ* hybridization with positive-strand ZIKV RNA probe set demonstrates ZIKV replication within villi from confirmed CZS cases but not in the placenta from the unaffected neonate (**A**: brown labeling, black arrowheads). Immunohistochemical staining for autophagic markers LC3B (**B**: dark red-brown labeling, black arrowheads) and SQSTM1/p62 (**C**: bright red labeling, black arrowheads) was noted in the same area of the villi where ZIKV replication was present. Probe-free controls confirm no non-specific binding in the absence of ZIKV, LC3B, SQSTM1/P62 probes, respectively. All images shown were obtained at 60X magnification. Micron bar scale in bottom right corner of images = 10 μm. CV, chorionic villus; IVS, intervillous space; ST, syncytiotrophoblast.

p62/SQSTM1 (red/brown labeling) was again noted within these same fetal neuro-ophthalmic structures (**Figures 4A–C**). Due to the breadth of tissue types present within these sections, analysis was limited to noting co-localization of ZIKV and autophagic activity rather than allowing quantification of autophagic activity.

Quantitative Comparison of Autophagy Protein Markers Between Infected and Uninfected Marmoset Placental Villi

To assess for a direct association between the viral infection and changing autophagy in an experimental *in vivo* primate model, post-imaging densitometric analysis of the bright field images was used to compare the levels of the autophagy proteins LC3B and p62/SQSTM1. Areas where infected and uninfected villous tissue was in close proximity were carefully chosen for comparison studies. **Figure 5A** labels ZIKV replication employing ISH. Stain thresholds were then set via post-processing densitometry to quantify the mean area of staining in each villi (defined as a region of interest) (**Figure 5B**). This allowed selection of villi with evidence of the most dense staining for ZIKV infection (**Figures 5A,B**: regions Z1–Z2) and least dense staining for ZIKV infection (**Figures 5A,B**: regions N1–N2). The same most ZIKV-infected and least infected villous regions of interest were identified in subsequent sections for LC3B and p62/SQSTM1 studies. Densitometry was again used to define autophagic protein stain area in each region for LC3B and p62/SQSTM1, respectively (**Figure 5B**, bright green color). Defined densitometric thresholds did not identify any stain in probe-free control images for ZIKV infection, LC3B, or p62/SQSTM1 (data not shown). Using the densitometric data, mean area density of staining was compared between ZIKV-infected and less-infected villi. Mean density of both LC3B and p62/SQSTM1 were significantly decreased in ZIKV-infected villi compared to less-infected areas [**Figure 5C**; LC3B mean (95%CI) 0.951 (0.930–0.971), $p = 0.018$; p62/SQSTM1 mean (95%CI) 0.863 (0.810–0.916), $p = 0.024$].

DISCUSSION

Principal Findings

In the current study, we observed that in the placenta, disruption of autophagy accompanies ZIKV replication *In vitro, In vivo,* and *In situ* in human and non-human primate infections. Specifically, we observed changes in gene expression of key autophagy genes *Atg5* and *p62/SQSTM1* accompanying ZIKV-infection and replication in human primary placental trophoblasts when compared to mock-infected controls. Similar reduction in autophagic gene expression has been shown to affect viral replication and infectivity in other models (45). When we additionally assessed measures of autophagy in experimental

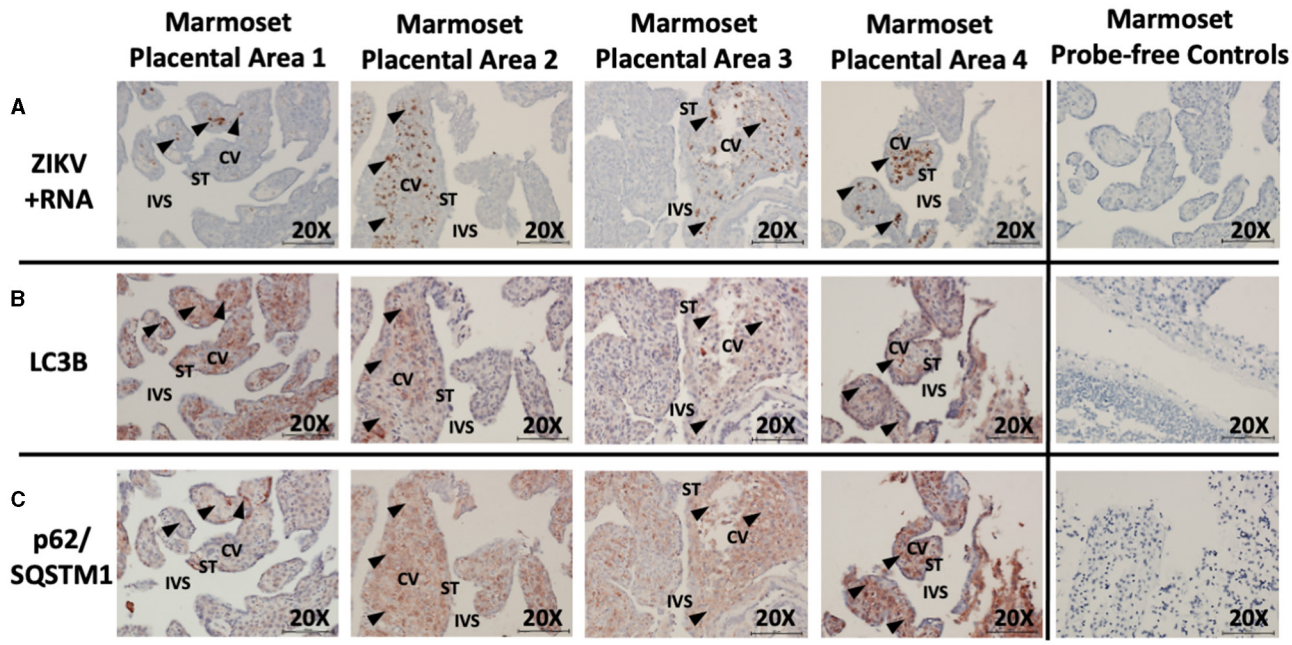

FIGURE 3 | *In situ* hybridization and immunohistochemistry staining demonstrates autophagy and ZIKV replication co-localize in ZIKV-infected marmoset placenta. *In situ* hybridization with positive-strand ZIKV RNA probe set demonstrates ZIKV replication (first passage Brazil ZIKV strain SPH2015) within some villi of the placenta from a ZIKV-infected marmoset (**A**: brown labeling, black arrowheads). Immunohistochemical staining for autophagic markers LC3B (**B**: red-brown labeling, black arrowheads) and SQSTM1/p62 (**C**: red-brown labeling, black arrowheads) was noted in the same areas of the villi where ZIKV replication was present. Probe-free controls confirm no non-specific binding in the absence of ZIKV, LC3B, SQSTM1/P62 probes, respectively. All images shown were obtained at 20X magnification. Micron bar scale in bottom right corner of images = 100 μm. CV, chorionic villus; IVS, intervillous space; ST, syncytiotrophoblast.

in vivo primate model and human cases of CZS, we observed significant spatial co-localization with the presence of key autophagic protein markers LC3B and p62/SQSTM1 proximal to ZIKV replication. When immunohistochemical staining in human and non-human primate placentae is quantified by densitometry, p62/SQSTM1, and to a lesser extent LC3B, are both significantly decreased in ZIKV infected placental cells when measured with an unbiased algorithmic method. These findings collectively suggest that dysregulated autophagy spatially and temporally accompanies placental ZIKV replication, providing the first *in situ* evidence in relevant pre-clinical models for human molecular therapeutic strategies aimed at agonizing/antagonizing autophagy. These studies have likely implications for other congenitally transmitted viruses, given the ubiquitous nature of autophagic disruption and dysregulation in host responses to viral infection during pregnancy.

Results

Our findings provide key evidence which reconciles seeming disparate prior studies demonstrating ZIKV-related induction of autophagic activity and seemingly pro-viral effects of autophagy. Specifically, we demonstrate that key autophagy gene transcription and protein translation are actually significantly **decreased** in foci of ZIKV-infected normal primary (i.e., non-transformed) human and primate cells with evidence of active replication. Prior suggestions for induction of autophagy and pro-viral effect of autophagy arose from transformed (i.e., high passage placental cancer) cell lines, where a marked increase in autophagy activity was found to accompany ZIKV infection (25, 28, 30). Induction of autophagy in these transformed cell lines seems to have pro-viral effects. In addition, inhibition of autophagy, via knock-out mouse models (40) or with use of pharmacologic inhibitors (28, 30), decreases viral infection. This is in contrast to the general tenet that induction of autophagy tends to confer placental cells with high resistance to viral infection, while inhibition of autophagy confers permissivity to viral infection (46, 47). Findings supporting an anti-viral role of autophagy have been seen in human neuroprogenitor and glial cells in culture (44) and Drosophila (48), where autophagy induction is associated with restricted ZIKV infection, and inhibition is associated with promotion of ZIKV infection.

Clinical Implications

In this current study, we demonstrate downregulation of autophagy in the endogenous setting of placental infection (e.g., *in vitro* infection of primary human trophoblasts or *in situ* measures of CZS-affected cases). Our observations reported herein show that key autophagy gene transcription and protein translation are actually significantly **decreased** in non-transformed placentae. This is further consistent with clinical observations of human cases. Namely, placental infection is not always (and even rarely) associated with either histologic nor molecular evidence of placental inflammation (49–51), but does

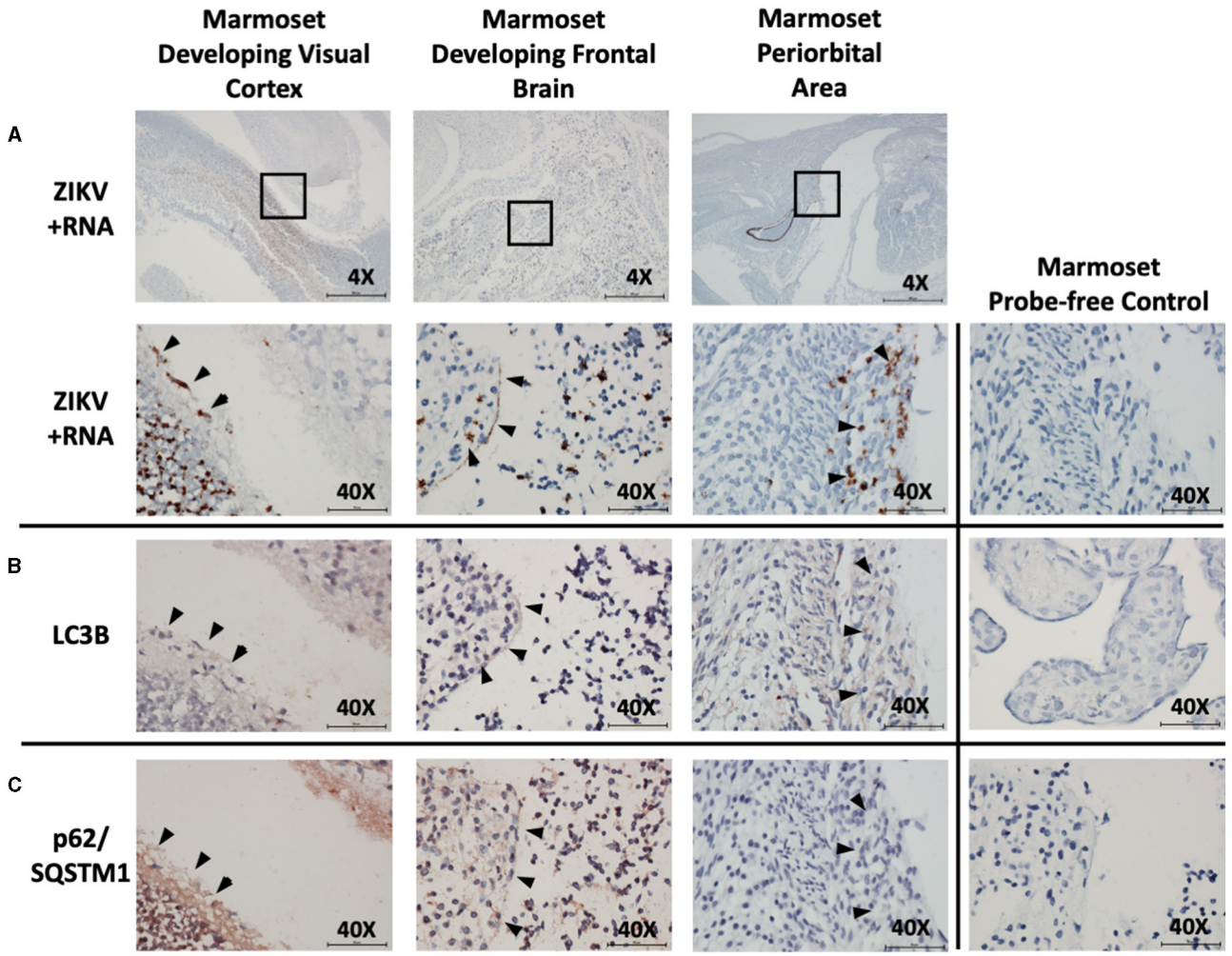

FIGURE 4 | *In situ* hybridization and immunohistochemistry staining demonstrate co-localization of autophagy and ZIKV replication in neuro-ophthalmic structures of ZIKV-infected marmoset abortus. *in situ* hybridization with positive-strand ZIKV RNA probe set demonstrates ZIKV replication within the developing visual cortex, frontal brain, and periocular structures in marmoset fetus (**A**: brown labeling, black arrowheads). Boxes indicate areas imaged further at 40X. Immunohistochemical staining for autophagic markers LC3B (**B**: red-brown labeling, black arrowheads) and SQSTM1/p62 (**C**: red-brown labeling, black arrowheads) was noted in the same neuro-ophthalmic structures where ZIKV replication was present. Probe-free controls confirm no non-specific binding in the absence of ZIKV, LC3B, SQSTM1/P62 probes, respectively. Images shown were obtained at 4X magnification (top row, **A**) or 40X magnification (all other images). Micron bar scale in bottom right corner of images = 500 μm (4X) or 50 μm (40X).

accompany pregnancy loss and fetal infection. There may be several biological reasons for our observations.

One explanation for these seemingly contradictory findings is suggested by Sahoo et al. (44), who similarly found evidence of a temporal relationship between diminished autophagy and persistence of infection. Initial autophagy induction (with limited viral replication during a brief period of <24 h post infection) was followed by mTOR activation and subsequent down regulation of autophagy with associated increased viral replication later in the course of ZIKV infection. Initial autophagic activation may serve to provide the substrates needed for initial viral replication resulting in the decreased p62/SQSTM1 levels (signal of autophagic flux) seen in this work. Subsequent autophagic downregulation prior to viral clearance may allow continued infection and is supported by the decreased p62/SQSTM1 gene expression and decreased LC3B presence seen in our non-human primate samples.

Second, support for subsequent downregulation of autophagy with ZIKV infection also arises from experiments conducted in primary human epithelial cells, primary skin fibroblasts, and non-transformed astrocytes where an alternate pathway for ZIKV-induced cell death was identified, making it unlikely that ZIKV requires autophagy later in infection for viral spread (52). This concept is not unheard of for flaviviruses, with DENV seemingly following a similar pattern of initial induction of autophagy with primary infection, followed by inhibition of autophagy with persistent infection (7). Our results are likely most consistent with downregulation of autophagy later in course

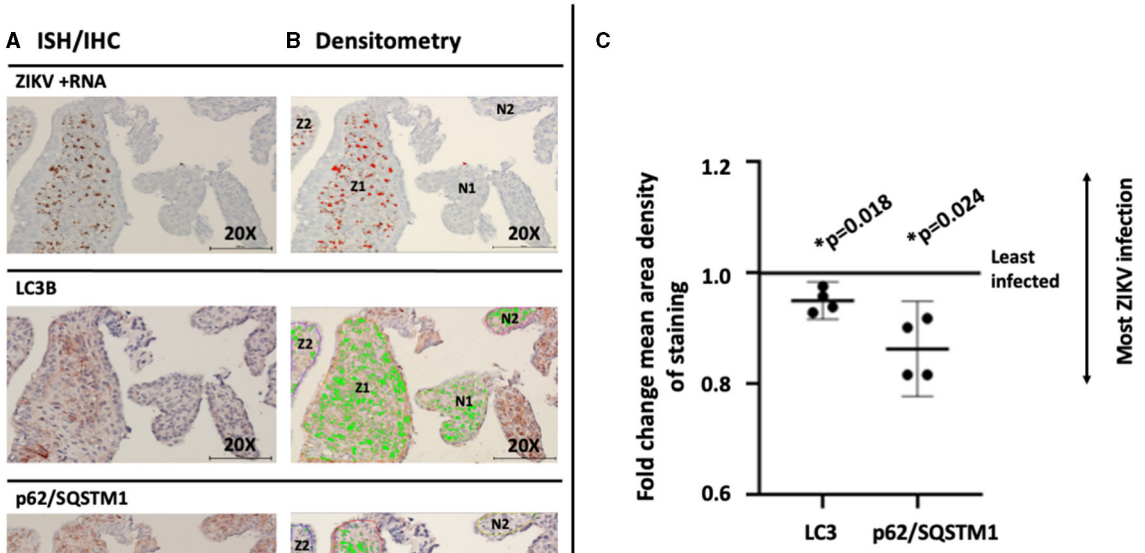

FIGURE 5 | Mean density of autophagic proteins LC3B and p62/SQSTM1 is significantly decreased in the greatest compared to the least ZIKV-infected marmoset placental villi. Villi with the greatest (regions Z1–2) and least (regions N1–2) dense staining for ZIKV infection were identified using stain thresholds and defined as regions of interest **(A)**. The same greatest and least infected regions of interest were identified in subsequent sections for LC3B and p62/SQSTM1 studies, and densitometry was again used to define autophagic protein stain area in each region (**B**, bright green color). Mean area density of staining was then compared between regions of interest. Mean density of both LC3B and p62/SQSTM1 were significantly decreased in the most ZIKV-infected villi compared to the least-infected areas [**C**; LC3B mean (95%CI) 0.951 (0.930–0.971), $p = 0.018$; p62/SQSTM1 mean (95%CI) 0.863 (0.810–0.916), $p = 0.024$]. All images shown were obtained at 20X magnification. Micron bar scale in bottom right corner of images = 100 μm. Statistical significance was determined using a p-value < 0.05 (*).

of the infection and disease process, since we observed that both the RNA from the primary human trophoblasts, and the human and non-human primate tissues were isolated past the 24 h of initial autophagy induction demonstrated by Sahoo et al. (44). p62/SQSTM1 as a pathogen recognition reception (PRR), and Atg5 which has been proposed to downregulate some PRRs, are both logical candidates for downregulation of autophagy by a virus trying to avoid normal anti-viral degradative pathways (3, 53), findings supported by this study. Put another way, if we could have sampled the same human and non-human primate placentae much earlier in infection (h), rather than days, weeks, or months later, we may have observed transient initial induction of autophagy, with subsequent downmodulation of autophagy. Such experiments will be the focus of future *in vivo* work, and our conclusions remain speculative.

Research Implications

While this study does demonstrate spatial and temporal co-localization between autophagy and ZIKV infection at a discrete point in time, further studies are necessary to define exactly when and how autophagy is induced and/or down-modulated during the entire course of ZIKV infection, especially within human placentae. This information will better inform the timing and pharmacologic basis (if any) of potential therapeutic options. In addition, it may help identify mechanisms and pathways by which viral persistence in the placenta might occur in some, but not all, cases of congenital Zika infection and resultant fetal disease.

Strengths and Limitations

Strengths of this study include the use of a highly relevant non-human primate model of congenital infection, with confirmation in human placental samples, both *in vitro* and *in vivo*. Limitations of this study include a small chance of cross-reactivity of the LC3B antibodies to other LC3 isoforms. In addition, the static nature of these experiments does not enable us to assess for longitudinal changes. Therefore, we cannot draw conclusions about autophagy in the initial phases of infection given our non-human primate model spontaneously aborted at 2 weeks with a highly advanced placental infection.

In general, reliance on specimens from other index studies constrained our experimental design and limited our biologic replicates. This is notably true in our *in vivo* infected marmosets (19). In the initial study, as we have previously published, we infected two pregnant marmoset dams with Brazil ZIKV at estimated gestational days 79 and 68, with a second inoculation 4 days later. As we initially detailed in the primary index study (19), spontaneous expulsion of intrauterine demised fetuses at

post-inoculation day 16 (dam 1, dizygotic twins) and 18 (dam 2, singleton) occurred. The placenta from dam 1 was unavailable for analysis, as it was mauled by the dam post-delivery. The second dam's expulsed singleton fetus and placenta were both collected and retained for analyses, and because they constituted a single exposure they were used in the current analysis (19). Recognizing the limitations of drawing meaningful conclusions based on a single pregnancies placenta and fetus, we attempted to mitigate these limitations with technical replicates by sampling different placental subsites and rigorous use of internal controls. In addition, we employed orthogonal approaches with use of human tissue specimens and primary trophoblasts in the current study. Nonetheless, we acknowledge the constraints in our primate specimens being derived from a single dam. Similarly, we acknowledge that architectural and histologic differences exist when comparing human and non-human primate placentae. However, the notable similarity of consequence for the current study is the hemochorial nature common to both human and non-human primate placentae whereby (unlike other species) the villi bathe in the maternal intervillous space. As such we feel that the marmoset is a highly appropriate model despite some anatomical differences. Moreover, we are further buoyed in the confidence of our findings and conclusions by the consistency of observations among our human and non-human primate subject's specimens.

CONCLUSIONS

Although the exact role of autophagy in ZIKV pathogenesis is still being determined, autophagy inhibitors, such as hydroxychloroquine (38), are being proposed as potential therapeutic agents to prevent vertical transmission and mitigate adverse fetal effects of the virus. Our results shed key light on this topic, and herald warning that such therapeutic approaches must be approached with thoughtful consideration of both timing and duration of therapy. Since our findings show that diminished autophagy later in the infection temporally and spatially co-localizes with ZIKV replication in non-human primate placentae from congenital infection cases, it cannot be concluded *de facto* that inhibition of autophagy will be of benefit. Conversely, we cannot assume that it would be of harm as our observations could be evidence of an adaptive response for maternal benefit. Rather, the appropriate interpretation and application of our findings suggest that future experimentation with anti-autophagic drugs as potentially effective in mitigating vertical transmission of ZIKV must consider the timing of administration with respect to infection. Likely subsequent downregulation of autophagic processes does not negate the benefits that such therapy may have, although does likely support the need for treatment to be limited to early in the course of infection.

SUMMARY

Dysregulated autophagy spatially and temporally accompanies placental ZIKV replication, providing *in situ* evidence in relevant pre-clinical models for human molecular therapeutic strategies aimed at agonizing/antagonizing autophagy.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation. Requests to access these datasets should be directed to Maxim D. Seferovic, maxim.seferovic@bcm.edu.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Baylor College of Medicine IRB H-257. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Southwest National Primate Research Center and approved by local Institutional Animal Care and Use Committee (IACUC) and Biohazard Committee.

AUTHOR'S NOTE

Poster presentation at Society for Maternal Fetal Medicine, the 41st Annual Pregnancy Meeting, January 25-30, 2021.

AUTHOR CONTRIBUTIONS

JM, MDS, MAS, and KA contributed to the conception and design of the study. JM, MDS, AM, ST, JP, EC, and KA performed the experiments. JM, MDS, AM, ST, JP, EC, and KA analyzed and interpreted the data. JM, MDS, and KA wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fviro.2021.720760/full#supplementary-material

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Fetal Central Nervous System Derived Extracellular Vesicles: Potential for Non-invasive Tracking of Viral Mediated Fetal Brain Injury

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Introduction: Extracellular vesicles derived from the fetal central nervous system (FCNSEs) can be purified from maternal serum or plasma using the protein Contactin-2/TAG1that is expressed almost exclusively by developing neurons in the hippocampus, cerebral cortex and cerebellum. We hypothesized that fetal CNSEs could be used to non-invasively detect and quantify viral mediated *in-utero* brain injury in the first trimester.

Materials and Methods: First trimester maternal samples were collected from a human clinical population infected with primary cytomegalovirus (CMV) and a non-human primate model of Zika (ZIKV) infection. In the CMV cohort, a nested case control study was performed comparing pregnancies with and without fetal infection. Cases of fetal infection were further subdivided into those with and without adverse neurologic outcome. ZIKV samples were collected serially following maternal inoculation or saline. All ZIKV cases had histopathologic findings on necropsy. Serum was precipitated with ExoQuick solution and FCEs were isolated with biotinylated anti-Contactin-2/TAG1 antibody-streptavidin matrix immunoabsorption. FCE Synaptopodin (SYNPO) and Neurogranin (NG) protein levels were measured using standard ELISA kits and normalized to the exosome marker CD81.

Results: Fetal CNSE SYNPO and NG were significantly reduced in cases of first trimester fetal CMV infection compared to those with infection limited to the mother but could not discriminate between fetal infection with and without adverse neurologic outcome. Following ZIKV inoculation, fetal CNSE SYNPO was reduced by 48 h and significantly reduced by day 4.

Discussion: These data are the first to suggest that first trimester non-invasive diagnosis of fetal viral infection is possible. Fetal CNSEs have the potential to augment clinical and pre-clinical studies of perinatal viral infection. Serial sampling may be needed to

discriminate between fetuses that are responding to treatment and/or recovering due to innate defenses and those that have ongoing neuronal injury. If confirmed, this technology may advance the paradigm of first trimester prenatal diagnosis and change the calculus for the cost benefit of CMV surveillance programs in pregnancy.

Keywords: exosomes/extracellular vesicles (EVs/ECVs), cytomegalovirus (CMV), Zika (ZIKV), Contactin-2, prenatal diagnosis, microcephaly

INTRODUCTION

Two percent (95% CI 2.1–2.4%) of seronegative women will acquire primary cytomegalovirus (CMV) infection during their pregnancy (1). At birth, 10–15% of congenitally infected neonates will have symptoms; of those 6% will die and up to 90% will develop sequelae such as sensorineural hearing loss (SNHL), developmental delay, cognitive impairment, neuromuscular dysfunction (cerebral palsy), epilepsy, impaired vision function, and possibly autism spectrum disorder. Asymptomatic infants remain at significant risk of developing SNHL before 2 years of age. Congenital CMV infection is responsible for more long-term sequelae than either Down syndrome or Fetal Alcohol Spectrum Disorder (2). Zika virus (ZIKV) is a mosquito borne flavivirus that has also been causally implicated in brain injury and microcephaly following *in-utero* infection (3). The estimated risk of clinically detected central nervous system (CNS) injury following first trimester ZIKV infection ranges from 1 to 13% (4). In an epidemiologic study based on Brazilian data, the peak risk of microcephaly appears to correlate with ZIKV infection between gestational week 14 and 17 (5), but findings of ventriculomegaly and cerebral calcifications have been seen with ZIKV infections as late as the 3rd trimester (6).

Significant barriers exist to the effective and timely diagnosis and treatment of fetal brain injury secondary to *in-utero* viral infections such as CMV and ZIKV. Many maternal viral infections are asymptomatic; therefore, detection of infection would require surveillance programs. However, enthusiasm for costly surveillance programs has been dampened by a combination of challenges in the accurate diagnosis of fetal brain injury, lack of effective *in-utero* interventions such as hyperimmune globulin (7), and limited means to monitor treatment effects in real time. In fetuses destined to develop microcephaly, head circumference measured on prenatal ultrasound may be normal prior to 22 weeks gestation age (weeks GA) and fetal MRI is of limited utility in diagnosing other intracranial abnormalities prior to 18 weeks GA resulting in late diagnosis in the absence of early surveillance programs (8, 9). Finally, definitive fetal diagnosis requires amniocentesis with amniotic fluid viral PCR. Although emerging data suggests that earlier amniocentesis may be accurate, the traditional recommendation is for amniocentesis to be performed 6 weeks from maternal exposure and at >21 weeks' gestation for CMV (10). This calculus may be shifting as data emerges regarding the efficacy of other early anti-viral therapies. A recent small randomized, double-blind, placebo-controlled trial demonstrated 71% reduction in the fetal infection rate with high dose oral valcyclovir in women with a primary CMV infection in the first trimester (3–12 weeks gestation) (11). Valganciclovir treatment was also associated with resolution of hydrops fetalis and minimal sequelae in a recent case report (12). The missing element to any first trimester strategy for the diagnosis and treatment of *in utero* viral infections is a non-invasive first trimester assay similar to cell-free DNA based tests for aneuploidy. An ideal first trimester test would be able to detect fetal injury, especially neurologic injury, prior to seroconversion and have improved sensitivity. Serial testing would ideally be available to determine if treatment was effective and to distinguish between those fetuses at low and high risk for adverse neurologic outcomes prior to mid-trimester ultrasound findings.

Exosomes/extracellular vesicles (ECVs) are membrane-bound endosome-derived nanovesicles that allow the intercellular transfer of genetic material, cytoplasmic and membrane proteins, lipids, miRNAs and other intracellular materials (13). Exosomes freely cross the blood-brain barrier and the placenta through unknown mechanisms, carrying proteins and micro RNAs (miRs) into the peripheral blood while protecting them from degradation (14, 15). We have published a series of foundational investigations demonstrating novel techniques to isolate ECVS derived from the fetal CNS from maternal serum using the protein Contactin-2/TAG1 (CTN-2/Tag-1) that is expressed almost exclusively by developing neurons in the hippocampus, cerebral cortex and cerebellum (16–18)[1]. A small proportion of CTN-2/TAG-1 ECVs may arise from tissues such as His-Purkinje cells (19) but the relatively small contribution of these tissues is unlikely to hinder assay performance. Based on comparisons of concentrations between pregnant and non-pregnant subjects, we have demonstrated that 97% of CTN-2/TAG-1+ ECVs isolated from maternal serum are fetal in origin (16); the 3% contamination by maternal neural ECVs reflects low levels of ongoing neural plasticity in adults. We have demonstrated that the placenta is not a source of CTN-2/TAG-1 (17). Taken together, our work demonstrates that CTN-2/TAG-1 can be used to isolate fetal CNS derived ECV (CNSEs) from maternal blood to generate novel biomarkers. We hypothesized that protein markers of neurologic injury quantified in CTN-2/TAG-1+ ECVs purified from maternal blood could identify fetal CNS injury secondary to *in-utero* viral infection with CMV or ZIKV.

MATERIALS AND METHODS

Clinical Recruitment CMV

Clinical recruitment was approved by the local ethics committee of Shaare-Zedek Medical Center and written informed consent

[1]https://www.proteinatlas.org/ENSG00000184144-CNTN2/tissue.

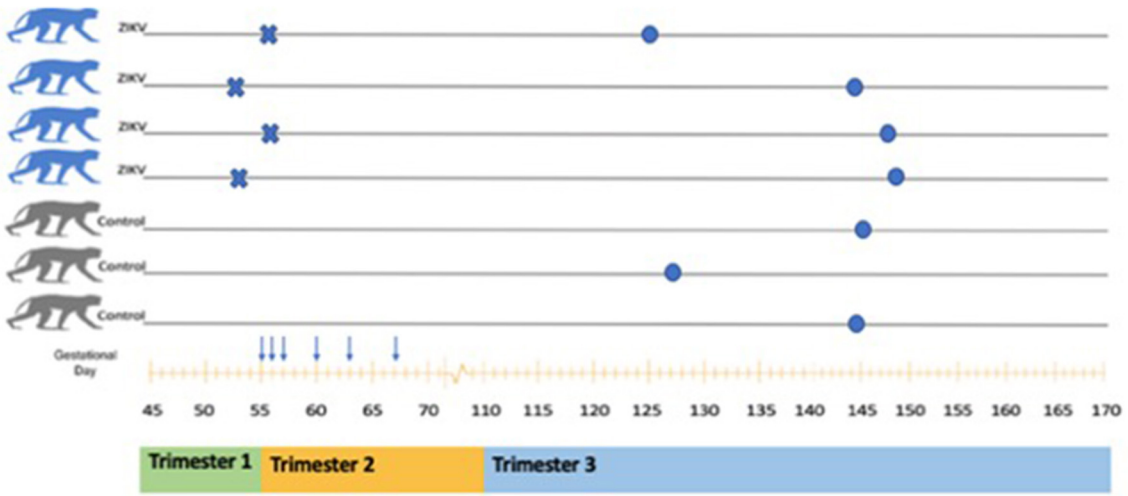

FIGURE 1 | Schematic of animals included in ZIKV analysis. A total of seven pregnant Rhesus Macaques were included (four ZIKV and three Controls). Inoculation with $ZIKV_{PRVABC59}$ is indicated by the symbol X. Serial dam blood sampling is indicated by the symbol ↓. Fetal delivery by cesarean is indicated by the symbol ●. A rough correlation with human pregnancy trimesters is shown below the x axis (gestational age in days).

was obtained from each participating woman. The study was performed according to Good Clinical Practice (GCP) guidelines.

Samples were collected from pregnant women who were diagnosed with primary CMV infection as previously described (20, 21). Briefly, the diagnosis was made by one of the following serological findings: CMV-specific IgG seroconversion or the presence of low avidity IgG antibodies or CMV-specific IgM with no previous IgG antibodies. The timing of primary infection was determined by the time of seroconversion and/or analysis of the increment of IgG avidity and/or by clinical symptoms. Intrauterine CMV transmission was determined by detection of viral DNA by real-time PCR, either in amniotic fluid or in the newborn's urine. The analysis of these specimens was performed by the treating physicians in their respective medical centers throughout Israel. Maternal samples collected at the time of diagnosis of primary infection were transferred for biomarker analysis under a uniform transfer of biologic material agreement and with the appropriate permit from the Centers for Disease Control.

Clinical Outcomes CMV

A nested case control study was performed among women who were infected with CMV and had both stored maternal serum samples and known pregnancy outcome.

Three outcome groups were defined. Non-Transmitters (NT) were defined by the absence of CMV detected in amniotic fluid or fetal urine at birth and by normal neurologic outcome. Asymptomatic Transmitters (AST) were defined by CMV detected in amniotic fluid and/or in fetal urine at birth in combination with the absence of adverse neurologic outcome. Symptomatic Transmitters (ST) were defined by CMV detected in amniotic fluid and/or in fetal urine at birth and abnormal findings in at least one of the following: brain ultrasound, retinal examination, or auditory brain-stem response.

Non-human Primate ZIKV Model

All Zika virus infection experiments utilizing non-human primates were performed in compliance with guidelines established by the Animal Welfare Act for laboratory animal housing and care and in accordance with Oregon National Primate Research Center (ONPRC) Institutional Animal Care and Use Committee approved animal protocol (IACUC #1099). NHP studies were performed in ABSL-2 containment facilities at the ONPRC. Samples used in this study were collected from animals that have been described in detail previously (22). Briefly, on GD 53–55, time-mated pregnant macaques were subcutaneously inoculated with 10^5 focus forming units (ffu) of $ZIKV_{PRVABC59}$ as previously described (ZD) or 1 mL of normal saline (CTL) (22, 23). Serial dam serum sample were collected post-inoculation via peripheral blood draws under ketamine sedation, and were sufficient for analysis on days 2–4, 7, 10, and 14. A schematic of the study design is shown (**Figure 1**). All ZD offspring had histopathologic findings consistent with ZIKV on necropsy.

Zika Virus Preparation

$ZIKV_{PRVABC59}$ was obtained from the CDC and passaged twice in C6/36 cells [American Type Culture Collection (ATCC)] as previously described (24, 25). Supernatant from infected C6/36 tissue culture was concentrated through a 20% sorbitol cushion and titrated in Vero cells (ATCC) through a focus-formation assay. The viral inoculum was sequenced as previously described (24, 25).

ECV Isolation and Protein Quantification

Fetal CNS ECVs were purified using previously published techniques (16–18, 26). Briefly, each ∼75–125 uL serum aliquot was incubated with thromboplastin-D and a cocktail of protease and phosphatase inhibitors. Supernatants were incubated with

TABLE 1 | Clinical characteristics of nested case control subjects.

Group	Maternal age	GA at CMV INFX (Weeks)	GA at sampling (Weeks)	Interval	Maternal symptoms	Fetal/neonatal findings
NT	25	8	12	4	Unknown	
NT	26	6	12	6	Unknown	
NT	21	10	16	6	Fever	
NT	26	6.5	10	3.5	Unknown	
NT	27	4	11	7	Fever	
AST	24	6.0	15	9	Unknown	
AST	33	10.5	15	4.5	Fever	
AST	28	5.5	11	5.5	Unknown	
AST	32	6	12	6	Fever	
AST	22	7.5	22	14.5	Unknown	
ST	32	6.0	12	6	Unknown	Microcephaly, intra-cranial calcifications TOP
ST	28	7	11	4	Unknown	Isolated severe unilateral hearing loss
ST	24	8	13	5	Fever	Brain Calcifications, normal development, normal hearing.
ST	28	4	11	7	Unknown	Severe brain damage–TOP

exosome precipitation solution (EXOQ; System Biosciences, Inc, Mountainview, California, USA). To isolate the subset of ECVs from neural sources, total ECV suspensions were incubated with monoclonal IgG1 anti-human Contactin-2/TAG1 antibody (clone 372913, R&D Systems, Inc, Minneapolis, Minnesota, USA) that had been biotinylated (EZ-Link sulfo-NHS-biotin System, Thermo Scientific, Inc), and antibody-bound ECVs were precipitated with Streptavidin-Plus UltraLink Resin (Pierce-Thermo Scientific, Inc). Contactin-2/TAG1 is a glycosyl-phosphatidylinositol anchored neuronal membrane adhesion protein of the immunoglobulin superfamily that is transiently expressed in human brain development to guide axonal connections and, in association with other proteins, promote molecular organization of myelinated nerves (27, 28).

The tetra spanning exosome marker human CD81 (American Research Products, Waltham, Massachusetts, USA: Cusabio), and neural markers Synaptopodin (SYNPO) and Neurogranin (NG) were quantified using standard ELISA methods (Reddot Biotech Inc, Kelowna, British Columbia, Canada). The mean value for all determinations of CD81 in each assay group was set at 1.0, and this value was used to normalize their recovery in individual samples.

Data Analysis

Data analyses were performed using SPSS (IBM SPSS Statistics for Windows, Version 26.0. Armonk, NY). Student's *t*-testing or ANOVA testing was used to compare means. Correlations were assessed using Spearman's rank correlation. The *p*-value for significance was set at 0.05.

RESULTS

Clinical Nested Case Control CMV Study

Subjects selected from within the original cohort included five non-transmitters (NT), five asymptomatic transmitters (AST), and four symptomatic transmitters (ST). The clinical characteristics of the study subjects are shown (**Table 1**). For subjects overall the mean maternal age (26.9 $\pm$ 3.7 years), mean gestational age (GA) at blood sampling (13.1 $\pm$ 3.1 weeks), mean GA at CMV infection (6.8 $\pm$ 1.7 weeks), or interval between infection and blood collection (6.3 $\pm$ 2.8 weeks) was not different between the three clinical groups ($p > 0.05$). Mean ECV SYNPO levels were significantly reduced in both groups with fetal infection (AST and ST) compared to pregnancies without fetal infection (NT; $p = 0.04$, **Figure 2**). While ECV SYNPO levels were lower in symptomatic *vs.* asymptomatic fetal CMV infections, this difference was not statistically significant ($p = 0.28$). While the lack of statistical significance might have been due to small samples size, it is clear that there is significant overlap between the two groups and that therefore, it is unlikely that their accurate discrimination/clinical classification at the time of the initial diagnosis of infection. Patient 1 had normal neurodevelopment at age 8 years. This was one of the oldest specimens in the cohort and may have been subject to degradation. Similar results were observed for NG (**Figure 3**). Of interest, 1 of 4 cases of ST was limited to severe isolated unilateral hearing loss without prenatal findings. Fetal CNSE SYNPO and NG were only partially correlated ($r = 0.56$, $p = 0.039$)

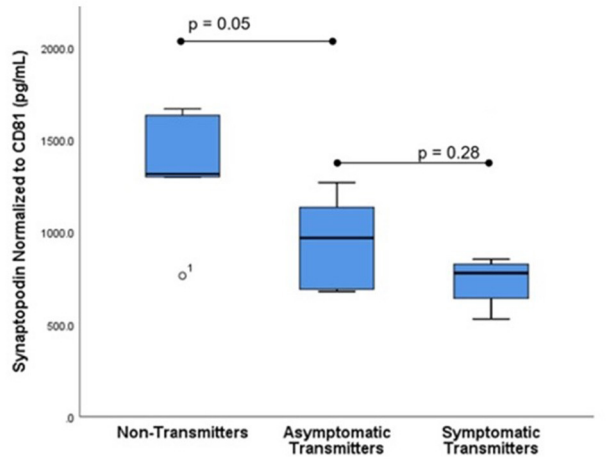

FIGURE 2 | Fetal CNS ECV synaptopodin levels by clinical outcome. ECV protein levels of synaptopodin normalized to CD-81 were compared in three groups: Non-transmitters (NT, $n = 5$), asymptomatic transmitters (AST, $n = 5$), and symptomatic transmitters (ST, $n = 4$). ANOVA testing was used to compare means. The p-value for significance was set at 0.05.

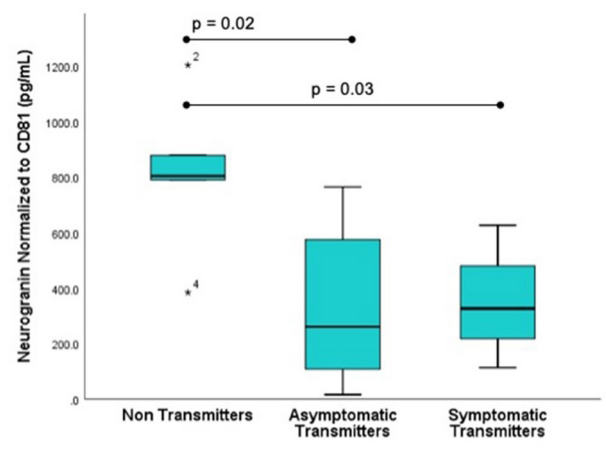

FIGURE 3 | Fetal CNS ECV neurogranin levels by clinical outcome. ECV protein levels of neurogranin normalized to CD-81 were compared in three groups: Non-transmitters (NT, $n = 5$), asymptomatic transmitters (AST, $n = 5$), and symptomatic transmitters (ST, $n = 4$). ANOVA testing was used to compare means. The p-value for significance was set at 0.05. * value outside the interquartile range.

suggesting a possible role for multiple markers to improved diagnostic accuracy.

Viral products could not be detected within fetal CNSEs (data not shown).

Non-human Primate Model ZIKV

Seven fetuses were evaluated for neurologic outcomes (three CTL and four ZD). Decreased levels of fetal CNSE SYNPO in ZIKV infected pregnancies was first observed on Day 2 (**Figure 4**; **Table 2**) but did not reach statistical significance until Day 4. Viral RNA could not be detected within fetal CNSEs (data not shown).

DISCUSSION

These exploratory data suggest that human fetal transmission of CMV can be detected non-invasively using fetal CNSEs isolated from maternal blood as early as 11 weeks gestation and that fetal CNS injury can be detected in a non-human primate model within 2 days of infection. Fetal CNSEs were useful at gestational day 53–55 in the non-human primate model described, which is roughly equivalent to the end of the first trimester in humans. Despite the sample size limitations, these data are the first to suggest that first trimester non-invasive diagnosis of fetal viral infection is possible. If confirmed, this finding could transform the paradigm of prenatal diagnosis for fetal viral infection. Our findings that human Contactin-2 Tag-1 antibody could be used to purify fetal CNSEs in a non-human primate model suggests that this methodology could be used to augment studies of perinatal viral infection in appropriate animal models. Of note, our samples likely represent a mixture of exosomes, other extracellular vesicles and some organelles and we did not attempt to confirm purity due to small sample volumes. However, as opposed to studies where purity is critical (mechanistic and functional exosome studies), biomarkers studies are judged on their ultimate test performance in discriminating between disease and non-disease states and purity is less critical. In future studies, if larger serum/plasma volumes are available, we plan to perform flow cytometry studies to investigate whether or not viral infection alters the proportion of exosomes that care Contactin-2 Tag-1 and/or CD-81.

One surprising finding from our human CMV study was that all fetuses with evidence of *in-utero* infection appear to have significantly reduced levels of injury markers. We hypothesize that all fetuses infected with CMV take an initial "hit" to the CNS but that some are able to rebound secondary to neuroplasticity, and that some are not and are born with neurologic sequelae. Therefore, the most important value of the early samples may be to discriminate between pregnancies with and without fetal transmission to determine which fetuses would benefit from potential interventions but would not provide sufficient information regarding prognosis. Serial sampling may be needed to discriminate between fetuses that are responding to treatment and/or recovering due to innate defenses and those that have ongoing neuronal injury. An addition benefit of fetal CNSE-based prenatal diagnosis is that both initial and serial sampling will be more acceptable to patients since amniocentesis is not necessary. Non-invasive testing would also negate the unavoidable delays in diagnosis using amniocentesis—where the highest sensitivity and specificity are reached after 21 weeks' gestation. At this gestational age, some CNS injury may be irreversible. If the positive and negative predictive value of CNSE-based prenatal diagnosis is confirmed, it may shift the cost-benefit analysis for CMV surveillance in pregnancy. A recent cost-benefit study concluded that surveillance might be cost effective if anti-viral interventions were more than 30% effective (29), however more precise targeting of those pregnancies that require intervention and serial evaluation would reduce excess costs of treatment and testing from unnecessary treatment.

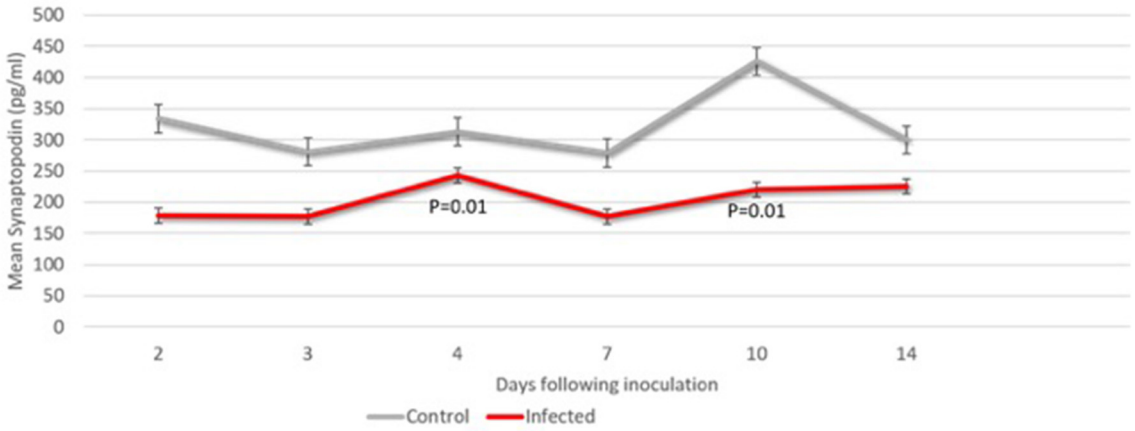

FIGURE 4 | Fetal CNS ECV synaptopodin levels days 2 through 14 following ZIKV vs. saline inoculation. ECV protein levels of synaptopodin normalized to CD-81 were compared in at each timepoint ($n = 2$–4 samples). The Students *t*-test was used to compare means at each time point. The *p*-value for significance was set at 0.05.

TABLE 2 | Mean fetal neural ECV synaptopodin by day following ZIKV inoculation (ZD) vs. saline injection (CTL).

Day	ZD/CTL (number per group)	SYNPO in ZIKV inoculation group (pg/ml ± SE)	SYNPO in saline inoculation group (pg/ml ± SE)	*p*-value
2	2/3	179.1 ± 39.3	334.4 ± 77.6	0.23
3	4/3	177.3 ± 14.2	281.3 ± 53.7	0.19
4	4/2	243.1 ± 13.6	313.3 ± 0.7	0.01
7	4/2	177.9 ± 23.4	279.3 ± 37.9	0.08
10	4/3	220.3 ± 31.2	426.0 ± 43.9	0.01
14	4/3	226.6 ± 19.1	300.5 ± 80.0	0.45

In addition to markers of neuronal injury, CNSEs may carry viral products or markers of CMV infection that reflects viral specific mechanisms. The similarities between the biogenesis of extracellular vesicles and the release of viral particles from infected cells are intriguing as both processes involved the ESCRT pathway (30). The CMV virus utilizes EVs to enhance viral spread, therefore it is not surprising that EVs can contain viral products (31). CMV specific viral products include the viral envelope proteins gB and gH as well as CMV generated miRs (32, 33). We have attempted to detect gB in CNSEs in our prior CMV studies and have found the results to be inconsistent. A recent study has demonstrated that only 15% of EVs released from CMV infected cells were positive for gB, 5.3% were positive for gH and 3.74% were positive for both gB and gH. The factors that determine the proportion of viral protein positive EVs are not known. Therefore, viral proteins appear to be imperfect biomarkers of CMV infection (34). We were unable to isolate viral genetic products from fetal CNSEs, which was disappointing; we had been hopeful that CNS "viral load" could be estimated non-invasively.

Although viral activity may not be able to be measured directly in fetal CNSEs—there are other potential markers that may reflect perinatal outcomes. EVs from infected cells are known to contain soluble DC-SIGN, a C-type lectin family molecule that increases the susceptibility of recipient cells to CMV but DC-SIGN is found largely in dendritic cells rather than neurons (35). Another study examined total EVs isolated from neonates with active CMV infection. EV levels of the CMV miRs US25-1-5p and UL112-3p were significantly higher in the setting of active CMV infection and were significantly associated with the degree of liver damage secondary to viral hepatitis as assessed by neonatal liver function tests (36). In addition, when free plasma levels of miRs from 13 infants with congenital CMV were compared to controls, levels of miR 183-5p and miR-210-3p were significantly higher (37). This suggests that CNSE miR levels may also be potentially useful in assessing degree of neurologic injury. In addition to the candidates above, there are several other miRs and proteins that have some premise from the literature. Human miR-21 is known to inhibit CMV viral gene expression by targeting Cdc25a, a cell cycle regulator, in neural progenitor cells and may act as an innate anti-viral defense (38). BclAF1 restriction factor, a cellular anti-viral protein, is down regulated by CMV miR-UL112-1 and is also found in ECVs released from human brain cells (39, 40). CMV miR UL112-1 down regulates the antiviral protein Interleukin-32 (IL-32), aiding in evasion of the host immune response (41). In turn, IL-32 is an important

regulator of TNF-α, which may adversely affect microglial-mediated neuroinflammation and potentiate fetal brain injury. While TNF-α has not widely been reported in exosomes, tumor necrosis factor-α 1 receptors (TNFR1) levels have been used as a biomarker of response to inhibitors of neuroinflammation in neuronal ECVs (42). Human miR-221 in neural precursor cells inhibits CMV replication by promoting type I interferon and upregulating the activation of NF-kB (43).

In summary, fetal CNSEs appear to have potential in the early identification of viral mediated fetal brain injury. Fetal CNSEs may be useful as early as 11 weeks gestation—and therefore may identify brain injury prior to irreversible damage. Fetal CNSEs offer an advantage as testing can be performed non-invasively, avoiding both the risk and the delay in diagnosis associated with amniocentesis. Fetal CNSEs may also be a useful adjunct to animal models of perinatal viral infection—where the timing of infection is more precisely known. However, larger studies are required to confirm the potential of fetal CNSEs both for endemic viral infections such as CMV and for episodic pandemic infections such as ZIKV and unknown future viral strains.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by IRB, Shaare-Zedeck Medical Center. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by IACUC, Oregon National Primate Research Center.

AUTHOR CONTRIBUTIONS

LG conceived of the scientific experiments related to exosome/ECVs as biomarkers, supervised the assays, and wrote the manuscript. AS and AM assisted in the scientific design, performed the Zika assays, and edited the manuscript. YS, YE-Y, and MH designed the CMV cohort study, collected biologic samples and outcome data, participated in the scientific design, and edited the manuscript. AH, DS, AF, VR, and NH designed and performed the primate studies, collected biologic samples and outcome data, participated in the scientific design, and edited the manuscript. ND and NM assisted in the scientific design, performed the CMV assays, and edited the manuscript. All authors agree to be accountable for the content of the work.

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Conflict of Interest: LG holds a patent for the methods for isolating fetal central nervous system extracellular vesicles/exosomes. This technology is not currently licensed for commercial use.

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

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Differential Type 1 IFN Gene Expression in CD14+ Placenta Cells Elicited by Zika Virus Infection During Pregnancy

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Zika virus (ZIKV) is an arthropod-borne Flavivirus that can also be transmitted vertically from infected mother to fetus. Infection of the fetus during pregnancy can lead to congenital malformations and severely impact fetal brain development causing a myriad of diseases now labeled Congenital Zika Syndrome (CZS). The mechanisms by which ZIKV crosses the placenta into the fetal circulation and the extent of ZIKV-induced changes remain unclear. We have previously shown that ZIKV infection of pregnant rhesus macaques results in abnormal oxygen transport across the placenta which may promote uterine vasculitis and placental villous damage. Changes in immune cell frequencies and activation status were also detected, as were distinct changes in the proportions of CD14+ cell subsets with an altered ratio of classical to non-classical CD14+ monocyte cells in both the maternal decidua and placental villous from ZIKV-infected animals compare to uninfected controls. In the current study, we performed single cell RNA sequencing on CD14+ cells isolated from the decidua of animals that were ZIKV infected at 31, 51, or 115 days of gestation (where term is ~168 days) compared to pregnant, time-matched uninfected controls. Bioinformatic analysis identified unique transcriptional phenotypes between CD14+ cells of infected and uninfected animals suggesting a distinct and sustained difference in transcriptomes between infected and uninfected CD14+ cells derived from the decidua. The timing of ZIKV infection had no effect on the CD14+ cell transcriptional profiles. Interestingly, ZIKV infection caused changes in expression of genes in pathways related to cellular stress and metabolism as well as immune response activation. Type 1 interferon response genes (ISGs) were among those that were differentially expressed following infection and these included members of the ISG12 family, IFI27 and IFI6. These ISGs have been recently described as effectors of the IFN response to flaviviruses. Supplementing our animal findings, in CD14+ cells isolated from human placenta, ZIKV infection similarly induced the expression of IFI27 and IFI6. Overall, our results showed that ZIKV infection during

pregnancy induces the stable expression of antiviral genes within CD14+ cells of the placenta, which may provide an immune shield to protect the placenta from further infection and damage.

Keywords: Zika virus, pregnancy, monocyte, macrophage, interferon

INTRODUCTION

Zika virus (ZIKV) is a flavivirus most commonly transmitted by the bite from an infected *Aedes* mosquito. Unique to ZIKV as a flavivirus, vertical transmission can occur from an infected mother to the fetus, and through sexual contact. ZIKV has emerged in recent years as a significant threat to humans primarily because of the effects it has on neonates. Historically, in healthy individuals, infection is most often either asymptomatic or results in mild symptoms. However, in the most recent epidemic, evidence indicates that ZIKV can cause neurological sequelae such as Guillain-Barre Syndrome in adults (1, 2). Prenatal ZIKV exposure was first associated with an increased risk for severe microcephaly in infants (3, 4). It is now clear that infection during pregnancy can result in a range of presentations with variable severity referred to as Congenital Zika Syndrome (CZS), divided into mutually exclusive categories: (1) brain abnormalities and/or microcephaly and (2) neural tube defects, eye abnormalities, or consequences of central nervous system dysfunction among fetuses or infants without evidence of other brain abnormalities or microcephaly (3, 5). The most severe phenotype appears to be associated with exposure during the first trimester (6), although confirmed CZS after exposure during all 3 trimesters has been reported. In fact, nearly half of the infants exposed to Zika *in utero* manifest abnormalities at birth or have symptoms during the first year of life (4, 7).

The severity and lasting effects of ZIKV infection on the developing fetus and pregnancy outcomes has attracted attention as a research topic. However, it is unknown what role placental dysfunction plays in ZIKV infection outcome. ZIKV RNA and/or antigen have been detected in multiple cell types within the placenta of human, non-human primate (NHP) and mouse models of infection (8, 9). For example, in human placenta from mothers infected with ZIKV, evidence of viral infection has been detected in the chorionic villi, placenta macrophages and histocytes in the intervillous space (9, 10). In a single pregnant pigtail macaque (*Macaca nemestrina*) infected with ZIKV, several aspects of CZS were characterized along with inflammation at the maternal-fetal interface, including mild decidual perivascular inflammation (not unusual in human decidua) and placental acute chorioamnionitis (11). Our group has shown that ZIKV infection of pregnant rhesus macaques (*Macaca mulata*), regardless of the severity of fetal effects, results in abnormal oxygen transport within the fetus with a dramatic effect on placental oxygen reserve (8). We hypothesize that these findings may be related to alterations in placental cell immune activation as well as a decrease in the ratio of classical (CD14+CD16-) to non-classical monocytes (CD14-CD16+) and an increase in intermediate monocytes (CD14+CD16+) in both the decidua and villous from ZIKV infected dams compared to uninfected controls (8).

Monocytes are a key immunomodulating cell during pregnancy these cells are critical for placental development and fruition (12). Infection of these cells is likely to alter their antiviral state and alter dissemination of the virus. For example, studies with human samples have shown ZIKV infects macrophages of the placenta inducing production of anti-viral factors including type 1 interferons (IFNs) (13). Beyond the placenta, human CD14+ monocytes are a primary site of ZIKV infection in pregnant and non-pregnant individuals (14). Analysis of human PBMCs infected *in vitro*, and PBMCs isolated from Zika virus positive Nicaraguan patients, demonstrated that CD14+CD16+ cells are the main targets of infection and this population of cells is expanded during infection (15).

Studies thus far support placenta CD14+ cells as an important site of infection and antiviral response during ZIKV infection. However, the impact of maternal ZIKV infection on CD14+ cell gene expression in the placenta and the effect that expression specific changes have on downstream cellular functions and viral infection is unknown. Viral infection is a dynamic process driven by an interplay between cellular pathways and viral mechanisms. In the current study, we performed single cell RNA sequencing on CD14+ cells isolated at 135 days of gestation (G) from the decidua of animals that were ZIKV infected at either G31, G51, or G115 and the results were compared to pregnant, time-matched uninfected controls. This method provided an unbiased characterization of transcriptional changes in individual CD14+ cells with insights into unique molecular signatures and discovery of specific cell functions. The overall objective of our study was to build upon our previous data from our NHP model of ZIKV infection during pregnancy by taking a targeted approach to determining immune cell characterization and function. Our data reveal the upregulation of multiple gene pathways involved in translation control, cellular stress and growth regulation, inflammation, and innate immunity that we suggest contribute to the antiviral response generated by CD14+ placenta cells following *in utero* ZIKV exposure.

METHODS

Ethics Statement Regarding Non-human Primate Research

Animal samples used in this study were collected from previous studies performed in compliance with local and national animal welfare bodies and in strict accordance with Institutional Animal Care and Use Committee (IACUC) protocols. Rhesus macaque studies were performed in a bio-containment facility at the Oregon National Primate Research Center (ONPRC), which is

accredited by the Assessment and Accreditation of Laboratory Animal Care (AAALAC) International. In our previous studies the dams and fetuses were humanely euthanized using a method that is consistent with the recommendation of the American Veterinary Medical Association.

Cells and Viruses

Zika virus isolate PRVABC59 received from the Centers for Disease Control (CDC) (16). PRVABC59 was passaged twice on C6/36 cells (ATCC CRL-1660) this working stock was concentrated by ultracentrifugation through a 20% sorbitol cushion, titered on confluent monolayers of Vero cells (ATCC CRL-1586), and sequenced and described previously (17). All cells were cultured in Dulbecco's modified eagle medium (DMEM) containing penicillin-streptomycin-glutamine and 5–10% fetal bovine serum (FBS) and cultured at 37°C.

CD14+ Cell Separation From Human Term and Experimental Rhesus Macaques Placenta

For our *in vitro* studies, CD14+ cells were isolated from experimentally infected rhesus macaque placenta and full-term human placenta that were processed as described below.

Rhesus Macaque Placenta

At the time of necropsy, ∼0.5–1g samples of rhesus macaque maternal decidua and fetal villous tissue were carefully dissected to avoid cross contamination ($n = 3$ infected and uninfected). Decidua and villous samples were collected separately into 5 mL of HBSS supplemented with 2% FBS and 10 mM Hepes (HBSS +). Tissues were dissected into a fine slurry using forceps prior to being digested with 50 mg collagenase in 35 mL HBSS + for 30 min at 37°C with continuous rocking.

Human Placenta

De-identified Human full-term placentas were obtained following non-laboring cesarian section (c-section) from OHSU patients (IRB# 15196). Multiple ∼0.5–1g samples of full thickness placenta were collected into HBSS+. Tissues were dissected into a fine slurry prior to being digested with 0.25% trypsin and 0.2% DNase in HBSS+ for 30 min at 37°C while rocking. Trypsin digested tissues were washed with HBSS + and further digested with 50 mg collagenase in 35 mL HBSS +, for 1 h at 37°C, with rocking.

Digested rhesus macaque and human placenta were washed with HBSS + and flushed through a 70 μM filter (Falcon). The cells present in the flow through were pelleted by centrifugation at 500 × *g*. Red blood cells were lysed with 5 mL BD Pharm Lyse lysing buffer (BD Biosciences) for 5 min followed by the addition of 10 mL HBSS+ to stop lysis. After a final wash in HBSS +, cells were resuspended in 5 mL HBSS +. Cells in 5 mL HBSS+ was centrifuged over lymphocyte separation medium (Corning) for 45 min at 3,000 rpm (1,459 x g) to isolate placental mononuclear cells. For magnetic bead isolation using MACS MicroBead Technology (Miltenyi Biotec), mononuclear were resuspended in MACS buffer at a concentration of approximately 1 x 10^7 cells/mL. Prior to magnetic separation, cells were passed through a 70 μM filter, to remove cell clumps. All cell types were isolated using a magnetic isolation method with species-specific reagents (MACS, Miltenyi Biotec). Approximately 1 x 10^7 cells were incubated with magnetic beads coated with anti-CD14 (Miltenyi Biotech) for 15 min at 4°C. The cells were then washed and resuspended in 500 μL MACS buffer before being loaded onto a LD magnetic separation column in the presence of a magnetic field. After sample loading the column was washed with 2 mL of MACS buffer. The magnetically labeled $CD14^+$ cells retained on the column were eluted by removing the column from the magnetic field and flushing with 1 mL MACS buffer using the provided plunger. The eluted cells were pelleted and resuspended in RPMI supplemented with 10% FBS and the purity of cells was demonstrated by flow cytometry (see below).

Flow Cytometry

Flow cytometry was used to demonstrate purity of CD14+ isolated cells. 1 x 10^4 cells were sampled from the 1 mL of cells eluted after magnetic separation (positive fraction) and from the column flow through during separation (negative fraction). Cells were stained with a CD14 specific antibody (HCD14 10 μg/mL Biolegend) and cellular surface staining was quantified using an LSRII instrument (BD Bioscience). Flow cytometry data was analyzed using FlowJo Software (TreeStar).

Single-Cell RNA-Seq and Data Analysis

Rhesus macaque placenta derived CD14+ cells were counted using a hemocytometry, and the cell number was normalized to a concentration of 1,000 cells/μL in chilled media. The cells were loaded into a 10x Genomics Chromium instrument (10x Genomics) and processed using the Single Cell 5′ kit Version 1.1 following the manufacturer's protocol. Generation of gene expression libraries was performed using manufacturer's instructions. To multiplex samples, cell hashing was performed using the MULTI-Seq lipid labeling system (18, 19). Libraries were sequenced using Illumina chemistry on either Novaseq or HiSeq instruments (Illumina). Raw sequence data are available under BioProject PRJNA639805 https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA639805.

The resulting sequence data were processed using cell ranger software (10x Genomics, version 4.0). Data were aligned to the MMul_10 genome build (release 98, assembly ID GCA_003339765.3), using Ensembl gene annotations (Build 10.103). The raw count matrix was imported directly into R and analyzed using the Seurat software package, version 4.0.2 (20). Briefly, the EmptyDrops algorithm was used to select droplets containing viable transcriptomes (21), followed by filtering on UMI count (discarding cells >20,000), feature count (discarding cells with <200 or >5,000). Passing cells were merged across samples and normalized using Seurat's "LogNormalize" method. Feature counts are divided by the total counts for that cell, multiplied by a scale factor, and natural-log transformed using log1p. The top 2,000 variable features were selected using Seurat's FindVariableFeatures function, using the "vst" method. Dimensionality reduction was performed with PCA followed by tSNE on the first 12 components. Differential expression was calculated using FindMarkers, using a Wilcoxon Rank

TABLE 1 | Primer and TaqMan probe sequences.

ZIKV	Forward: 5′-TGCTCCCACCACTTCAACAA
	ZIKV PRVABC59 genome sequence nucleotides 9797–9816)
	Reverse: 5′-GGCAGGGAACCACAATGG
	complement of nucleotides 9840–9857
	TaqMan probe: 5′ FAM-TCCATCTCAAGGACGG-MGB
	nucleotides 9819–9834
Rhesus macaque	
IFIT3	Forward: 5′-GAGAAGCGACAATCCCATCAG
	Reverse: 5′-CCACGTTGGAGAGCAGTGTCT
	TaqMan probe: 5′ VIC-TATTGCAACCTTCAGGAATA-MGB
IFI27	Forward: 5′-CGGTGTCATGGGCTTCACT
	Reverse: 5′-CTGCTGCGGACATCATCTTG
	TaqMan probe 5′ VIC-TCACCTCTTCCTCCATAGC-MGB
GAPDH	Forward: 5′-GCACCACCAACTGCTTAGCAC
	Reverse 5′-TCTTCTGGGTGGCAGTGATG
	TaqMan probe: 5′ FAM-TCATCCATGACAACTTTGGTA-MGB
Human	
IFIT3	Forward: 5′-GGCAATTGCGATGTACCATCT
	Reverse: 5′-TGGCCTGCTTCAAAACATCA
	TaqMan probe: 5′ FAM-TCACCCAGAGAAACAGT-MGB
IFI27	Forward: 5′-CCTTGTGGCTACTCTGCAGTCA
	Reverse: 5′-TGGAGCCCAGGATGAACTTG
	TaqMan probe: 5′ FAM-CTGGACTCTCCGGATTG-MGB

Sum test, and then filtered using adjusted $p < 0.001$ and average log fold-change >0.5. Data were visualized using the heatmap R package (1.0.12). Pathway analysis of the differentially regulated genes was performed using Qiagen's Ingenuity Pathway Analysis (IPA), Gene Ontology (Go) Term analysis, and String analysis.

qRT-PCR Analysis

RNA from *in vitro* infected placental samples was isolated using TRIzol reagent (Invitrogen) as previously described (8, 17, 22). Isolated RNA was quantified using a Nanodrop spectrophotometer, diluted to a concentration of 100 ng/μl and DNAse treated with ezDNase (Invitrogen). Single-stranded cDNA was generated using 1 μg of total RNA, random hexamers and Superscript IV reverse transcriptase (Invitrogen, Carlsbad, CA). qRT-PCR was performed with Taqman Fast Advanced Master Mix using the following cycling conditions: 95° for 2 min followed by 40 cycles of two steps with the first at 95° for 1 s followed by 60° for 20 s (Applied Biosystems, Foster City, CA). Forward and reverse primers directed against ZIKV and rhesus GAPDH were used at 300 nM in the reaction and the probe at 150 nM (Primer/Probe sequences are listed in **Table 1**). The results from each assay were analyzed with Applied Biosystem's QuantStudio 7 Flex Real Time PCR System software. Expression was normalized across all samples using the Rhesus macaque housekeeping gene GAPDH. The sensitivity of this qRT-PCR assay is <100 copies (8, 17, 22).

IFI27 Constructs

Total rhesus placenta RNA was used to generate cDNA by qRT-PCR using methods described above. The gene for rhesus IFI27 containing an in frame C'terminal HiBiT tag was amplified from cDNA and the product was ligated into pGEM-Teasy in *E. coli* DH5? (Promega). Individual colonies were screened for inserts and plasmid DNA was digested with restriction digested with EcoRI and HindIII and cloned into pCDNA-3.1(-). Resultant clones were sequenced verified and plasmid DNA was produced using a Qiagen midi-prep kit (Qiagen) and quantified using a Nanodrop spectrophotometer.

Cell Transfection and ZIKV Quantification

An 80% confluent flat bottom 96-well plate of HEK293 cells was transfected with 100 μg/well of the DNA constructs described above using lipofectamine 2,000 (Life Technologies) in replicates of ten. After overnight incubation at 37°C with 5% CO_2, the supernatant was removed and fresh media containing ZIKV [multiplicity of infection (MOI) =1] was added. At 72 h post infection (hpi), supernatants were collected in a 96-well plate and stored at −80°C. ZIKV virus in supernatant was quantified using a focus forming assay. Stored samples were thawed and serial dilutions of supernatant were plated in 96-well plates seeded with Vero cells, allowed to adsorb for 1h, followed by overlay with 0.5% carboxymethyl-cellulose (CMC; Sigma). At 30 hpi, cells were fixed with 4% paraformaldehyde, washed twice with PBS and blocked/ permeabilized for 1 h in PBS supplemented with 2% normal goat serum (NGS; Sigma) and 0.4% triton X-100. Cells were then washed twice with PBS followed by incubation with 0.3μg/ml anti-flavivirus monoclonal antibody 4G2 (23) in PBS supplemented with 2% NGS for 1h. The plates were washed twice with PBS and then incubated with anti-mouse IgG-horseradish peroxidase (Santa Cruz Biotech) for 1 h, after which they were washed twice with PBS. ZIKV foci were visualized by incubation with the Vector VIP peroxidase substrate kit (Vector Labs) according to manufacturer's specifications and counted using an ELIspot reader (AID).

Statistical Analysis

Statistical analysis of single cell sequencing data is described in Single-cell RNA-seq and Data Analysis. All other statistical analysis was performed using Prism v6 software (GraphPad Software, Inc). For gene expression experiments, data was analyzed by either a Student's *t*-test or a two-way ANOVA with secondary analysis to determine significance.

RESULTS

CD14+ Placental Cells From ZIKV Infected Rhesus Macaques Exhibit a Unique Gene Expression Profile

In our previous study, pregnant rhesus macaques (RMs) were infected with ZIKV at G31, G51, or G115 followed by cesarean section delivery at G135, at which time maternal and fetal tissues and the placenta were collected and processed for further analysis (8). Uninfected control RMs from ongoing studies underwent similar delivery and tissue collection at G135. Our previous

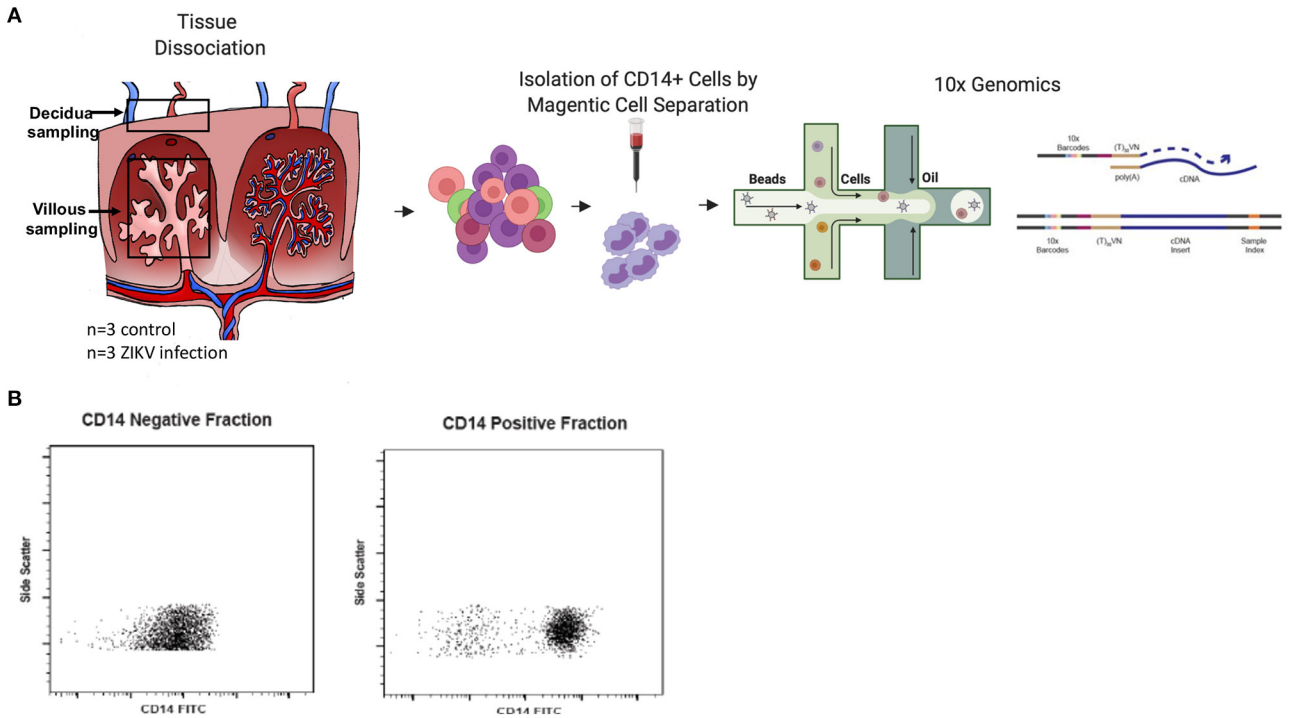

FIGURE 1 | Experimental design. **(A)** Decidua samples were collected after C-section delivery at G135 of pregnant dams either infected with ZIKV at G31, G51, or G115 or time matched uninfected control dams ($n = 3$). Tissues were dissociated and processed to single cells. For isolation of CD14+ cells, cells were positively selected using CD14+ microbeads and magnetic cell separation. Isolated cells were loaded into a 10x Genomics Chromium instrument and libraries were sequenced using Illumina chemistry. Resulting sequence data was processed using cell ranger software and further analyzed in the Seurat software package of R. **(B)** Both the CD14+ positive cells and negative flowthrough cells were analyzed by flow cytometry to determine purity of the CD14+ cell fraction.

analysis of these tissues confirmed the presence of persistent ZIKV nucleic acids in the placenta and other tissues of the RM. We also described substantial virus specific changes in the innate and adaptive immune responses within the placenta. Of primary interest to us was the increased activation of CD14+ cells and changes in monocyte subset proportions reported in both the decidua and villous (8). To further understand the role of CD14+ cells in the response to ZIKV infection during pregnancy, single cell sequencing was used to identify unique transcriptional signatures in CD14+ cells isolated from the maternal decidua of RM infected with ZIKV during gestation (**Figure 1A**). To isolate CD14+ cells, previously collected single cell RM decidua samples were bound to CD14+ labeled magnetic beads and processed through a separation column, resulting in a >90% pure cell population (**Figure 1B**).

To examine the state of placental monocytes/macrophages in ZIKV-infected RM, we isolated CD14+ cells and performed single cell RNA-seq (scRNA-seq). Using single-cell transcriptomes, we first performed unsupervised clustering to determine whether there were particular transcriptional states over-represented in cells from ZIKV-infected RM that subdivided into five phenotypic clusters (**Figure 2A**). We found clearly distinct phenotypes between the ZIKV and uninfected animals, with relatively homogenous transcriptional phenotypes within each cohort regardless of duration of animal infection (**Figures 2B–D**). Notably, the majority of CD14+ cells from ZIKV-infected animals formed two clusters (1 and 3, **Figure 2C**). To determine transcriptional differences between ZIKV-infected and control samples, we identified differentially expressed genes (DEGs) between each cluster (**Figure 2E**). Cluster 1, which represents 80.8% of cells from ZIKV-infected RM, shows a strong signal of immune activation, including up-regulation of chemokines CCL4L1/MIP-1-B and CCL2, MHC class II (Mamu-DPA), and dual-specificity phosphatase genes (DUSP1/2). This signature also includes ENSMMUG00000054210, an uncharacterized lncRNA. A subset of CD14+ cells from ZIKV-infected animals did not display this immune activation signature; however, these cells showed strong activation of genes involved in oxidative phosphorylation (**Figure 2E**, Cluster 3). CD14+ cells from ZIKV-infected RM also displayed a near universal down-regulation of a gene module including HMOX1, which is a macrophage modulator associated with anti-inflammatory M2 macrophages (24). Overall, we found expression profiles from infected animals did not significantly overlap with those from uninfected animals suggesting a distinct and sustained difference in transcriptomes between CD14+ cells derived from the decidua of animals infected during gestation.

DEG Pathway Analysis of Type 1 IFN Pathway Response

Gene ontology enrichment analysis (GO) and String: Functional Protein Association Networks online analysis tools were used

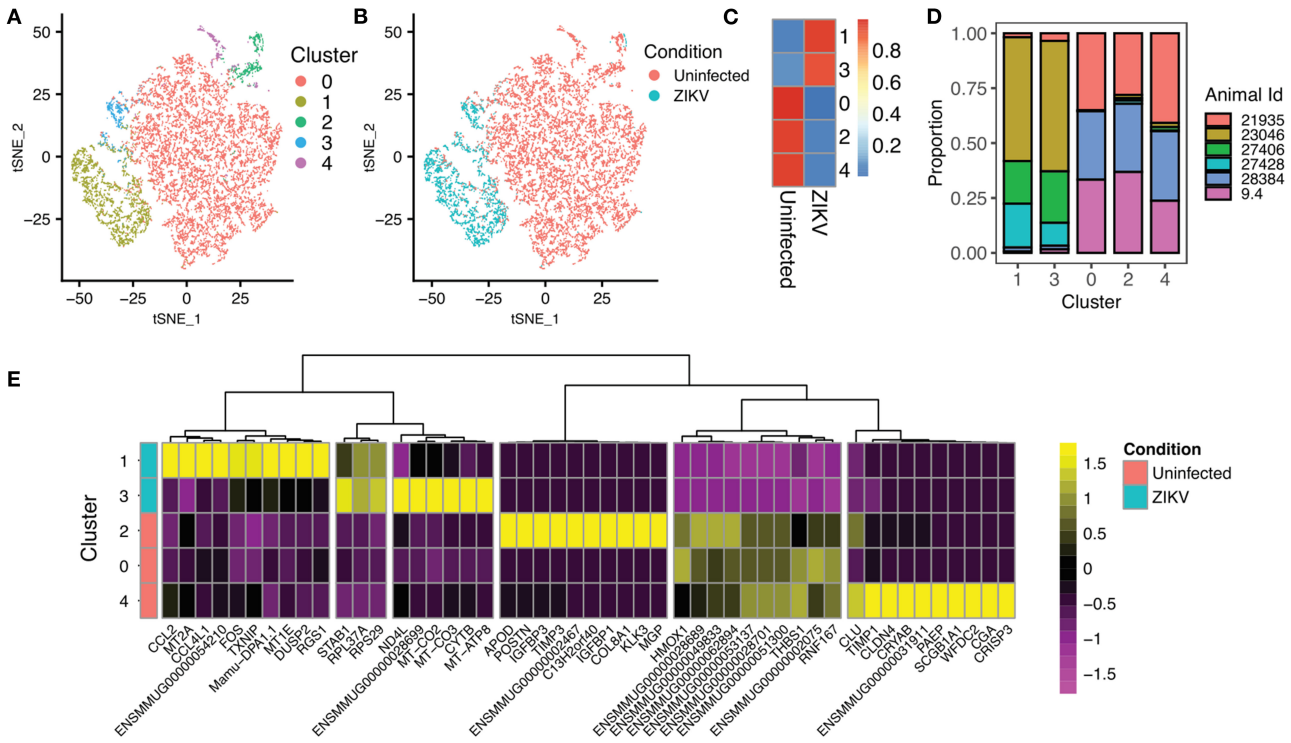

FIGURE 2 | Single-cell RNA sequencing of CD14+ cells from the placenta of ZIKV and uninfected RM. **(A)** Unsupervised clustering of single-cell transcriptomes from 11,752 CD14+ placental monocytes. Cells are colored based on cluster assignment. **(B)** Identical dimensionality reduction as **(A)**, with cells colored by the ZIKV of the RM, with cells from ZIKV-infected RM colored blue, and cells from uninfected controls colored red. **(C)** The heat map illustrates the proportion of cells from ZIKV vs. uninfected RM in each cluster. There is very little overlap between cells from ZIKV-infected and uninfected animals. **(D)** Contribution of samples to each cluster. **(E)** Heatmap showing the top differentially expressed genes for each cluster. Clusters 1 and 3 (top) are primarily from ZIKV-infected animals. Clusters 0, 2, 4, and 5 are primarily from uninfected RM.

to identify enriched biological processes and gene nodes within the significantly differentially expressed genes (DEGs) from ZIKV *vs* uninfected placenta samples (**Figures 3A,B**). The top enriched processes identified by these two analyses fit into the following broad categories: (1) translation control (*e.g.*, EIF2 signaling; Regulation of eIF4 and p70S6k); (2) stress response (e.g., Mitochondrial dysfunction; HIF1α signaling; Sirtuin signaling); (3) control of cellular growth (e.g., mTOR signaling; HER-2 signaling); (4) inflammatory responses (e.g., Granulocyte adhesion and diapedesis; Antigen processing); (5) signaling pathways mediated through cytokines/chemokines (IL-17 signaling; IL-10 signaling; OX40 signaling); and (6) innate immunity and antiviral responses (e.g., Role of PKR in interferon induction; Communication between innate and adaptive immune cells) (**Figure 3B**). Ingenuity pathway analysis (IPA) also demonstrated a clear relationship for interferon signaling in placenta derived CD14+ cells with nodes formed around IRF1 and IFNα (**Figure 4**). Among the genes upregulated in tissues from CD14+ cells of decidua from infected RM were type 1 IFN stimulated genes (ISGs) from the ISG12 family IFI6 and IFI27, which have been recently described as effectors of the IFN response to flaviviruses (25) (**Figure 4**). To confirm the upregulation of the ISG12 family during ZIKV infection of the placenta, we directly measured expression of IFI27 by qRT-PCR in CD14+ cells isolated from the villous and decidua of RMs infected with ZIKV at various time points during gestation. These samples were from additional, previously published, animal cohorts to bolster sample size ($n = 6$) (8, 22). IFIT3, a well-studied type 1 IFN ISG, was used as a positive control. CD14+ cells from the decidua or villous of ZIKV infected RM expressed higher levels of IFI27 compared to cells from placentas from uninfected controls (**Figures 5A,B**). The villous showed higher expression of both IFI27 and IFIT3 compared to the decidua, suggesting a more robust IFN response in villous tissue. These data confirm ZIKV infection during gestation induces IFI27 expression in the decidua and villous. Importantly, we did not see the same dysregulation of IFI27 in peripheral blood mononuclear cells from infected animals compared to uninfected controls indicating a specific and persistent response to infection by cells in the placenta (**Figure 5C**).

ZIKV Infection Induces IFI27 Expression in Rhesus Macaque and Human Placenta *in vitro*

To validate ZIKV infection-induced expression of IFI27 in placental monocyte/macrophages, we explored gene expression in ZIKV naïve cells from RM and human placenta that we

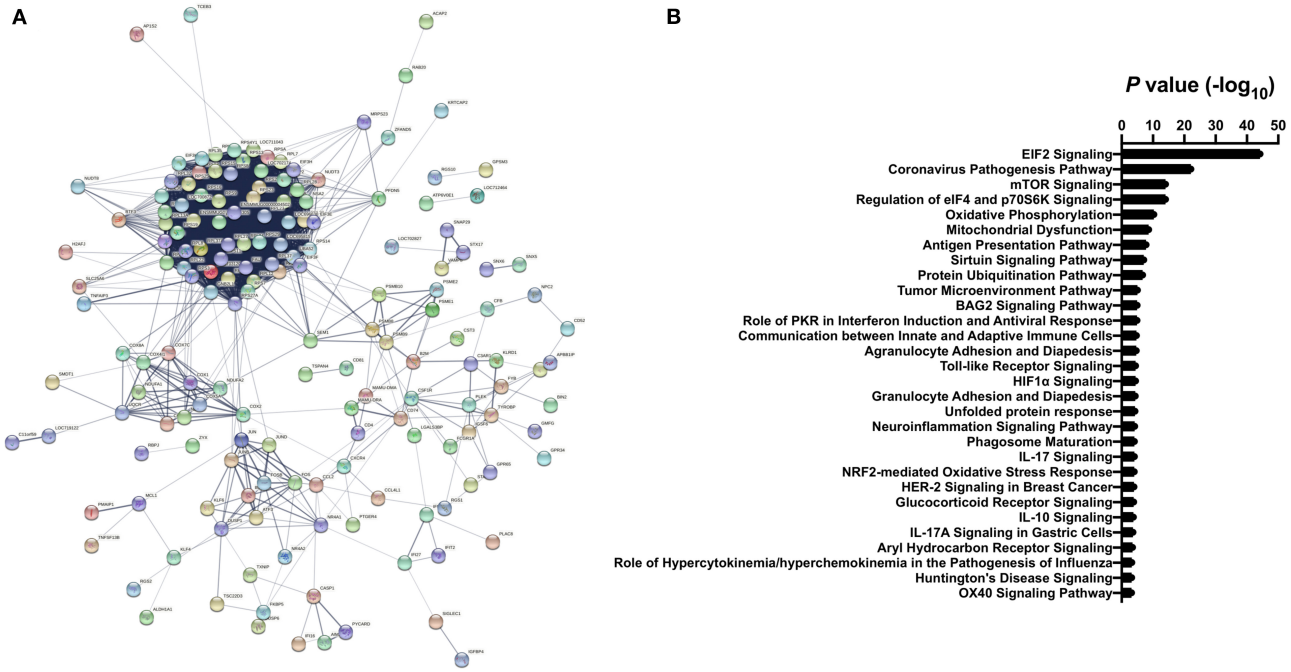

FIGURE 3 | Differential gene expression analysis of CD14+ villous cells **(A)** STRING analysis of significant differentially expressed genes from ZIKV infected vs. uninfected clusters. Lines with different colors represent the existence of different types of evidence applied to predict associates between proteins. **(B)** The top Gene Ontology (GO) terms from differentially expressed genes represented as -$\log_{10}$ (*P* value). Differentially expressed genes between ZIKV infected and uninfected clusters were identified using a Wilcoxon Rank Sum and filtered using adjusted $p < 0.001$ and average fold-change >0.5.

infected with $ZIKV_{PRABC}$ *in vitro*. For this experiment, CD14+ cells were isolated from the decidua and villous of control RM using the methods described above. In the human placenta, the division between decidua and villous is not as distinct and consequently is difficult to definitively isolate these cell types, therefore whole thickness samples from human placenta were digested followed by CD14+ cell isolation using antibody-conjugated magnetic beads. Isolated CD14+ cells were infected with $ZIKV_{PRABC}$ *in vitro* at 0.5 or 5 FFU/cell. Cells were collected into Trizol reagent at 48hpi or 72hpi, RNA was isolated gene expression analyzed by qRT-PCR of ZIKV, IFI27, and IFIT3. CD14+ cells from both RM and human placenta tissues were readily infected with ZIKV (**Figures 6A**, **7A**). ZIKV infection resulted in a MOI dependent increase in the level of IFI27 and IFIT3 transcripts in both RM and human CD14+ placenta cells (**Figures 6B,C** and **7B,C**). These results confirm the ability of ZIKV infection to induce IFI27 and IFIT3 as well as the transferability of the model to a human infection scenario.

IFI27 Overexpression Reduces ZIKV Replication *in vitro*

To confirm the role of IFI27 as an antiviral factor against ZIKV, we constructed a plasmid expressing rhesus IFI27 to allow exogenous expression of the ISG in ZIKV-infected cells. IFI27 was transfected into 293 cells and expression from the plasmid was confirmed by western blot (**Figure 8A**). HEK293 cells were transfected with an IFI27 expressing or control plasmid one day prior to infection with ZIKV. Supernatants were collected 3dpi and infectious virus was quantified by focus forming assay. In this system, exogenous expression of IFI27 significantly decreased the production of infectious virus by nearly a log (**Figure 8B**), confirming that IFI27 plays a role as an antiviral factor against ZIKV.

DISCUSSION

The rhesus macaque model of *in utero* ZIKV infection recapitulates many of the hallmarks of the human disease caused by this neurotropic flavivirus including fetal neuropathy and demise. We previously showed that ZIKV infection in Rhesus macaques during pregnancy induces placental damage associated with increased vascular damage that leads to altered nutrient perfusion across placental membranes, as well as increased inflammation and remodeling (8). Placental monocytes and macrophages (especially of the classical lineage) are key cells for normal placental vascular development and actively patrol the placenta for pathogenic invaders as the first line of defense. We previously reported dramatic alterations in the activation profiles for both monocyte/macrophages and CD4+ T cells that were consistent with ongoing inflammatory processes with possible correlations with ZIKV persistence. This long-term cellular activation was not observed for either dendritic cell or CD8+ T cell populations indicating that there is a unique effect of ZIKV infection on placenta immunopathology. Since

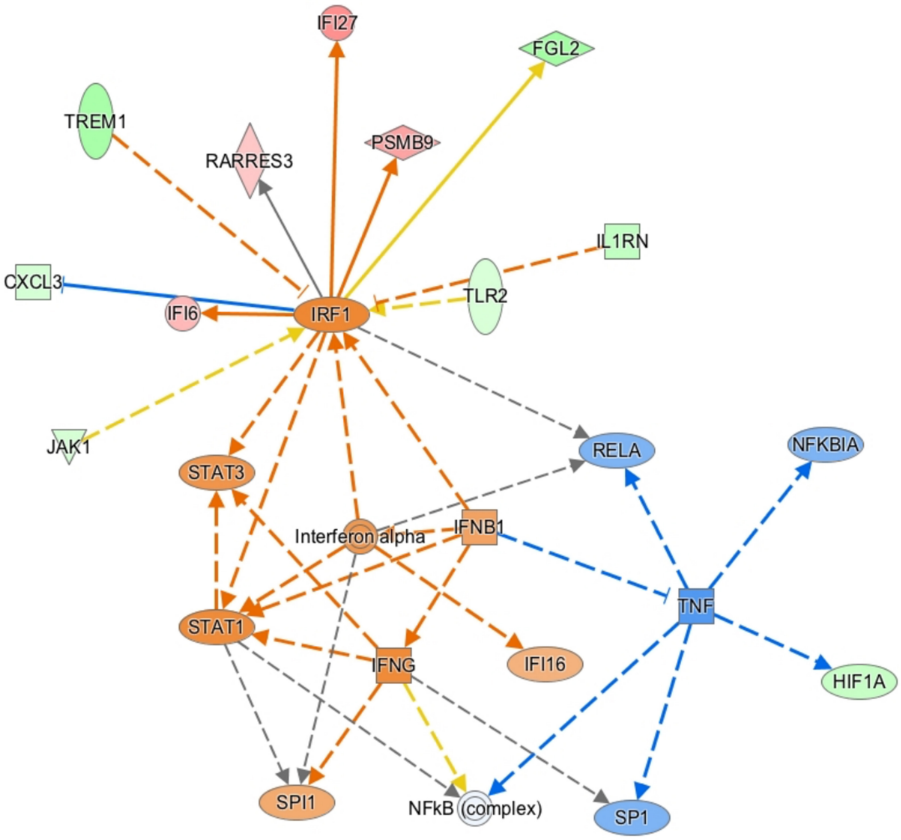

FIGURE 4 | Ingenuity pathway core analysis was used to identify enrichment in biological processes (ZIKV Infected *vs.* Uninfected). Statistically significant alterations in differentially expressed genes from SC-RNAseq analysis of decidua CD14+ cells are represented by labeled shapes depending on their molecular function. Expression and predicted activation levels determined by fold-change between ZIKV infected vs. uninfected are presented using the following convention on color scales with increasing saturation associated with a greater absolute fold-change value: red = increased detection in CD14+ cells from ZIKV infected decidua; green = decreased detection; orange = predicted activation. Predicted relationships are indicated by arrow-heads and blue lines to indicate predicted inhibition; yellow lines indicate inconsistent findings that make it difficult to predict outcomes of interactions.

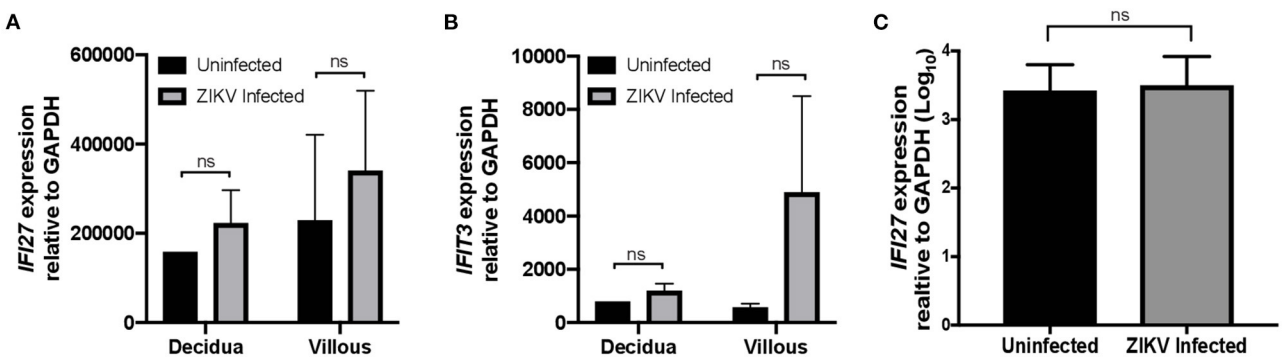

FIGURE 5 | IFI27 is highly expressed in CD14+ decidua and villous cells after ZIKV infection. Validation of increased expression of IFI27 in CD14+ decidua and villous cells collected from pregnant dams infected with ZIKV and uninfected controls from multiple study cohorts ($n = 6$ per condition). RNA was isolated and gene expression analyzed by qRT-PCR of **(A)** IFI27 and **(B)** control IFIT3 relative to GAPDH. **(C)** RNA was isolate from the PBMCs of dams infected with ZIKV and uninfected controls from multiple study cohorts ($n = 12$ per condition) and analyzed for IFI27 gene expression using GAPDH to normalize the data across samples. Student's *t*-test was used to determine statistical significance of expression data obtained from infected and uninfected animals.

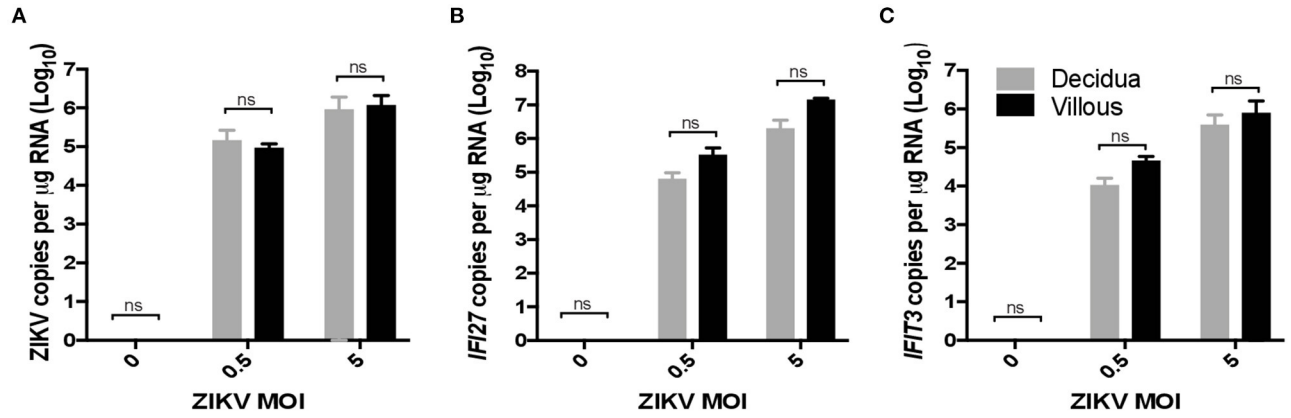

FIGURE 6 | ZIKV infection *in vitro* induces IFI27 expression in rhesus macaque decidua and villous. CD14+ cells were isolated from the decidua and villous of uninfected control rhesus macaque and infected with ZIKV (MOIs = 0.5 and 5 FFU/cells) at 72hpi supernatants were collected and analyzed by qRT-PCR for expression of **(A)** ZIKV **(B)** IFI27 **(C)** IFIT3 using species specific primers. Student's *t*-test was used to determine statistical significance.

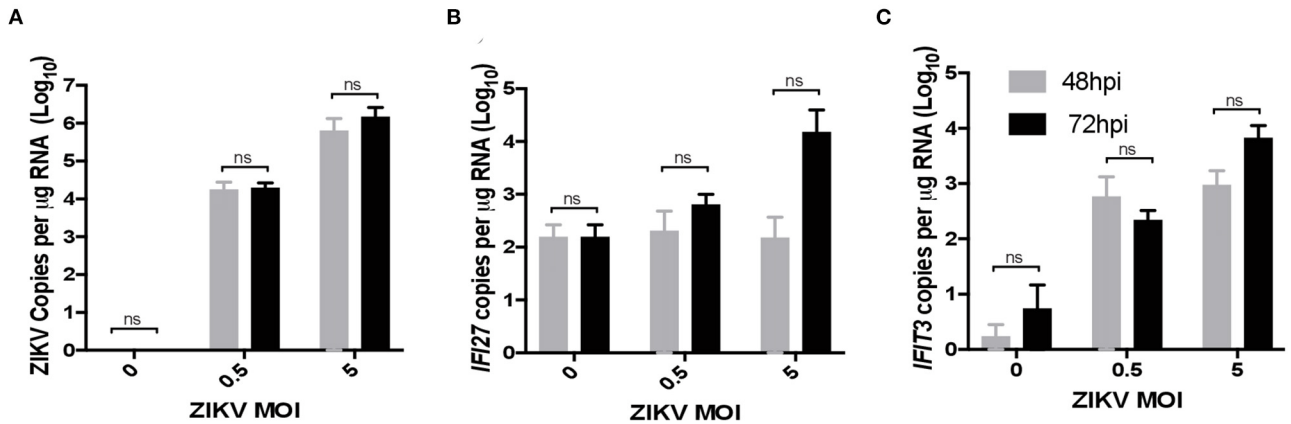

FIGURE 7 | ZIKV infection *in vitro* induces IFI27 expression in human placenta. CD14+ cells were isolated from de-identified human full-term placentas and infected with ZIKV (MOIs = 0.5 and 5 FFU/cells) at 48 and 72 hpi supernatants were collected and analyzed by qRT-PCR for expression of **(A)** ZIKV **(B)** IFI27 **(C)** IFIT3. A two-way ANOVA with secondary testing was used to determine statistical significance for these data.

macrophages are a key cell type in placental immunobiology and development, we interrogated the changes to the placenta macrophage population following infection. Herein, we utilized a single cell sequencing approach to further investigate the CD14+ monocyte/macrophage cell population to identify transcriptional signatures in cells isolated from the maternal decidua of ZIKV infected animals. Our objective was to identify mechanistic pathways that are dysregulated in the placenta following ZIKV infection. Our study findings reveal significantly altered gene expression in placental macrophages isolated from ZIKV-infected animals and demonstrate the sustained upregulation of macrophage functions even in the absence of positive viral detection indicating a prolonged response to viral exposure. This may be an adaptive response to put up an immune shield to prevent further infection or disease in the placenta and to limit the adverse impacts on fetal growth and development.

Specifically, in the samples analyzed in this study, the DEGs in CD14+ villous cells of ZIKV infected RM were strongly associated with eIF2 signaling, eIF4 signaling, mTOR signaling, oxidative phosphorylation, and mitochondria dysfunction. eIF2, EIF4, and mTOR are regulators of protein translation and the cellular stress unfolded protein response (UPR). These pathways are tightly interrelated with inflammatory pathways leading to production of proinflammatory molecules (26). The mitochondrion also plays a role in regulating the antiviral immune response, a function that viruses can regulate to evade the immune response, leading to host cell damage through changes in ROS and cell metabolism (27–29). Altered bioenergetics and mitochondrial dysfunction of peripheral blood monocytes have been described for other viral infections in connection to changes in CD14/CD16 monocyte population distribution (30).

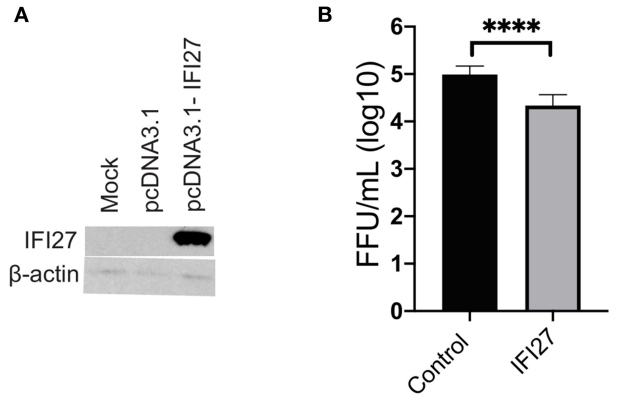

FIGURE 8 | IFI27 overexpression reduces ZIKV replication *in vitro*. **(A)** To confirm IFI27 protein expression 100 μg of pcDNA3.1 or pcDNA3.1-IFI27 DNA was transfected into HEK293 cells. At 7 h post-transfection cell lysates were analyzed for IFI27 or actin by western blotting. **(B)** HEK293 cells were transfected with 100 μg of a control vector constitutively expressing GFP or pcDNA3.1-IFI27 24 h post-transfection cells were infected with ZIKV (MOI = 1). At 72 hpi supernatants were collected and virus quantified by focus forming assay. P values were determined by paired student's *t*-test $^{****}p < 0.0001$, all other comparison were not significant.

Our results are in accordance with a recent a study by Lum et al. using placenta from pregnant women infected with ZIKV during the first, second, or third trimester. Only ZIKV infection during the first trimester resulted in a gene expression profile that was different from healthy controls, and the resulting DEGs were associated with eIF2 signaling, oxidative phosphorylation, and mitochondrial dysfunction, which is very consistent with DEG found in our study. Additionally, there was a distinct relationship between the degree of infection and level of CD14+ monocytes in the patients (31). While in our study we did not see an effect of infection gestational age on the transcriptional program in CD14+ cells, this could be a result of the fact that we looked at this cell type specifically at only G135 or possibly due to the RM model or ZIKV virus strain used for infection. The similar DEGs between the studies do suggest the importance of these conserved macrophage-centric pathways in ZIKV infection on the immunopathology of the placenta. Our findings implicate that CD14+ cells play an important role in regulating the antiviral response through multiple pathways following infection with ZIKV. Notably flaviviruses possess multiple mechanisms to modulate aspects of innate immunity that may affect infection of cells including CD14+ monocytes (32–38). Future experiments will focus on understanding the role of these interactions in placental pathology and effects on outcomes of ZIKV infection during pregnancy.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The name of the repository and accession number can be found below: National Center for Biotechnology Information (NCBI) BioProject, https://www.ncbi.nlm.nih.gov/bioproject, PRJNA639805.

ETHICS STATEMENT

The animal study was reviewed and approved by OHSU Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

NH, DS, AH, and VR: designed, coordinated, and implemented NHP experiments. NH, DS, AH, VR, BB, and AF: reviewed the data, aided in experiment designed, and drafted and edited manuscript. AH and JS: provided and prepared viral stocks and performed plaque assays. NH and HS: performed rhesus macaque and human cell isolation. BB and KO: performed 10x genomics statistical analysis. NK, MD, HS, and CK: performed cloning and gene expression quantification. All authors contributed to the article and approved the submitted version.

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REVIEW
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Human Anelloviruses: Prevalence and Clinical Significance During Pregnancy

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Although the bacterial microbiota of various compartments (e.g. vagina, amniotic fluid, and placenta) have been studied in pregnancy, there has been far less emphasis on normal and pathological viral communities. Cumulative evidence shows the presence of a number of apathogenic viruses in various tissues of healthy people, including pregnant individuals. What role, if any, these viruses play in human physiology is unknown. Anelloviruses (family *Anelloviridae)* are circular, single-stranded DNA viruses commonly detected with high prevalence in vertebrate hosts, including primates. Humans are nearly always colonized with at least 1 of 3 *anellovirus* subtypes, namely *Alphatorquevirus* (torque teno virus, TTV), *Betatorquevirus* (torque teno midi virus, TTMDV), and *Gammatorquevirus* (torque teno mini virus, TTMV). In healthy pregnant people, the prototype anellovirus, TTV, has been found in maternal and (variably) fetal blood, amniotic fluid, cervical and vaginal secretions, breast milk, and saliva. Nonetheless, the relevance of human anelloviruses in pregnancy and labor is unclear. There is evidence suggesting a link between anellovirus colonization and preterm birth. In this review, we discuss what is known about this family of commensal viruses in health and disease, and specifically the roles they might play during pregnancy and in the timing of delivery.

Keywords: human virome, *Anelloviridae*, anelloviruses, commensal virus, pregnancy, preterm birth

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INTRODUCTION

The human body serves as a host to a highly diverse community of microorganisms. These microorganisms may benefit the host (creating a "mutualistic" relationship), harm the host (forming a "pathogenic" relationship), or have no apparent effect (a "commensal" relationship). From time to time, mutualistic or commensal microorganisms may assume a pathogenic character (for example, in the case of vaginal yeast infections). The genomes that constitute the human microbiome include bacteria, archaeans, other eukaryotes, and viruses (1). These microbial communities are highly dynamic and vary based on the individual's age and health status, the biology of the anatomical site, diet, and hygiene (2).

While research on the human microbiome has focused mainly on bacterial populations, much less is known about viral communities residing at different sites in and on the human body and their roles in health. Advances in sequencing have uncovered myriad novel viruses in humans, many of which cause no apparent illness (3). Most humans are colonized in almost every tissue type by members of *Anelloviridae*, a family of diverse, non-enveloped, circular, single-stranded

DNA eukaryotic viruses (4, 5). Thus far, anelloviruses have not been linked definitively to any disease states (6), although there is some evidence suggesting a link to human disease (7). This review discusses this novel class of human viruses, including their prevalence, genome diversity, transmission routes, and potential association with human health and disease. We focus on pregnancy, including a possible role in the timing of delivery. Anelloviruses have been detected in maternal and—to a lesser and highly variable extent, depending on the study—fetal tissues (8–10). We discuss the potential mechanisms by which anelloviruses may interact with and modulate maternal immune responses and influence pregnancy outcomes.

DISCOVERY AND NOMENCLATURE

In 1997, while searching for a viral agent responsible for non-A to E hepatitis, Nishizawa et al. found a novel DNA virus in the serum of a Japanese patient with post-transfusion hepatitis of unknown etiology (11). The viral clone was designated TT virus (TTV) after the patient from whom it was recovered. Subsequent studies revealed TTV as a small, non-enveloped, single-stranded, circular DNA virus (12). After the discovery of the original TTV isolate, smaller variants of TTV were identified and subsequently named torque teno mini virus (TTMV) (13), and torque teno midi virus (TTMDV) (14), derived from the Latin terms *torque* meaning "necklace" and *tenuis* meaning "thin" (15). Recent changes in nomenclature have classified the 3 anellovirus genera found in humans: *Alphatorquevirus* (TTV), *Betatorquevirus* (TTMV), and *Gammatorquevirus* (TTMDV), which together comprise the human *Anelloviridae* family (16).

ANELLOVIRUS AND HUMAN DISEASE

A clear link between anellovirus positivity and human disease has not been established (6). On the one hand, the fact that anelloviruses are rarely detected earlier than 3 months of age and are acquired later in life in healthy individuals (17–19) suggests that anellovirus acquisition over the lifespan is normal. On the other hand, recent studies have suggested that certain anellovirus subtypes are associated with various illness and diseases such as unexplained fever (20), diabetes (7), cirrhosis in liver transplant patients (21), respiratory disease (22–26), cancer (27–30), and autoimmune disorders (31–33). There is some evidence suggesting a high occurrence of anellovirus with *Epstein-Barr virus* (34) and hepatitis B or C (5). Whether this means that anelloviruses have a role in enabling pathological viral infections remains to be elucidated. Given the prevalence of TTV in organ transplant recipients, TTV load has been suggested as a candidate indicator of immune suppression (35–37).

PREVALENCE OF ANELLOVIRUS BY AGE AND GENDER

Anelloviruses are reported at a high prevalence in the general population across the globe (38). TTV, the prototypical anellovirus, is multitropic, i.e., found in nearly every body site, fluid, and tissue tested, as summarized in **Table 1**.

A plethora of evidence suggests that anelloviruses are detected by PCR in all age groups. A study analyzed fecal specimens collected longitudinally from day of life 1–4 (month 0) and at 3, 6, 12, 18, and 24 months of age from 4 healthy twin pairs (18). Anelloviruses were rarely detected earlier than 3 months of age. Thereafter the prevalence increased significantly, peaking at 6–12 months of age, and began to decline at 18 and 24 months of age. Among 8 infants enrolled in the study, 1 infant harbored no less than 47 anellovirus species at 12 months of age. In some infants, the same anelloviruses could be detected from fecal samples collected up to 12 months apart, suggesting persistence and expansion of anellovirus richness in the gut of infants. Another study of 20 twin pairs (0–30 months of age) showed the abundance of anellovirus species increased until 15–18 months of age, after which time abundance diminished (66). A prospective single-center study of 98 clinically healthy breastfeeding infants (1–12 months of age) demonstrated a significant increase in whole blood anellovirus load during the first year of life, reaching a plateau after 6 months of age (17).

A study investigated the epidemiology of anellovirus in blood samples derived from healthy children (1–14 years) and healthy blood donors (18–59 years) (67). Among 208 children, 141 were

TABLE 1 | Tissues tested for anellovirus.

	Citations
Tissues with detectable anellovirus	
Whole blood, plasma, or serum*	(3, 39–48)
Peripheral blood mononuclear cells (PBMCs)	(43, 49–51)
Exosomes-enriched vesicles from plasma	(52)
Bone marrow, lymphoid tissue, thyroid gland, muscle, pancreas, spleen, kidney, lung	(48)
Bronchoalveolar lavage	(53)
Nasal or throat swabs	(24, 53)
Saliva*	(10, 40–42, 54)
Liver	(43, 55)
Bile	(56)
Feces	(41, 42, 46, 49, 56–59)
Urine	(60, 61)
Skin, hair follicle	(62)
Tears	(42)
Semen	(42, 54)
Amniotic fluid*	(9)
Cervix*	(63, 64)
Vaginal secretions*	(65)
Umbilical cord blood*	(9, 10)
Breast milk*	(9, 45, 47)
Tissues without detectable anellovirus	
RBCs	(50, 51)
Platelets	(50, 51)
Sweat	(42)

**Tissues tested in pregnant or post-partum people.*

TTV-positive. TTV prevalence was highest in 1–2-year-olds, lower for 8-year-olds, and higher again in 14-year-old children. Among 196 healthy blood donors representing the normal population, 103 were TTV-positive; there was no difference in the TTV DNA prevalence with age. However, other studies (68–70) with larger sample sizes have consistently demonstrated positive correlations between anellovirus prevalence and age in healthy populations. Phylogenetic analyses did not find associations between anellovirus genotypes and particular age groups (67) or geographic locations (68, 71). One study (68) noted viral loads were highest in blood donors more than 50 years old, but a longitudinal analysis of plasma TTV loads after 2 years showed minimal changes in TTV viremia (70). The findings suggest that although anelloviruses are acquired over the lifetime, healthy aging causes only minimal increases in TTV viremia.

Anellovirus prevalence and viral load may be gender-specific. One study found TTV prevalence was significantly higher in males than in females (70). A separate study found that young women (20–30 years) had lower plasma loads of anellovirus than men in the same age group (19).

Substantial evidence suggests that anellovirus load is governed by the immune system (72). Although the mechanisms by which the immune system reacts to anellovirus colonization are unknown, studies have shown that people receiving a solid organ transplant (73–77), and those with cancer (47), HIV infection (78), and sepsis (79) have higher plasma anellovirus loads than healthy donors. Other studies have shown an inverse correlation between levels of TTV and CD4+ lymphocytes in HIV-positive patients (80) and pediatric lung transplantation patients (81). The latter study findings revealed that patients with low anellovirus genome copies are at risk of transplant rejection or death. There is also evidence of increased anellovirus DNA concentrations after antiviral therapy (6). Thus, it appears that anellovirus load is inversely correlated to and may serve as a marker of general immune status.

ANELLOVIRUS GENOME

Despite their nucleotide sequence diversity, anelloviruses share virion structure and genomic organization (13). Electron microscopy of the prototype anellovirus, TTV, isolated from serum specimens (82) and a TTV-infected HEK293 cell line (83) demonstrate TTV as an unenveloped icosahedral virus with a diameter between 30 and 50 nm. As indicated by their names, the human anelloviruses differ in genome size: 3.9 kb for TTV, 3.2 kb for TTMDV, and 2.8–2.9 kb for TTMV. The TTV genome consists of an untranslated region (UTR) of ∼1.2 kb and a potential coding region of ∼2.6 kb. The non-coding UTR of the TTV genome contains a GC-rich segment (>60% GC) flanked by a TATA box upstream of the coding region and a poly-A sequence downstream (84), and multiple stem-loop structures that facilitate virus replication (71). The coding region consists of 3–5 overlapping open reading frames (ORF1-5) which encode at least 6 proteins with structural (85), host immune suppression (86, 87), cell cycle regulation, and apoptosis-inducing properties, respectively (88). ORF1 also contains hypervariable regions where mutations occur more frequently than in other regions. These hypervariable regions help the virus evade the immune system (89).

Genetic Heterogeneity

In addition to size, the 3 *Anelloviridae* genera can be grouped according to their degree of genetic similarity in the ORF1 region. TTV, TTMV, and TTMDV have at least 105, 68, and 34 species, respectively. Phylogenetic analysis of TTV isolates recovered from disparate locations have identified 7 major clusters, with genomic sequence differences of up to 35% (90). It has been hypothesized that in a given individual, genetic variability within a viral group is high, and that coinfection by distinct viral strains in blood and other tissues is common (91, 92). A study investigated possible relationships between the number of genogroups carried and the total TTV load present in 239 TTV-positive subjects (93). Individuals with high viral loads tended to possess more TTV genogroups than those with low viral loads. TTV genogroups 1 and 3 were the most prevalent, followed by genogroups 4 and 5, while genogroup 2 was rather infrequent.

DETECTION AND QUANTITATION OF ANELLOVIRUSES

To date, polymerase chain reaction (PCR) is the most prominent method used to detect anellovirus. Because of the extensive heterogeneity among the genomes of anelloviruses, detection of the entire spectrum of the anellovirus variants is impossible using a single set of primers. For genotyping, primer pairs designed either in the ORF1 region or the sequences spanning 5' or 3' UTRs are widely used (93, 94). Taking advantage of these regions, nested and semi-nested PCR assays are developed in which the genomic DNA of all anelloviruses is amplified by first-round PCR with universal primers, and then species-specific DNA are amplified by using a second set of primers (7). In a number of studies, sequences spanning N22-ORF1 regions are utilized for the detection of anellovirus DNA (11, 92). However, this strategy allows detection of only some genotypes of TTV, a genus with more than 30 genotypes (95). For example, N22 primers can efficiently amplify genotypes in group 1, but amplifies certain genotypes in group 2 less efficiently (96) and fails to amplify many genotypes in groups 2, 3, and 4 at all. Over time, studies have increasingly focused on utilizing degenerate primers and highly conserved regions located just downstream of the TATA box to potentially detect all known genetic forms of anelloviruses (97–99). The results are validated across multiple iterations followed by phylogenetic analysis (94, 100, 101). In recent studies 5'UTR primer sets are often used, but these primers differ in their abilities to detect TTV and related genotypes by PCR (95). Therefore, differences in primer selection could explain some of the considerable variation in estimates of anellovirus prevalence between studies. Even within a single healthy cohort, TTV detection ranged from 53% (251/471) to 90% (90/100) depending on which primers were used (68). In addition, measuring prevalence of detectable TTV is highly dependent on the type of specimen analyzed—for example, TTV titer is higher in whole

blood than in plasma (70). Therefore, TTV negativity in a sample could be a laboratory artifact due to sub-optimal sensitivity of the detection methods. A study validated the commonly used PCR primer sequences to detect TTV and TTV-like virus in different populations (102). Primer alignment and PCR product characterization consistently indicated that a minimum of five primer sets (NG, TT, TLMV-S, TLMV-L, and a genotype 21-specific set of primers) are required to detect all known genotypes of TTV and TTV-like viruses in healthy individuals.

In addition to the PCR method, antibody-based detection of TTV has also been developed and used for the diagnosis of TTV colonization (103).

SITES OF ANELLOVIRUS REPLICATION

Despite decades of research, the main site of anellovirus replication remains unknown. Studies have indicated the association of TTV with peripheral blood mononuclear cells (PBMCs) and distinct distribution of TTV subtypes between plasma and PBMCs (104, 105). Research has also shown that TTV is abundant in granulocytes compared with other peripheral blood cell types in healthy individuals (51). Given the reported evidence of elevated TTV titers with immunosuppression and transplant-related complications (6, 106), a study investigated TTV levels in plasma samples and potential sites of TTV replication in individual blood cell types derived from pediatric allogeneic hematopoietic stem cell transplant (HSCT) recipients (107). Among 43 HSCT patients enrolled in the study, 34 had detectable TTV in plasma before transplantation, and all patients tested positive for TTV by day+50 post-transplant. TTV copies reached peak titer around day+100, and then gradually declined to pre-transplantation levels over a period of about 2 years. TTV DNA was not present in NK cells, B- and T-cells. On the other hand, granulocytes isolated from peripheral blood or bone marrow were invariably positive in post-transplant samples of all patients. Until day+30 post-transplantation, TTV tested either near or below the detection limit in granulocytes, but dramatically increased between days +30 and +100 days post-transplantation in peripheral blood and bone marrow granulocytes. At the same time, TTV DNA was absent in granulocytes derived from healthy immunocompetent controls throughout the study period. Together, these findings suggest granulocytes as potential TTV replication sites, particularly in immunosuppressed individuals.

Evidence comparing viral titers between different tissues within a single patient suggests anellovirus replication can occur in bone marrow (108), liver (109, 110), lungs (111), lymphoid tissue (112), oropharyngeal and/or salivary glands (40). These findings suggest that viral replication takes place in multiple tissues at distinct levels in infected individuals (48).

Attempts to replicate anellovirus *in vitro* have been unsuccessful thus far. Human cell lines, including Chang liver (109), HEK293TT (113), lymphoma and T-cell leukemia (83), and the Raji cell line (109), have demonstrated TTV infection in initial passages, but the virus did not propagate to later passages (83, 113, 114).

IMMUNOBIOLOGY OF HUMAN ANELLOVIRUSES

Toll-like receptors (TLRs) are members of a family of cell-surface proteins responsible for recognition of a diverse spectrum of pathogens and generation of an innate immune response. TLR9 recognizes intracellular unmethylated heterodimers of guanosine and cytosine (CpGs), which are abundant in the genomes of DNA viruses. Depending on the number of nucleotides flanking CpGs, this may stimulate the production of either pro- or anti-inflammatory cytokines (115). It has been reported that the genome as well as the replicative intermediates of anellovirus are unusually rich in CpG sequences (116). The DNA of 1 genogroup of anellovirus (ViPiSAL strain) was found to provoke robust activation of TLR9 and the production of proinflammatory cytokines in *ex vivo* mouse spleen cells (117). Nevertheless, the genomes of other anellovirus strains failed to promote inflammatory responses. These findings may indicate that the effects of anelloviruses on the host's inflammatory status vary depending on genogroups.

Due to the lack of an efficient culture system to support TTV replication, the transcription profile of TTV has been largely gained from human cell lines (COS1, HEK293, and L428) transfected with TTV plasmids (87, 118). Three spliced mRNAs of TTV that produce at least 6 proteins by alternative translation initiation have been reported (85). At present, the functional role of ORF2 protein is well-characterized. Overexpression of TTV ORF2 encoded protein has been shown to suppress NF-κB activation elicited by TNFα in various human cancer cell lines, including HeLa and HepG2, and in the mouse macrophage line RAW 264.7 (86). Further analyses revealed that TTV ORF2 protein has the ability to suppress NF-κB activity *in vitro* in a dose-dependent manner, affecting translocation of NF-κB p65 and p50 subunits to the cell nucleus, thus inhibiting the transcription of downstream genes such as interleukin (IL)-6, IL-8, and cyclooxygenase-2. Together these findings indicate that TTV ORF2 protein may be involved in negative regulation of host cell inflammatory responses.

Evidence suggests that TTV encodes microRNAs (miRNA) that cooperate with viral proteins to regulate the expression of viral genes involved in replication, pathogenesis, inflammation, and immune evasion (119). The functional relevance of proteins translated from other TTV ORFs and TTV-encoded miRNAs warrant further study.

ROUTES OF TRANSMISSION

Numerous studies have suggested horizontal and vertical TTV transmission routes. Horizontal transmission includes parenteral, fecal-oral, and sexual. Vertical transmission involves the possible passage of virus from mother to fetus during pregnancy and breast feeding.

Parenteral Route

Since bone marrow cells and activated PBMCs are recognized as potential sites of TTV replication (120, 121), blood and blood products could be among possible routes of TTV transmission.

Therefore, people with blood-related diseases such as hemophilia and thalassemia (122–124), blood donors (5), patients having multiple blood transfusions (124–126), and patients who have undergone organ transplantation (73, 127–131) are more likely to have TTV colonization.

Fecal-Oral Route

To examine patterns of anellovirus shedding into the circulation and the GI tract after new infection, 2 naïve chimpanzees were injected intravenously with bacteria-free (filtered) fecal supernatant or serum from human newborns with documented acute TTV infection (132). Serum and fecal specimens obtained weekly from experimentally infected chimpanzees were tested for TTV DNA by nested PCR. In the chimpanzee that received TTV-positive human serum, TTV DNA was detected in serum starting 5 weeks post-inoculation (PI) and remained positive until 15 weeks PI. In the chimpanzee that received fecal supernatant, TTV DNA was detected in serum samples 7–12 weeks PI and peaked at 14–16 weeks PI and continued to be positive for longer than 30 weeks. TTV DNA was detected in fecal specimens from the chimpanzee inoculated with TTV-positive human fecal supernatant after 16 weeks PI (coincident with high-titer TTV DNA in the serum). However, fecal specimens obtained at 24 weeks PI (when serum titers were low) were negative for TTV DNA.

Sexual Contact

Detection of TTV DNA in semen (54), and vaginal fluid (64, 133), suggests possible TTV transmission during sexual intercourse.

Transplacental Route

The published information on transplacental TTV transmission is inconsistent. In a prospective cohort study, paired maternal and cord bloods were examined for the presence of TTV DNA (69). Among 105 participants enrolled in the study, 37 mothers were TTV DNA-positive, and 7 cord blood samples from the 37 TTV-positive mothers were also TTV-positive. All cords from TTV-negative mothers were TTV-negative. In another study (134) TTV DNA was present in the blood of 57 of 138 mothers. Among the 57 TTV-infected mothers, 19 cord sera were positive for TTV DNA. A follow-up of 3 randomly selected infants with TTV sequences in their cord blood showed positivity persisting for 8 weeks after birth. The finding of TTV in the cord blood of between 1/5 and 1/3 of colonized mothers is consistent with transplacental passage of virus, however other routes are possible, as is contamination of the cord specimens by maternal blood.

A separate study analyzed plasma samples from 54 mothers and their newborns for TTV DNA (135). Though TTV-DNA was detected in 49 of 54 mothers, only 4 (8%) infants tested positive.

By contrast, another study analyzed TTV DNA in maternal and fetal cord blood collected postpartum from 100 mother-child pairs (44). TTV DNA was detected in 84% of maternal samples, while cord blood was devoid of TTV.

The sum of these findings call into question whether transplacental transmission of TTV occurs in human pregnancy.

Breast Feeding

Several studies provide evidence of anellovirus transmission by breast feeding (9, 134, 136). In a cohort study, blood was sampled from 300 normal pregnant people (60 of whom were TTV-positive). Twenty infants born to TTV-positive women in the cohort who delivered vaginally ($n = 10$) or by C/S ($n = 10$) were sampled at both 5 days and 3 months after birth. Half the infants in each group were also tested at 6 months after birth. Additionally, breast milk was collected from 30 TTV-positive nursing women (137). All infants from TTV-positive mothers were TTV-negative at both 5 days and 3 months after birth, regardless of delivery method, arguing against TTV transmission either transplacentally or during the birth process. By 6 months after birth, 4 of the 10 infants born to TTV-positive parents were TTV-positive. TTV DNA was detected in the breast milk of 7 of 30 TTV-positive patients.

An earlier study in Germany looked for TTV in 46 women who collectively birthed 47 children. Of this cohort, 22 maternal serum samples tested positive for TTV. Notably, TTV DNA was detected in 22 of 23 serum samples of 1-week-old infants who were born to TTV-positive parents. Twenty four TTV-negative individuals gave birth to 24 TTV-negative children who remained negative throughout the study period of 28 months. TTV DNA was detected in 77% of breast milk samples from TTV-positive patients and in none from TTV-negative individuals (45).

A prospective single-center study in Russia analyzed whole blood TTV load in 98 clinically healthy breastfeeding infants of 1–12 months of age to determine TTV dynamics during the first year of life (17). The findings revealed a significant increase in TTV copy number for the first 60 days, before plateauing after 6 months, with viral loads correlating with age.

In sum, these findings suggest that newborns can acquire TTV through breast milk, but acquisition from either parents or others via alternative routes was not ruled out. There is some evidence that among infants who are breast-fed, the prevalence of TTV positivity increases with prolonged lactation (136).

Horizontal Transmission Could Be the Major Route of Anellovirus Colonization in Infants

A study determined whether the predominant route of transmission of TTV in children is horizontal, vertical, or both, by testing infants born to TTV-positive mothers (138). Serum samples were obtained from 12 mothers on the day of delivery or within 1 month after delivery. Among 12 mothers, TTV DNA was detected in 10 (83%) cases. Serum samples were obtained from infants at 0.5–3 month intervals from 1 to 12 months of age. All infants, aside from 1 born by C/S, were delivered vaginally. The prevalence of TTV in infants born to TTV-positive and TTV-negative mothers were 9/10 (90%) and 0/2 (0%) respectively. Serum TTV DNA was not detected in any infant at 1 month of age but was detected for the first time at 1.5–8 months of age, and thereafter TTV positivity persisted throughout the follow-up period. Detection of TTV in 9/10 infants born to TTV-positive mothers and 0/2 infants born to TTV-negative mothers suggests that

TTV transmission from mothers to their infants postpartum is possible.

To confirm the transmission route, a homology search was performed in 7 randomly selected TTV-positive mother-infant pairs. Although only a few clones tested for each case were sequenced, the degree of homology varied considerably in most matched mother-infant pairs. One of the 7 mother-infant pairs showed a high degree of similarity for all TTV clones (98.7–100%), 2 pairs had 88–99% homology, and the remaining 4 showed 83.6–89% nucleotide identity. While these findings indicate that colonization with maternal TTV can occur, most acquired TTV is not identical to maternal strains.

These findings suggest a predominance of horizontal, rather than vertical transmission of TTV to infants, whether from their mothers or from other sources.

HUMAN ANELLOVIRUS COLONIZATION AND PREGNANCY-RELATED COMPLICATIONS

Although the bacterial microbiota of various compartments (e.g., vagina, amniotic fluid, and placenta) have been studied in pregnancy (139–143), there has been far less emphasis on the normal or pathological viral community (144, 145). Given the prevalence of anelloviruses in various tissues and body sites of healthy asymptomatic pregnant individuals, several studies have attempted to understand what impact, if any, TTV colonization has on pregnancy, labor, and birth.

Anellovirus Colonization May Have a Role in Determining the Timing of Parturition

Evidence suggests that overt maternal viral infection with influenza (146), hepatitis (147, 148), HIV (149), and herpes (150) can lead to preterm labor and delivery. Although the mechanisms underlying these associations are not clear, it has been suggested that maternal viral infection may predispose toward an exaggerated pro-inflammatory response to a secondary inflammatory stimulus (such as bacterial infection), leading to labor through a "double-hit" mechanism (151, 152). With this premise, we examined the association of virus colonization with a preterm "initiating event of labor" [either spontaneous labor with intact membranes or premature rupture of membranes in the absence of labor (PROM)] using a prospective case-control study (153). We hypothesized that patients experiencing a preterm initiating event of labor (< 37 weeks, "cases") would be more likely to harbor viruses than patients who enter labor at term ("controls"). An initial unbiased screen for viruses performed with next-generation sequencing in serum pooled from 8 cases identified 7 unique viral sequences, all TTVs. Subsequently, 72 patient samples were analyzed individually by nested and semi-nested PCR to identify other anellovirus subtypes. Among patients experiencing spontaneous labor, TTV and TTMV were significantly more prevalent in cases than controls, while TTMDV was not different between the 2 groups. Cases were more likely to harbor at least 1 member of the anellovirus family (91% vs. 68%). In the subgroup of subjects experiencing spontaneous labor with intact membranes, the incidence of TTV was significantly higher in preterm patients (23 of 24 cases) than in controls (8 of 13), whereas there was no difference in TTMDV and TTMV. There were no significant differences in viral subtypes in serum from patients with PROM.

These observations led us to hypothesize that anelloviruses may have a role in determining parturition timing. A potential mechanism for such a phenomenon is through modulation of the inflammatory and immune landscape (154), lowering the threshold for a labor response to stimuli, such as subclinical bacterial infection or non-infectious stimuli, that on their own would be insufficient to induce parturition. It is also possible that, due to the predilection of anelloviruses for leukocytes and the changes in leukocyte populations induced by labor, premature onset of the parturition process is a cause, rather than a consequence, of increased anellovirus recovery in these subjects. Given that the findings are qualitative and were made in a small group of subjects, confirmatory studies are needed.

Anellovirus May Associate With Other Maternal Microbiomes to Precipitate Preterm Birth

A nested case-control study analyzed the vaginal eukaryotic DNA virome and its associations with the bacterial vaginal community and preterm birth (155). Viral communities were analyzed according to diversity, dynamics over time, and association with bacterial community in vaginal swabs collected longitudinally from 60 subjects across pregnancy. Overall, 6 families of human DNA viruses were detected in vaginal samples from pregnant patients, including *Papillomaviridae, Polyomaviridae, Herpesviridae, Poxviridae, Adenoviridae,* and *Anelloviridae*. Anelloviruses were the most common viruses, detected in more than 40% of the patients. Viral richness diminished through the trimesters of pregnancy in subjects who had term delivery. Changes in vaginal virome diversity were similar to changes in the vaginal bacterial microbiome over pregnancy. The 24 pregnant subjects who delivered preterm showed higher viral richness compared to term birth patients. Although higher viral richness was significantly associated with both spontaneous and indicated preterm birth subtypes, no single virus or viral community was associated with preterm birth. Nonetheless, individuals who had both high bacterial diversity [as is seen in bacterial vaginosis, itself associated with preterm birth (156)] and high viral diversity early in pregnancy had the highest risk for preterm birth.

Evidence links the composition of the vaginal microbiome with immune status and variations in cervical length in pregnant people (157, 158). Specifically, when *Lactobacillus crispatus* is dominant, the vaginal level of D-lactic acid isomer is high, matrix metalloproteinase (MMP)-8 is low, and vaginal inflammation tends to be absent. Conversely, when *Lactobacillus iners* or bacteria other than lactobacilli are dominant, D-lactic acid levels are low, and MMP-8 levels are high, which is associated with a more pro-inflammatory vaginal environment and overall shorter cervical lengths (159). A recent cohort study of 121 pregnant subjects investigated TTV presence in vaginal secretions, and

how its occurrence and/or titer varies with the dominant bacteria in the vaginal microbiome (65). Vaginal secretions collected from pregnant individuals in their first trimester (≤12 weeks), third trimester (28–38 weeks), and 28–45 days postpartum were analyzed for TTV DNA by quantitative PCR. Approximately 40% of pregnant individuals who delivered a healthy baby at term had TTV detected in their vaginal secretions during at least 1 of these time points. In subjects who were tested at all time points ($n = 33$), those who were TTV-positive in the first trimester were equally likely to became negative or remain positive throughout the other sampling time points. These findings suggest that vaginal TTV colonization is most often associated with healthy gestation and normal outcomes. However, the correlation between vaginal TTV and features of bacterial vaginosis provides a mechanism by which anellovirus colonization may lead to preterm delivery. In the first trimester, *L. crispatus* was dominant in 66.7% of pregnant individuals who were negative for TTV, as opposed to 25% of those who were TTV-positive, and D-lactic acid levels were diminished in TTV-positive patients. Similarly, in the third trimester, *L. crispatus* was dominant in 50% of pregnant individuals who were TTV-negative and only 6% of those who were TTV-positive. In summary, vaginal TTV colonization appears to correlate with features of bacterial vaginosis (diminished predominance of *L. crispatus*, higher MMP-8, and lower D-lactic acid levels).

Adverse Pregnancy Outcomes May Not Be Associated With Anellovirus Presence or Quantity

A study determined the prevalence of viruses in matched maternal-infant preterm cohorts and ascertained whether viral presence or load correlates with histologic chorioamnionitis, spontaneous preterm labor, and preeclampsia (160). Preterm labor was defined as spontaneous preterm labor or preterm premature rupture of membranes that resulted in very premature delivery <31 weeks. Histological chorioamnionitis was determined by placental pathology, and preeclampsia was based on clinical diagnosis. Whole blood or plasma collected from 56 matched mothers and premature infants was analyzed for the presence and quantity of anellovirus and 8 other viruses by qPCR. Twenty-nine of the 56 maternal samples contained viral nucleic acid, of which anellovirus was most prevalent (26 samples). However, there was no association of presence or quantity of viral load in samples from mothers with or without preeclampsia, histological chorioamnionitis, or preterm labor. Taken together, this study suggests no clear relationship between TTV load and perinatal morbidity or spontaneous preterm labor, though its small size and focus only on extreme prematurity are limitations that require validation.

A MECHANISM BY WHICH ANELLOVIRUS COLONIZATION COULD INFLUENCE THE TIMING OF PARTURITION

The link between infection and preterm labor has long been recognized. In some instances, this may entail the initial presence of microorganisms (whether bacterial, viral, or fungal) which creates a favorable environment or amplifies the effect of a secondary infection. As noted above, experimental models illustrate the potential for synergy between viral and bacterial infections leading to amplification of host responses. Polyinosinic:cytidylic acid [poly(I:C)] is a TLR3 ligand and synthetic analog of double-stranded RNA, which is a replication intermediate for most viruses, including DNA viruses. Poly(I:C) induces preterm delivery when injected either into the uterus (152) or systemically (161) in mid- to late gestation and greatly amplifies the potency of bacterial products in mice when injected into the uterus (162). In a mouse model, it has been demonstrated that viral infection of the cervix during pregnancy reduces the capacity of the female reproductive tract to prevent bacterial infection of the uterus (163). Similarly, sub-clinical viral infection in pregnant mice has been shown to sensitize them to bacterial infection, leading to preterm delivery (151). These findings suggest the existence of synergism during combined viral and bacterial infection. This "2 hit" trigger and existence of synergism might be a beneficial strategy to a host, as it would blunt the maternal response to mild insults (such as subclinical infection), while providing for rapid and efficient amplification of the labor response in cases of a superimposed or more severe infection. Given the higher prevalence of circulating anellovirus in preterm than in term patients (153), and TTV's association with other bacterial communities linked to preterm birth (65, 155), we propose that anellovirus colonization during gestation might affect the onset of labor through lowering the threshold for a response to stimuli, such as subclinical bacterial infection, that on their own would be insufficient to induce parturition.

On the other hand, pregnant patients who have a normal term pregnancy and give birth to a healthy infant may harbor viral sequences or genogroups that protect against preterm labor. Functional studies have revealed that apathogenic, endogenous retroviruses (ERV), and ERV-derived proteins found in the placenta mediate cell-cell fusion, suppress maternal immunity, and protect the fetus from exogenous viruses (164). Given the evidence that TTV ORF2 protein suppresses NF-κB pathways and inhibits transcription of proinflammatory cytokine genes (86), it is possible that TTVs may act as "little helpers" in shaping the gene networks of innate and adaptive immune responses to maintain normal pregnancy.

In the majority of human body sites, microbial diversity is considered a signature of health (1). If multiple variants or genotypes of anellovirus ("anellome") found in healthy humans remain stable for a long time, they may make up the personalized and healthy part of the host microbiome (92). The gene products of anellovirus might help to maintain the composition and fitness of other (beneficial) microbial communities by preventing colonization by pathogens. At the same time, the host immune system, through immunosurveillance, may maintain a safe balance, thus protecting the body from the pathogenic effects of the virus (165). However, as noted above, microbial diversity (including anellovirus) in the pregnant vagina is associated with premature timing

of delivery. In summary, under physiological conditions, human anellovirus is unlikely to be pathogenic *per se*. Nonetheless, perturbations in host defense and microbial composition may allow anellovirus to achieve an opportunistic pathogen status.

FUTURE DIRECTIONS

At present, the quality and number of studies on the association of anelloviruses with pregnancy outcomes are limited. Large cohort studies are important to clarify the role, if any, of anellovirus colonization in the timing of labor. Investigations are warranted with a focus on determining the kinetics of anellovirus colonization over the course of pregnancy, and whether certain genogroups promote or suppress preterm birth. Studying anellovirus abundance in other conditions associated with pregnancy, such as miscarriage, preeclampsia, and gestational diabetes, will provide more detailed insight in the relationship between anellovirus colonization and clinical outcomes.

CONCLUSIONS

The impact of anelloviruses on human health remains incompletely characterized. Although the possible pathogenicity of anelloviruses is still an open question, further study of anellovirus colonization during pregnancy and in mother-infant pairs will help determine whether and how these ubiquitous viruses affect microbial infection-associated preterm labor and preterm birth.

AUTHOR CONTRIBUTIONS

CK, MS, and EH contributed to the literature review and composition of the present text. All authors reviewed and approved the final version of the manuscript.

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High Dose Vardenafil Blunts the Hypertensive Effects of Toll-Like Receptor 3 Activation During Pregnancy

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The maternal innate immune system plays a central role in preeclampsia (PE). Toll-like receptors (TLRs) are innate immune system receptors that recognize characteristics of extracellular endogenous ligands or pathogens, and their activation leads to a pro-inflammatory immune response. We and others have reported that excessive activation of TLRs causes pregnancy-dependent hypertension in animals and is associated with PE in women. Activation of TLR3 by poly I:C mimics the innate immune system activation by viruses that women who develop PE encounter during pregnancy. Vardenafil was approved by the FDA for erectile dysfunction but has recently been examined as a potential PE medication due to studies done with a similar drug, sildenafil. Preclinical as well as recent clinical studies demonstrate the potential effectiveness of sildenafil for PE. However, vardenafil is more potent than sildenafil and acts by increasing expression of placental growth factor in addition to increasing cGMP levels. We hypothesized that vardenafil will be more potent and effective in reducing the negative health effects in a mouse model of virus-induced PE. Pregnant mice were injected with the TLR3 agonist poly I:C (PPIC) on gestational days 13, 15, and 17. We treated PPIC mice with a high dose of vardenafil (50 mg human equivalent), a lower dose of vardenafil (20 mg human equivalent), or sildenafil (50 mg human equivalent) on gestational days 15–17 after hypertension was established. Daily i.p. injections of either high dose or low dose vardenafil significantly decreased systolic blood pressure in PPIC mice whereas sildenafil had no effect. There were no differences in body weight between the groups. The splenomegaly induced in PPIC mice was ameliorated in high dose vardenafil-treated PPIC mice, while low dose vardenafil-treated and sildenafil-treated PPIC mice still exhibited splenomegaly. High dose vardenafil-treated PPIC mice also did not exhibit any fetal demise characteristic of PPIC mice, while low dose vardenafil-treated and sildenafil-treated PPIC mice still had significantly increased incidences of fetal demise. These data support the notion that high dose vardenafil may be safe and effective at reducing blood pressure during a virus-associated hypertensive pregnancy.

Keywords: vardenafil, immunity, virus, pregnancy, hypertension

INTRODUCTION

Hypertensive disorders of pregnancy, including preeclampsia (PE), are high-risk conditions diagnosed in the latter stage of pregnancies (1, 2). Due to the limited availability of effective therapeutic options, PE often leads to a high rate of fetal, neonatal, and maternal morbidity and mortality (2, 3). The underlying pathophysiology of PE is poorly understood and a continued search for novel and effective drugs to manage the condition is needed (4, 5). PE is multifactorial in its pathophysiology, but is widely associated with reduced placental perfusion, immune system activation, and systemic vascular endothelial dysfunction (6). The resultant placental ischemia induces the renin-angiotensin-aldosterone system (RAAS), inflammation, and oxidative stress that may propagate PE. With respect to immune system activation, we and others have reported viral activation of the maternal immune system during pregnancy is associated with PE in women and induces a PE-like syndrome in rodents (7–11). Given the recent rise in viral pandemics, therapies to aid in managing PE associated with a viral infection are needed.

In the clinic a variety of therapeutic agents have been used to acutely lower blood pressure in PE including calcium channel blockers, methyldopa, diazoxide, hydralazine, prostacyclin, prazosin, and isosorbide (5). In recent years oral extended release nifedipine, oral labetalol, and methyldopa are the generally accepted first-line agents for non-severe hypertension in women with PE. Beta-blockers and diuretics are acceptable, while RAAS inhibitors remain contraindicated (5). However, these agents have their own side effect profile and have been shown to be relatively ineffective (12). Thus, the clinical management of PE is still a challenge and needs effective treatment options (13). One of the quickest ways to develop an effective drug for the treatment of PE is to identify new effects of the drugs that are already approved for other indications and have been found clinically safe (14).

Vardenafil is a phosphodiesterase 5 (PDE5) inhibitor that is closely related in function to sildenafil, a PDE5 inhibitor commonly used to treat male erectile dysfunction and pulmonary vascular diseases (15–17). During pregnancy, nitric oxide (NO) is synthesized in *in utero* placental tissues and endothelial cells and activates the intracellular second messenger cyclic guanosine monophosphate (cGMP). cGMP causes vasodilation and thus maintains low vascular resistance in the uterus and fetoplacental circulations (12, 18). However, cGMP is metabolized by PDE5 and leads to vasoconstriction and may contribute to the development and maintenance of PE. Thus,

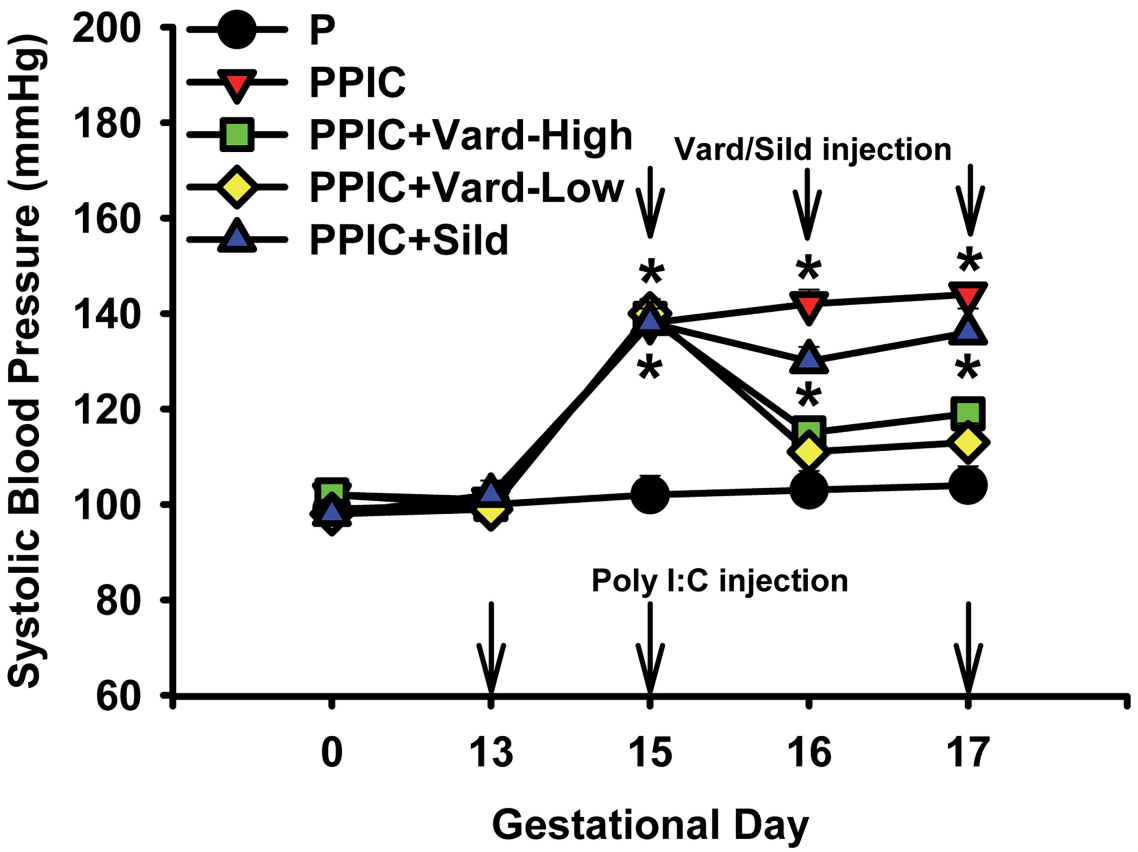

FIGURE 1 | Systemic blood pressures of control pregnant (P) mice ($n = 10$), P mice treated with the TLR3 agonist and viral mimetic poly I:C (PPIC) ($n = 10$), PPIC mice treated with a high dose ($n = 6$) and low dose ($n = 6$) of vardenafil (Vard), and PPIC mice treated with sildenafil (Sild) ($n = 6$). *$p < 0.05$ by ANOVA compared to P mice.

inhibition of PDE5 may enhance the vasodilatory effect of NO by preventing cGMP degradation (12). It has been demonstrated that PE is, in part, related to decreased NO-mediated vasodilation of the uterine circulation (18). Earlier studies demonstrated that sildenafil treated PE in mouse models (19–21). However, in clinical studies, it was reported to be relatively ineffective as a treatment for PE, although it was shown that it could be used safely during pregnancy (22). Since vardenafil is a potent PDE5 inhibitor and available clinically as a safe and effective drug (17, 23, 24), we hypothesized that vardenafil will decrease blood pressure and improve fetal outcomes in a mouse model of PE induced by a viral mimetic.

MATERIALS AND METHODS

Mice

Male and female C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME). All animal use protocols were approved by the Texas A&M University Institutional Animal Care and Use Committee and were performed in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals.

Viral Mimetic Induced Hypertension (PPIC), Treatments, and Measures

Pregnant (P) mice were injected i.p. with the TLR3 agonist and viral mimetic poly I:C 20 mg/kg body weight (PPIC) on gestational days 13, 15, and 17 as described previously (8–10, 25–30). These PPIC mice develop hypertension by day 14, which remains when poly I:C is injected every other day until parturition. We treated PPIC mice with a high dose of vardenafil (50 mg human equivalent), a low dose of vardenafil (20 mg human equivalent), or sildenafil (50 mg human equivalent) on gestational days 15–17 after hypertension was established. All PDE5 inhibitors were injected i.p. Mice had free access to normal chow (Teklad 8604, with NaCl content roughly 0.5%) and drinking water. Systolic blood pressure was determined using the tail-cuff method after acclimatization and training

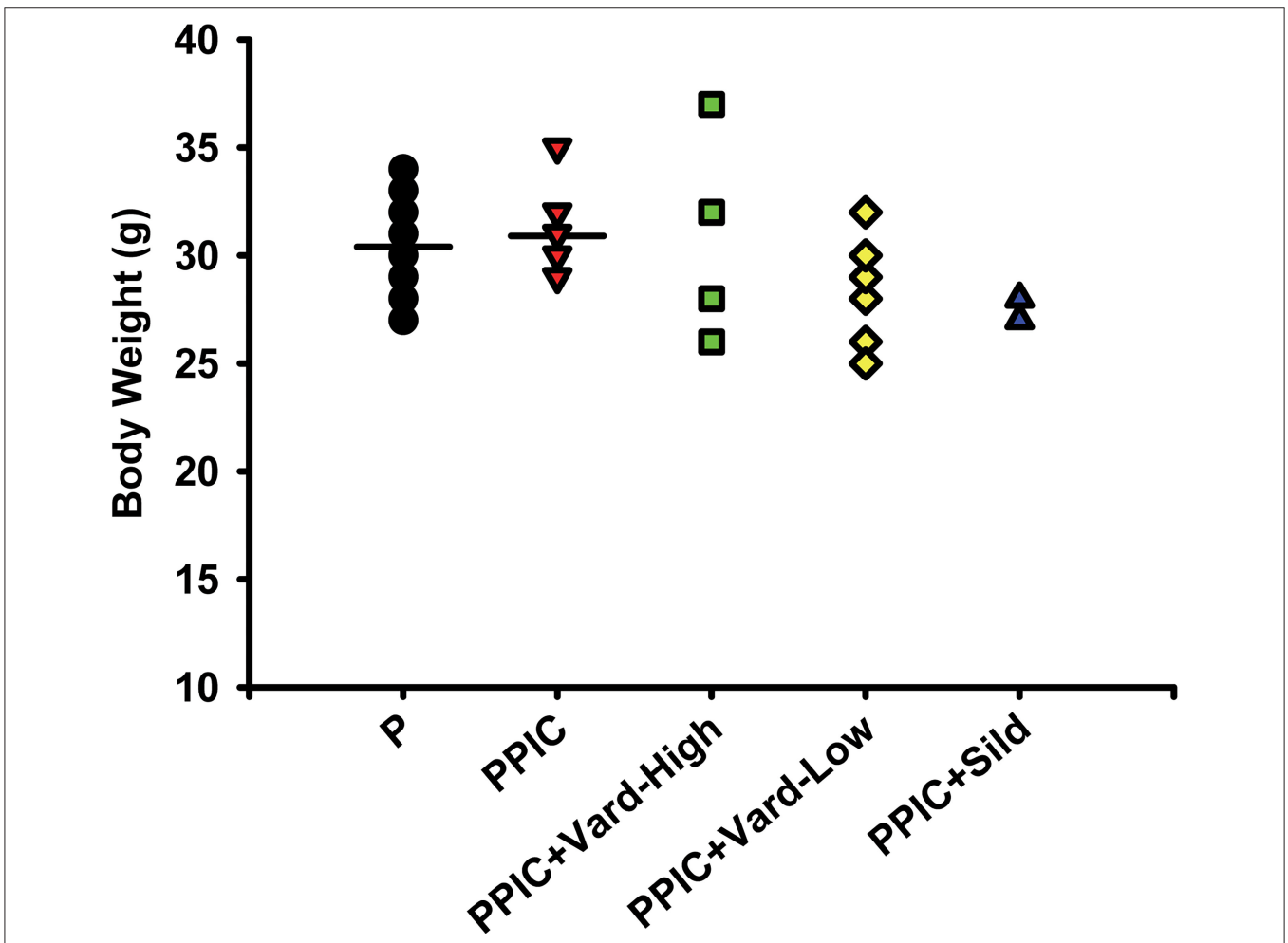

FIGURE 2 | Body weight measures of control pregnant (P) mice ($n = 10$), P mice treated with the TLR3 agonist and viral mimetic poly I:C (PPIC) ($n = 10$), PPIC mice treated with a high dose ($n = 6$) and low dose ($n = 6$) of vardenafil (Vard), and PPIC mice treated with sildenafil (Sild) ($n = 6$).

as described previously (31). All mice were anesthetized and euthanized on gestational day 18 and body weight, spleen weight, and the number of fetuses and morphology were counted and noted. The P and PPIC groups had 10 mice in each, while the PPIC groups treated with vardenafil or sildenafil had six mice in each group.

Statistics

A one-way ANOVA followed by a Student–Newman–Keuls *post-hoc* test was used to compare groups and the significance level was set at $p < 0.05$ compared to P.

RESULTS

Daily i.p. injections of either high dose ($n = 6$) or low dose ($n = 6$) vardenafil significantly decreased systemic blood pressure in PPIC ($n = 10$) mice to P ($n = 10$) levels, whereas sildenafil ($n = 6$) had no effect (**Figure 1**). There were no differences in body weight between the groups (**Figure 2**). The splenomegaly induced in PPIC mice was ameliorated in high dose vardenafil-treated PPIC mice, while low dose vardenafil-treated and sildenafil-treated PPIC mice still exhibited splenomegaly (**Figure 3**). High dose vardenafil-treated PPIC mice also did not exhibit any fetal demise characteristic of PPIC mice, while low dose vardenafil-treated and sildenafil-treated PPIC mice still had significantly increased incidences of fetal demise (**Figure 4**). The fetal demise incidence in sildenafil-treated PPIC mice was significantly increased compared to that of PPIC mice (**Figure 4**).

DISCUSSION

In the current study, like our previous reports (7–11), we confirm that administration of a viral mimetic during pregnancy induces hypertension, immune system activation as measured

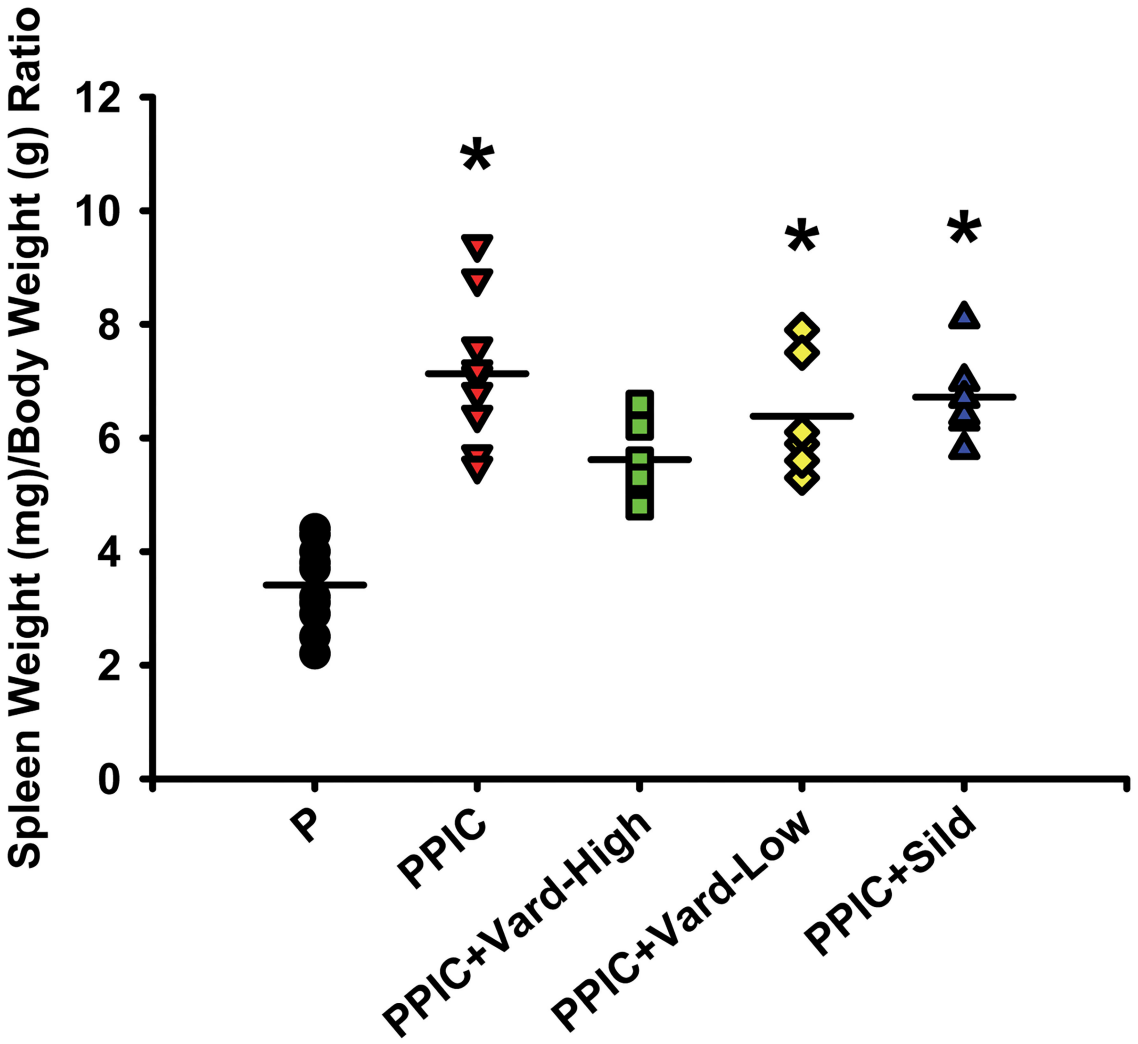

FIGURE 3 | Spleen weight to body weight ratios of control pregnant (P) mice ($n = 10$), P mice treated with the TLR3 agonist and viral mimetic poly I:C (PPIC) ($n = 10$), PPIC mice treated with a high dose ($n = 6$) and low dose ($n = 6$) of vardenafil (Vard), and PPIC mice treated with sildenafil (Sild) ($n = 6$). *$p < 0.05$ by ANOVA compared to P mice.

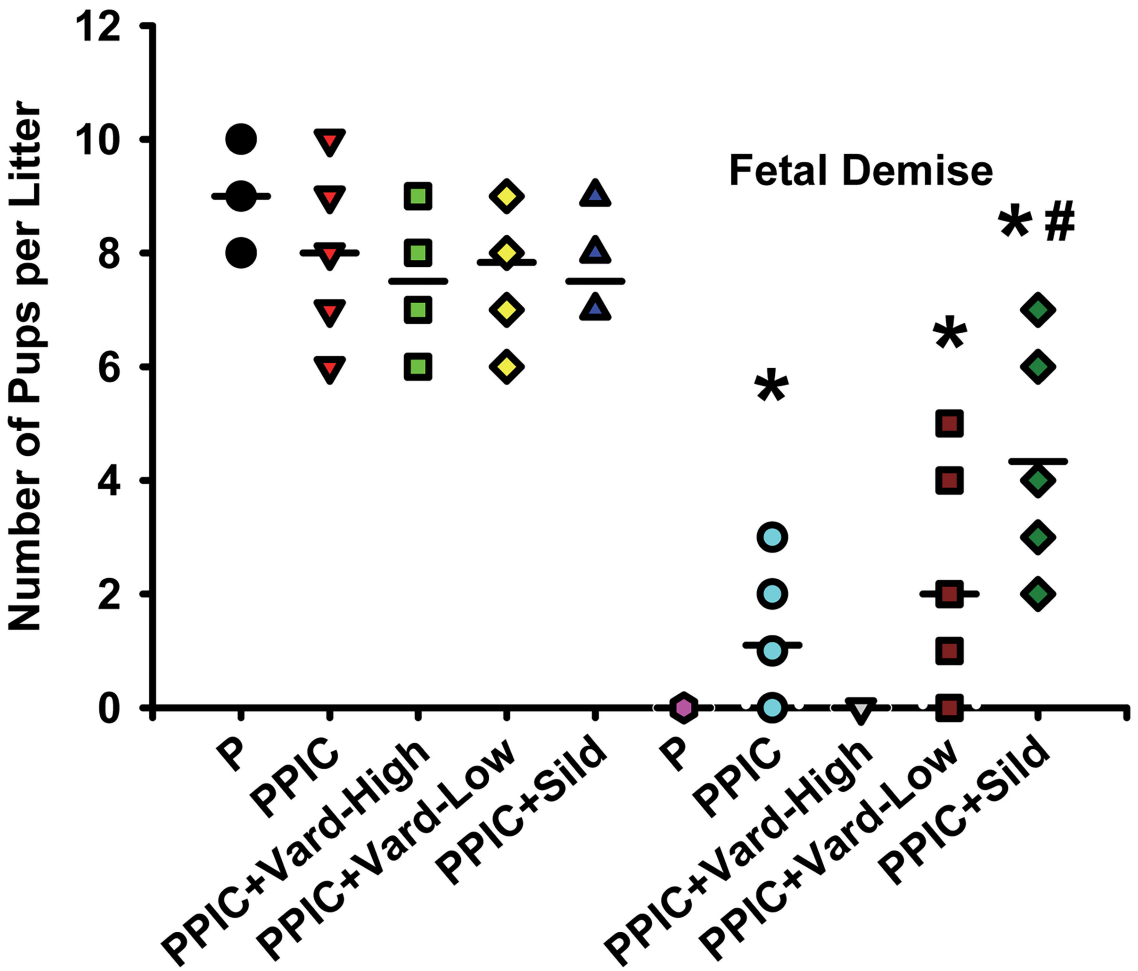

FIGURE 4 | Number of pups per litter as well as number of pups undergoing fetal demise per litter of control pregnant (P) mice ($n = 10$), P mice treated with the TLR3 agonist and viral mimetic poly I:C (PPIC) ($n = 10$), PPIC mice treated with a high dose ($n = 6$) and low dose ($n = 6$) of vardenafil (Vard), and PPIC mice treated with sildenafil (Sild) ($n = 6$). $^{*}p < 0.05$ by ANOVA compared to P mice, $^{\#}p < 0.05$ by ANOVA compared to PPIC mice.

by splenomegaly, and fetal demise in mice. These results are consistent with PE in women of which there are known associations with various viruses, and these are associated with hypertension, immune system activation, and intrauterine growth restriction. We also report that treatment with a high dose of vardenafil, but not sildenafil, was able to attenuate these negative effects in pregnant mice.

A PubMed search of "vardenafil preeclampsia" only reveals 2 papers. In 2011, Karasu et al. (32) examined vardenafil and sildenafil effects on relaxation responses of human umbilical arteries taken from women with PE and healthy pregnant women. They reported that in all sets of experiments, including the absence and presence of various NO pathway inhibitors, vardenafil induced a maximal relaxation response while sildenafil did not. These results suggest vardenafil acts through NO/cGMP dependent and independent mechanisms. The other paper reported in 2015 by Kakigano and colleagues found that vardenafil markedly increased placental growth factor through various endothelial cell screens and suggested that vardenafil may be useful to treat PE given its pro-angiogenic and vasodilator properties (1).

A number of previous reports demonstrate beneficial effects of the PDE5 inhibitors sildenafil and tadalafil in isolated vessels from women with PE and animal models of PE (32–35). Others have also reported beneficial blood pressure and/or fetal effects of sildenafil in pregnant hypertensive animals (21, 36–42). However, these promising pre-clinical data have not translated smoothly into clinical trials (19, 43). Earlier studies reported no beneficial effects of sildenafil treatment on pregnancy duration, isolated vessels, or fetal outcomes (22, 44, 45). The more recent STRIDER (Sildenafil TheRapy In Dismal prognosis Early-onset fetal growth Restriction) clinical trial not only reported no beneficial effect of sildenafil on pregnancy duration but that treatment increased the risk of neonatal pulmonary hypertension and mortality and was subsequently ended (46–48). While the negative effect of sildenafil on neonatal pulmonary pressure has not been directly

determined, it is possible that too much NO at certain time points of gestation may be harmful, similar to that found with high doses of antioxidants during pregnancy. Together, these would support the notion that a proper NO-ROS balance is needed for a healthy fetal outcome.

The significantly increased incidence of fetal demise in our sildenafil-treated PPIC mice supports these findings. Additionally, our results in sildenafil-treated PPIC mice showing no beneficial effects on fetal demise, blood pressure, or splenomegaly contrast those in other animal models of PE such as the COMT –/– mouse, Dahl salt-sensitive rat, L-NAME-treated mice, sFlt-1-treated mice, and others (19–21, 39, 42, 49). This may suggest that viral infection during pregnancy resulting in a PE-like syndrome does not affect the NO pathways that sildenafil targets, but rather that vardenafil improves both NO and placental growth factor and together these contribute to the beneficial effects.

Cellular calcium handling may also play a role in the detrimental effects of a viral infection during a hypertensive pregnancy, as well as the beneficial effects of vardenafil but not sildenafil. Viral infections have been reported to alter calcium dynamics (50). Vardenafil, but not sildenafil, was reported to block caclium channels resulting in vasorelaxation (51, 52). This mechanism may also contribute to the decreased blood pressure and improved fetal development.

Taken together, the beneficial maternal and fetal effects of vardenafil treatment during a virus-induced hypertensive pregnancy in mice may overcome the once promising effects of sildenafil for treating PE that turned out to be somewhat harmful. In pregnancies complicated by viral infection, vardenafil therapy may be beneficial in decreasing blood pressure, dampening immune system activation, and improving fetal outcomes.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Texas A&M University IACUC.

AUTHOR CONTRIBUTIONS

MK, ZR, and BM: study concept, design, and obtained funding. DB and SD: acquisition, analysis, and interpretation of data. DB, MT, ZR, and BM: drafting of the manuscript. All authors carried out critical revision of the manuscript for important intellectual content, read, and approved the final manuscript.

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Placental Macrophages Following Maternal SARS-CoV-2 Infection in Relation to Placental Pathology

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Introduction: In December 2019, a novel coronavirus, SARS-CoV-2, was identified. Whilst pregnant women appear to be at risk of severe infection, pre-term birth, and stillbirth, it is unclear whether placental dysfunction is a consistent feature of maternal SARS-CoV-2 infection during pregnancy. We aim to describe the immune response in placentas of women who had COVID-19 infection during pregnancy and investigate whether there are any associated morphological changes.

Methods: The placentas of women testing positive for COVID-19 during their pregnancy were compared to contemporaneous controls who were not known to have had COVID-19 during pregnancy. Samples of each placenta were sent for histopathological analysis or underwent immunohistochemical staining for CD163, CD20, CD3, CD31, and SARS-CoV-2 spike protein. A subset of samples were sent for transmission electron microscopy.

Results: There was a significant increase in the number of $CD163^+$ macrophages in the Post COVID group ($p = 0.0020$). There was no difference in the percentage of $CD3^+$, $CD20^+$ cells, but there was an increase in placental vascularity in the Post COVID group compared to controls ($p = 0.026$).

There were no structural differences observed between the samples sent for EM analysis. However, one of the placentas from the Post COVID group was seen to have several large sub-apical vacuoles in the syncytiotrophoblast. We did not observe any virions within the vacuoles and SARS-CoV-2 spike protein staining was negative for the sample. Histopathological investigations indicated that there was no specific placental pathology caused by maternal COVID-19 infection in this cohort of samples.

Conclusions: This study did not confirm previous studies which describe a possible increase in cases of both maternal and fetal vascular malperfusion, and placentitis in women who had COVID-19, which were seen in association with adverse pregnancy outcomes. It remains unclear whether observed abnormalities are caused by maternal infection, or whether maternal infection exacerbates existing placental pathology; understanding why some placentas generate these abnormalities is a key goal.

Keywords: COVID-19, SARS-CoV-2, placenta, histopathology, macrophages

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1. INTRODUCTION

SARS-CoV-2 was first detected in Wuhan, China, in December 2019. The resulting disease COVID-19, became a worldwide pandemic, with nearly 200 million people catching the virus which to date has claimed the lives of over four million people (1). A systematic review of international studies suggests that SARS-CoV-2 infection during pregnancy is associated with an increased risk of maternal and fetal deaths (2); data regarding spontaneous pre-term births are equivocal.

Although currently there appears to be no specific placental pathology associated with maternal SARS-CoV-2 infection (3), several cases of placentitis have been described in association with stillbirth, suggesting that placental inflammation may occur after SARS-CoV-2 infection and could be associated with fetal death. There is a paucity of research investigating the presence of macrophages, and other immune cells in the placenta following infection. Whilst macrophages form part of the innate immune system, T and B lymphocytes form the adaptive immune system which can tailor the immune response to the pathogen (4). Placental macrophages, otherwise known as Hofbauer cells, reside in the stromal core of the placental villi (5). In other infectious diseases, such as ZIKA virus, placental macrophages are thought to be a reservoir for viral replication (6).

There are conflicting data available as to whether the number of Hofbauer cells are increased following maternal SARS-CoV-2 infection. Whilst some studies have found no increase in Hofbauer cell number (7, 8), other studies report an increase (9). There are limited data regarding the effect of SARS-CoV-2 infection on T or B lymphocyte numbers, although one study reported no difference following maternal infection (8).

As there are still no conclusive data as to whether maternal SARS-CoV-2 infections leads to placental pathology, we aimed to investigate if there was an increase in the number of immune cells (macrophages, T and B lymphocytes) and an altered placental morphology in the placentas of women who had tested positive for SARS-CoV-2 during their pregnancy, or those who tested positive shortly before or at delivery, compared to healthy controls. We also performed histopathological analyses on the placentas, to identify if any pathologies were more likely to be present following maternal SARS-CoV-2 infection compared to controls.

2. MATERIALS AND METHODS

Placentas were collected from women who tested positive for COVID-19 <12 days prior to delivery or at delivery (Active COVID-19) and women who had tested positive >12 days prior to delivery (Post COVID-19). Women who to their knowledge had never had COVID-19 infection during their pregnancy and who had a negative PCR test on admission to hospital for delivery as per hospital policy were included in the control group. Women in the Active and Post COVID-19 groups had PCR tests following symptom onset, or had a positive antibody test. None of the women included in the study had severe symptoms requiring admission to hospital or enhanced care. Due to the low availability of samples, participants were included in spite of any maternal pregnancy complications or if the fetus was small or large for gestational age. Written, informed consent was given by all participants (18/NW/0451).

2.1. Immunohistochemistry

Sections of formalin fixed, paraffin-embedded placenta and umbilical cord were immunostained with the following antibodies: CD163 for macrophages (BioRad, 1mg/mL at 1:100), CD3 for T lymphocytes (Dako, 138mg/L at 1:100) CD20 for B lymphocytes (Invitrogen, concentration not determined at 1:200) or CD31 for endothelial cells (Dako, 201mg/mL at 1:50). Tissue sections were also stained with haematoxylin and eosin (H&E) to allow for structural analysis. Immunostaining for the SARS-CoV-2 spike protein (Stratech (1:1000)) was performed at the Department of Pathology, Alder Hay Hospital, Liverpool on a Dako automatic platform following their standard histopathology staining procedure. A placenta previously identified as testing positive for SARS-CoV-2 was used as a positive control sample for every staining run.

Slides were imaged using Pannoramic 250 slide scanner (3D HISTECH, Bioimaging Facility, University of Manchester). Positive staining was analyzed using QuPath (version 0.2.3) as previously described (10). Briefly, CD163 analysis was performed using the positive cell detection function in QuPath, areas of quantifiable tissue were highlighted and thresholds were set to detect positive DAB staining compared to haematoxylin staining. Identification of T and B lymphocytes was performed by selection of 10 random 1 x 1 mm regions of tissue, manually counting the number of lymphocytes and automatic detection of haematoxylin-stained nuclei. Vascularity was determined by calculating the area of CD31 positive DAB staining as a percentage of tissue area. Placental macrophages ($CD163^+$), T ($CD3^+$), and B ($CD20^+$) lymphocytes were expressed as a percentage of number of nuclei. Quantification of staining was performed excluding any areas of folded or poor quality tissue and a minimum of two tissue sections per tissue per immunostain were analyzed.

Histopathological investigations were performed by a consultant perinatal pathologist (GB) who was blinded to the maternal COVID-19 status. Histopathological description and diagnosis of the placentas was made following the Amsterdam Placental Workshop Group Consensus Statement (11).

2.2. Transmission Electron Microscopy

Approximately 2 mm^2 sized pieces of placental villous tissue were fixed in 4% paraformaldehyde (PFA) and 2.5% gluteraldehyde in 0.1 M HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) for 24 h. The samples were then transferred to 0.5% PFA until they were processed by the staff in the EM Core Facility in the Faculty of Biology, Medicine and Health, University of Manchester. Semi-thin sections were produced by CJPJ.

Two samples from each group were chosen for transmission electron microscopy (TEM), due to their percentage of $CD163^+$ cells being close to the median for their group. Samples were analyzed on an FEI Tecnai12 BioTwin transmission electron microscope (TEM).

2.3. Statistics

Statistical analyses were performed in Graphpad Prism (Version 8, Jolla, CA, USA). Mann-Whitney U or Kruskal-Wallis, with Dunn's *post hoc*, or Fischer's exact tests were performed as appropriate. The threshold of statistical significance was set at $p < 0.05$.

3. RESULTS

3.1. Participant Demographics

3.1.1. Maternal Data

The demographic characteristics of the women included in the study were similar between the groups (**Table 1**). The parity of the women in the control group was significantly higher than that of the women in the Active and Post COVID groups ($p = 0.03$ and 0.007, respectively) than controls. In the control group, approximately two thirds of women were Caucasian, compared to 55% in the Post COVID group and 38% in the Active COVID group.

Due to the poor availability of samples and comparatively low rates of COVID-19 infection in pregnant women (who were advised to shield in the lockdown periods), women were included in the study regardless of any co-morbidities. In the Active COVID group, one woman had pre-existing diabetes and chronic hypertension, two women were Rhesus negative, one having additional gestational diabetes. In the Post COVID group, one woman had hyperthyroidism and a second woman had recurrent reduced fetal movements (RFM). One woman in the control group also had recurrent RFM.

The mean gestation at maternal infection was 271 and 202 days for Active COVID and Post COVID pregnancies ($p > 0.99$ and $p < 0.0001$, respectively, compared to controls), although the exact date of a positive COVID test was only known for 18 Post COVID women. The median interval between a positive COVID-19 test result and delivery was 2.5 days (range 0–12 days) in the Active COVID group ($p = 0.0011$) and 76 days (range 19–211 days) in the Post COVID group ($p < 0.0001$).

There were some differences in the method of delivery with 50% of women being delivered by Cesarean section in the Active COVID group, compared to 55% in the Post COVID group and 81% of the control group.

When the notes of one participant in the control group were reviewed to obtain missing data, a positive COVID-19 test the day after delivery was found, with an inconclusive test on the

TABLE 1 | Participant demographics for Active COVID, Post COVID, and control groups.

Demographics	Active COVID	Post COVID	Controls	Active COVID vs. Controls	Post COVID vs. Controls
Number	8	20	16		
Gestation (days) median (range)	274 (250-287)	274 (257-287)	273 (255-285)	n/s	n/s
Gestation at COVID-19 test (days) median (range)	271 (245-282)*	202 (63-254)**	270.5 (252-285)	n/s	<0.0001
Maternal age (yr) median (range)	29 (21-45)	30 (24-41)	34.5 (25-40)	n/s	n/s
Maternal BMI (Kg/m^2) median (range)	25 (22.4-39.0)	27 (19-42)	27.5 (18-43)	n/s	n/s
Parity	0.5 (0-2)	0.5 (0-4)	2 (0-8)	0.0325	0.0065
Ethnicity					
Caucasian	3	12	10	n/s	n/s
Non-Caucasian	5	8	6		
Birthweight (g) median (range)	3240 (2882-4100)	3237 (2664-4466)	3384 (2156-4250)	n/s	n/s
IBC median (range)	58.6 (18.5-89.8)	39.85 (5.6-99.8)	44.85 (5.1-96.4)	n/s	n/s
Smoking status					
Smokers *n* (%)	1 (12.5)	1 (5)	2 (12.5)	n/s	n/s
Sex *n* (%)					
Male	4 (50)	12 (60)	8 (50)	n/s	n/s
Female	4 (50)	8 (40)	8 (50)		
Mode of delivery *n* (%)					
VD	4 (50)	9 (45)	3 (19)	n/s	n/s
C/S	4 (50)	11 (55)	13 (81)		

*Statistics: Kruskal-Wallis or Fisher's exact test. Significance level $p < 0.05$. IBC = individualized birthweight centile, VD = vaginal delivery, C/S = Cesarean section. *one participant tested positive for COVID-19 one day post delivery, value recorded as 0 for purposes of analyses. **two women self-reported a positive test result, exact date of test is unknown.*

day of delivery. Therefore, this participant was moved to the Active COVID group, and the positive test was recorded as date of delivery for the purposes of analyses.

3.1.2. Neonatal Data

A summary of neonatal data can be found in **Table 1**. All infants were live-born, with 50% of infants being male in the Active COVID group, compared to 60% in the Post COVID and 50% in the healthy control groups. The median gestational age at delivery of infants from the Active COVID group was 274 days, whilst in the Post COVID and normal pregnancies, median gestational age were 274 and 273 days, respectively. There was no significant difference in the median birth weight of the infants between groups (Active COVID 3240g, Post COVID 3237g, control group 3384g).

No infants were born $\leq 10^{th}$ or $\geq 90^{th}$ individualized birthweight centile (IBC) in the Active COVID group. In contrast, four infants were born $\leq 10^{th}$ IBC in the Post COVID group, and five were born $\geq 90^{th}$, whilst two infants were born $\geq 90^{th}$ IBC and two $\leq 10^{th}$ IBC in the healthy control group. No infants in any group were born $\leq 5^{th}$ IBC, and, therefore, classed as having fetal growth restriction (FGR).

3.2. Histopathology Reports

Histopathology reports were obtained for 41 placentas. A summary of results can be found in **Table 2**. Full results can be found in **Supplementary Table 1**.

The most common observations were excessive syncytial knots, distal villous hypoplasia and villous immaturity. Villous dysmaturity was identified in one of the Active COVID placentas, three Post COVID placentas, and two of the negative control group placentas. One placenta from the Post COVID group had inflammation and one had infarcts. One of the negative controls had sub-chorionic chronic histiocytic inter-villositis and one Active COVID placenta had a chorangiosis-like appearance. There were no significant differences in the presence of histopathological lesions in placentas from the Active of Post COVID groups compared to healthy negative controls.

TABLE 2 | Summary of histopathology findings in placentas from Active COVID, Post COVID, and healthy control groups.

Pathology	Active COVID	Post COVID	Healthy control	*p* value
Excessive syncytial knots n (%)	5 (71)	10 (56)	8 (50)	0.63
Distal villous hypoplasia n (%)	4 (57)	8 (44)	3 (19)	0.14
Villous immaturity n (%)	2 (29)	6 (33)	6 (38)	0.91
Villous dysmaturity n (%)	1 (14)	3 (17)	2 (13)	0.94
Chorangiosis-like appearance n (%)	1 (14)	0	0	0.083
Sub-chorionic chronic histiocytic intervillositis n(%)	0	0	1 (6)	0.45
Calcifications n (%)	0	2 (11)	0	0.26
Inflammation n (%)	0	1 (6)	0	0.52

Summary of histopathological lesions identified in placentas from the Active COVID (n = 7), Post COVID (n = 18), and healthy controls (n = 16). Two Post COVID placentas were not available for analysis. Chi-squared test, $p < 0.05$ considered statistically significant.

3.3. Placental Histochemical Analysis

3.3.1. Immune Cells

Analysis of the percentage of intra-villous $CD163^+$ Hofbauer cells found that there was a significant increase in the placentas from mothers who had tested positive for SARS-CoV-2 during their pregnancy (Post COVID) compared to controls ($p = 0.002$) (**Figure 1A**). There was no difference between the fetal sex within each group (data not shown). Representative images can be seen in **Figure 1D**.

Analysis of the percentage of both $CD3^+$ and $CD20^+$ lymphocytes found that there was no difference between the placentas of women who had COVID-19 and those who had not (**Figures 1B,C**). There was no difference when the groups were split by fetal sex (data not shown). Representative images can be seen in **Figures 1E,F**.

3.3.2. Vascularity

There was a statistically significant increase in the percentage area of $CD31^+$ endothelial cells in the placentas of the Post COVID group compared to controls ($p = 0.026$). There was no difference observed in the Active COVID group compared to the control group ($p = 0.06$) (**Figure 2**).

3.3.3. SARS-CoV-2 Spike Protein Staining

Forty one placentas were sent for SARS-CoV-2 spike protein staining. None of the placentas stained positive for the spike protein; there was one area of staining in the syncytiotrophoblast of a placenta from the Active COVID group, but we could not be confident that this was not artefactual as there was staining of the stroma (**Figures 3A,B**, positive and negative controls **Figures 3C,D**).

3.4. Ultrastructural Analysis

A subset of samples were sent for ultrastructural analysis by TEM. There were no specific morphological abnormalities observed in either of the COVID-19 groups compared to controls.

In the Active COVID group, one sample had numerous dilated cisternae of the endoplasmic reticulum (**Figure 4A**). However, the rest of the samples looked healthy with normal basal lamina and mitochondria apart from one sample with an area of degenerate syncytium (**Figure 4B**) lacking normal microvilli. Another Active COVID sample had widely dilated cisternae of rough endoplasmic reticulum and some apical vacuoles with cytoplasmic contents; mitochondria were swollen and there were several cytoplasmic lamellar bodies. The basal lamina was thickened and the underlying Hofbauer cell had multiple inclusions. The capillary looked normal although the basal lamina was multi-layered, which has previous been observed in diabetes and Rhesus incompatibility and is a considered to be a sign of high cell turnover (12) (**Figure 4C**). Multiple ferritin particles were observed in Hofbauer cell inclusions (**Figure 4D**).

One placenta from the Post COVID group had several large sub-apical vacuoles in the syncytiotrophoblast (**Figure 4E**). The same sample had fibrotic stroma, a thick basal lamina and some malformed, swollen microvilli with another area of microvillous breakdown (**Figure 4F**). Another sample contained

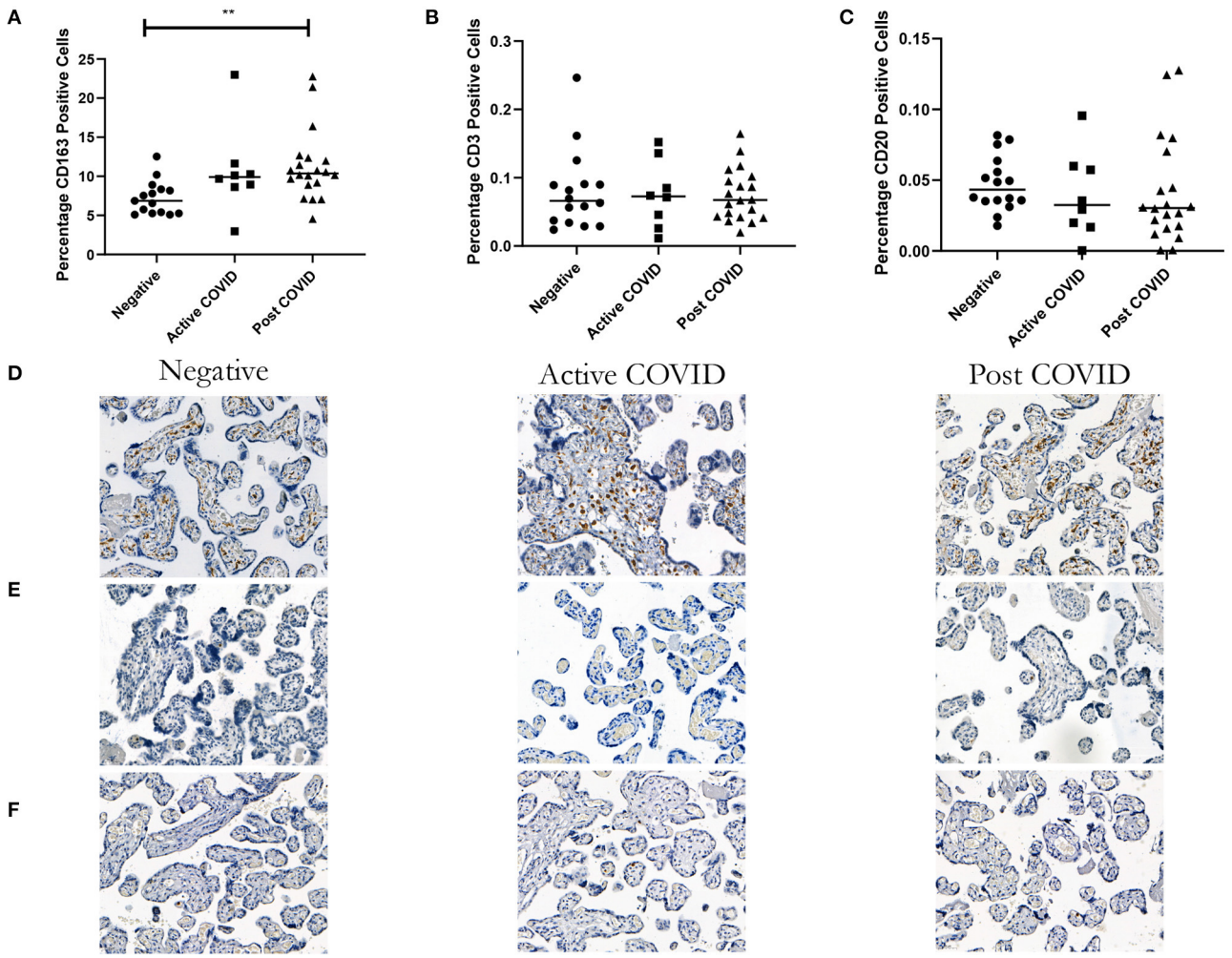

FIGURE 1 | Increase in the percentage of macrophages in placentas from women who tested positive for COVID-19, but no difference in the percentage of T or B cells. **(A)** A significant increase in the percentage of CD163 macrophages in the Active and Post-COVID groups compared to negative controls. **(B)** No difference in the percentage of CD3 cells between groups. **(C)** No difference in the percentage of CD20 cells between groups. Representative images, magnification X20 **(D)** CD163, **(E)** CD3, **(F)** CD20. Kruskal-Wallis, $^{**}p < 0.009$. Active COVID n = 8, Post COVID n = 20, and controls n = 16.

small capillaries for gestational age (**Figure 4G**) and alongside a thickened basal lamina, the tips of the microvilli were swollen (**Figure 4H**).

Almost all the samples had lamellar bodies (noted in **Figures 4H,I** by *), but this may be an artifact of fixation. Other observations included small capillaries in a term terminal villus in a healthy control, whilst another control had numerous infoldings/interdigitation in the syncytium basal plasma membrane and the basal lamina was thickened and cribriform (**Figure 4I**). The former has been observed in many pathological conditions including pre-eclampsia and maternal hypertension while basal lamina thickening is a very non-specific response and has been described in most pathologies (13). Additionally, in controls, focal sites of necrosis with a total loss of microvilli and breakdown of the apical membrane and washed out cytoplasm were occasionally found (**Figure 4J**).

4. DISCUSSION

Whether placental pathology is associated or not with maternal SARS-COV-2 infection is of interest to obstetricians, histopathologists, and scientists attempting to delineate the effects of SARS-CoV-2 infection on pregnancy outcome. Our study has shown a significant increase in the percentage of $CD163^+$ macrophages in the Post COVID group compared to controls. We have previously shown an increase in intra-villous macrophages in several placental complications, including villitis of unknown etiology (VUE) (14) in the placentas of infants with a decreased growth rate, but not in those classified as FGR, which were thought to be small-for-gestational-age (SGA) (15). However, in contrast to previous studies, the observed increase in $CD163^+$ cells in this current study is not associated with low birth weight, with no infants being classed FGR ($\leq 3^{rd}$ IBC) and

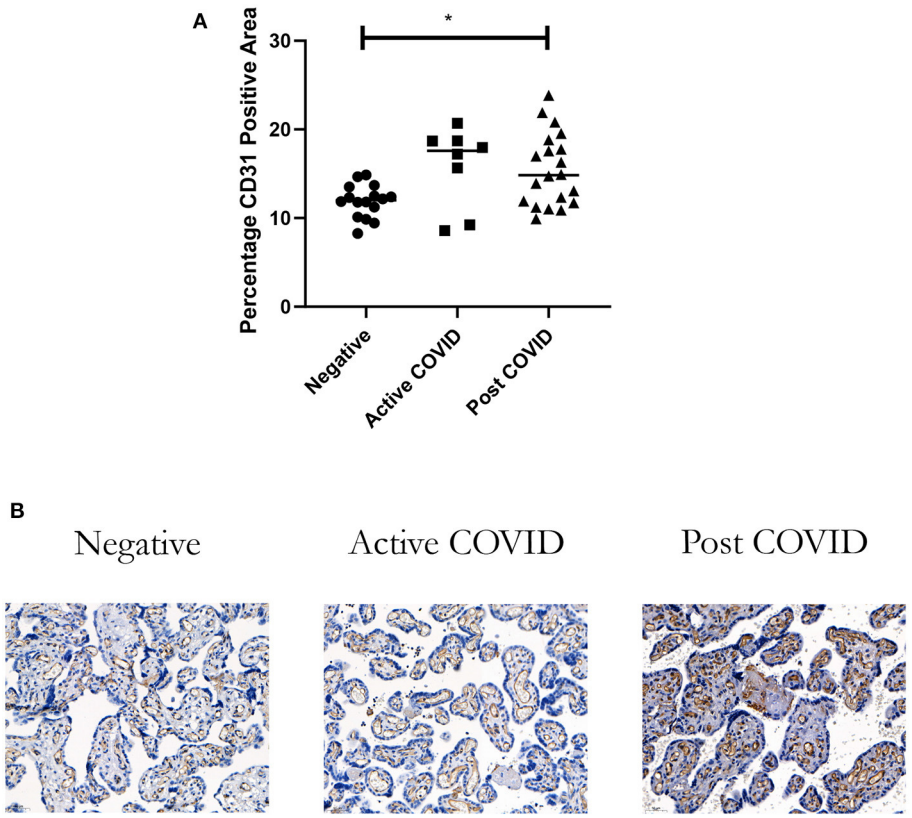

FIGURE 2 | Vascularity of placentas from women who had COVID-19 during their pregnancy. **(A)** There was a significant increase in the percentage vascularity in the placentas of women in the Post COVID group compared to controls (*$p = 0.026$). There was no difference in placental vascularity in the Active COVID group compared to controls ($p = 0.06$). **(B)** Representative image magnification X20. Kruskal-Wallis, Active COVID $n = 8$, Post COVID $n = 20$, and controls $n = 16$.

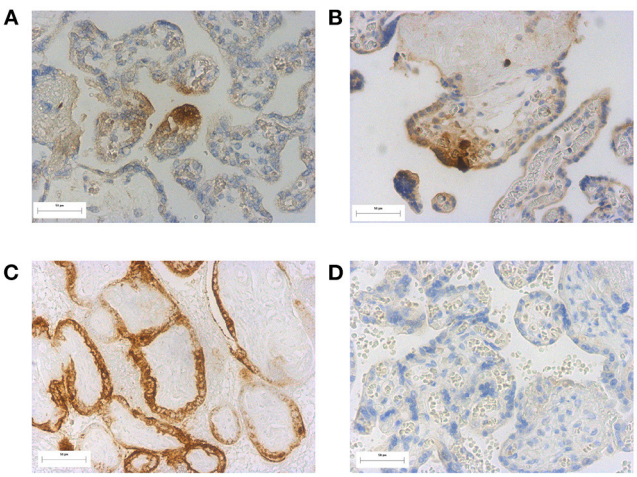

FIGURE 3 | SARS-CoV-2 spike staining in placentas **(A)** and **(B)** possible positive staining in a placenta from a participant within the active COVID group, **(C)** positive staining of a previously identified SARS-CoV-2 positive placenta (positive control) **(D)** negative SARS-CoV-2 spike protein staining in an unaffected placenta. Magnification X20.

only four infants $\leq 10^{th}$ IBC in the Post COVID group. One of the roles of Hofbauer cells is to protect against pathogens, partly through expression of Toll-like Receptors (16). Hofbauer cell hyperplasia has been identified in 3 out of 20 SARS-CoV-2 positive placentas with an additional diagnosis of chronic histiocytic inter-villositis (CHI) (17). Hofbauer cell hyperplasia was not present in all CHI placentas, indicating that the increase was not due to CHI. The cause of the hyperplasia both in the study by Schwartz et al. and our study remain unknown. Further investigations into placental macrophage role/function following maternal SARS-CoV-2 infection are required to understand any potential adverse downstream placental changes as a result of the increase in Hofbauer cell number.

The number of macrophages may be linked to underlying pregnancy pathology as in the Active COVID group, one participant has diabetes mellitus and chronic hypertension, whilst another had gestational diabetes mellitus. In the Post COVID group, one participant had recurrent RFM, and one had hyperthyroidism. Previous research has identified an increase in the number of placental macrophages in the presence of maternal diabetes (type 1, 2 and gestational) (10). However, as the majority of participants in the Post COVID group did not have any

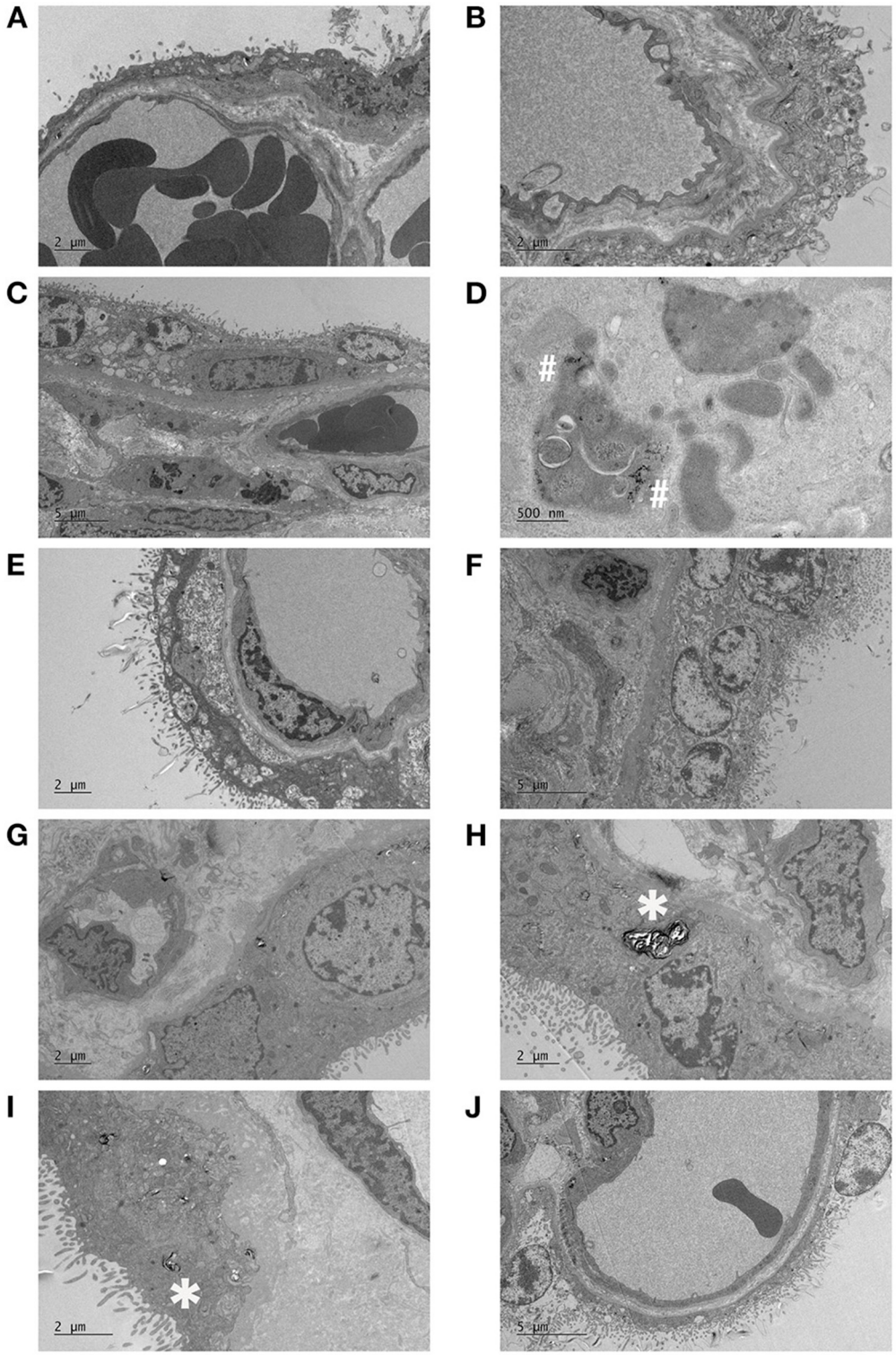

FIGURE 4 | Electron micrographs of placentas following maternal SARS-CoV-2 infection. Active COVID samples: **(A)** dilated cisternae of endoplasmic reticulum and sparce microvilli, **(B)** necrotic syncytium, **(C)** dilated syncytial endoplasmic reticulum, and swollen mitochondria, with multiple inclusions in an underlying Hofbauer cell and a multi-layered capillary basal lamina, **(D)** high power image of the ferritin particles within a Hofbauer cell inclusion. Post COVID samples: **(E)** large sub-apical vacuoles in the syncytiotrophoblast, **(F)** fibrotic stroma, thick basal lamina, malformed swollen villi, **(G)** small capillaries and **(H)** thickened basal lamina, swollen microvilli with possible loss of actin. Negative control samples: **(I)** numerous infoldings in the syncytial basal plasma membrane and thickened and cribriform basal lamina, with dispersed lamellar bodies **(J)** focal loss of microvilli (top right) and breakdown of the apical membrane and washed out syncytial cytoplasm (bottom left). The vasculosyncytial membrane appears relatively normal. *Indicates lamellar bodies, #indicates ferritin particles.

pregnancy complications, this is not likely to been the cause of the observed macrophage increase in all placentas.

4.1. Poor Pregnancy Outcomes and Placental Pathology

There are mixed findings as to whether maternal SARS-CoV-2 infection leads to an increased risk of poor pregnancy outcomes, particularly stillbirth. Whilst there are reports of an increased risk of stillbirth (18, 19), other studies have reported no difference (20, 21). There have been reports of an increase in neonatal deaths (7, 19); however, in our study, there were no poor pregnancy outcomes, although this is partly by design as women with Active COVID and a poor pregnancy outcome (e.g., a prior fetal death) were not approached to take part in the study.

Whilst we identified an increase in both maternal and fetal vascular malperfusion in our systematic review of studies reported between December 2019 and July 2020 (3), Girolamo et al., reported similar findings (22), and placental hypoperfusion and inflammation were identified in a more recent systematic review and meta-analysis. Other research has identified no associated placental pathology following third trimester infection, at either the macro- or microscopic level (23), or statistically significant increases in features, such as fibrin deposition (7). Our histopathology reports identified excessive syncytial knots, distal villous hypoplasia and villous immaturity in all study groups and there did not appear to be a preponderance of these features in women with SARS-CoV-2 infection. Whilst there may be a placental pathology in pregnancies with a poor pregnancy outcomes, or declining fetal health *in utero* indicated by RFM (24–26), there does not appear to be any specific pathology in placentas from women with normal pregnancy outcomes, having mild symptoms and not requiring enhanced care in ICU. Although there was an increase in placental macrophages, this was not accompanied by an increase in other immune cells that are responsible for resolving viral infections, namely T and B lymphocytes (27). The syncytiotrophoblast acts as a placental barrier and prevents viruses passing from the maternal blood into the fetal circulation. Staining of placentas with the SARS-CoV-2 spike protein revealed that none of the samples evaluated here tested positive. As a result, an increase in immune cells would not be expected if there has not been a break in the syncytiotrophoblast.

Analysis of the placenta from the participants who tested positive for SARS-CoV-2 the day after delivery showed a low number of placental macrophages compared to the other placentas in the group (average of 2.97% across the three placental sections compared to between 8.67 and 22.98% for the remainder of the samples in the group). The most likely explanation for this difference is that the woman was possibly asymptomatic and was earlier in the course of COVID-19 infection, thereby reducing inflammation and consequent stress on the placenta.

There was a significant increase in vascularity between the Post COVID group compared to healthy controls. The high proportion of cases with fetal vascular malperfusion reported in the literature would have reduced the number of fetal vessels, whereas there was an increase in the Post COVID group and a trend toward an increase in the Active COVID group. It is likely that this observation in the Active COVID group may be affected by confounding factors, as the Active COVID group contained one infant who was large-for-gestational-age (LGA), two women with maternal diabetes (one with gestational diabetes) and one SGA infant. As the interval between a positive maternal COVID-19 test and delivery was between 2 and 12 days, it would be unlikely that there would be sufficient time for additional capillaries to develop in the placenta, in response to maternal SARS-CoV-2 infection, despite angiogenesis continuing until term (28), however, an increase in vascularisation has previously been observed in pregnancies with diabetes mellitus (10). The increase in vascularity in the Post COVID group is less likely to be affected by confounding factors as only two women had additional pregnancy pathologies; one women had RFM during her pregnancy and another had hyperthyroidism. However, this may be a chance finding, as there was no statistically significant difference in the histopathological findings.

The placentas analyzed in our study were collected between May 2020 and May 2021. During this period, women in the UK were recommended to shield as they were classed as being clinically vulnerable and SARS-CoV-2 vaccinations for pregnant women were not approved by the Joint Committee on Vaccination and Immunization (JCVI) until April 2021 (29). Maternal vaccination has been found to reduce severity of SARS-CoV-2 infection (30), and should be encouraged. Whilst our cohort of women did not have severe symptoms of COVID-19, despite being unvaccinated, the lack of any associated placental pathology and the minimal changes observed in the number of placental immune cells indicates that mild maternal COVID-19 infection is unlikely to cause harm to the developing fetus. This study demonstrates that in general, women who have mild symptoms are unlikely to have a poor pregnancy outcome, although why some women do go onto have a poor outcome remains unclear.

None of the placentas sent for SARS-CoV-2 spike protein staining showed definitive positive immunostaining, indicating that in most cases SARS-CoV-2 does not enter the placenta itself, unlike other viral infections which manage to cross the syncytium including ZIKV (causing Zika virus disease). The mechanism of cellular entry for SARS-CoV-2 is thought to be mediated by TMPRSS2 and ACE2 receptor, with minimal co-expression found in the placenta and chorioamniotic membranes (31). Another study reported strong staining using immunohistochemistry for ACE2 in the placenta, particularly in the syncytiotrophoblast, but there was minimal staining of TMPRSS2 and any identifiable weak staining was on the endothelium of the villous capillaries (9). ZIKV does infect Hofbauer cells (32) and can replicate inside them (6). Hofbauer cells, however, have been shown not to express ACE2 (9) and, therefore, it would be unlikely for SARS-CoV-2 to replicate similarly to ZIKV, even if there is a break in the syncytiotrophoblast to allow viral entry to the villous core.

There are multiple antibodies that can be used to identify positive SARS-CoV-2 staining in the placenta. We chose to use an antibody against the SARS-CoV-2 spike protein (clone 1A9) which has also been used by (33–35). However, alternative

clones are available, such as 007 used by Facchetti et al. (36). Alternatively, antibodies for the SARS-CoV-2 nucleocapsid protein can also be used, such as those used by Debelenko et al. (35), Facchetti et al. (36), and Morotti et al. (37). Positive staining of the placenta has been identified using antibodies against the SARS-CoV-2 spike protein and the nucleocapsid.

4.2. Strengths and Limitations

The inclusion of women who had active COVID-19 infection at the time of delivery is a strength of this study, as it allows for any acute impact of maternal infection to be observed. We also collected contemporaneous healthy controls which is important as this provides controls for confounding issues such as the background stress of having a pregnancy during a pandemic (38), alongside other pregnancy complications such as diabetes mellitus. Ultrastructural analyses of a subset of placentas was also conducted. Whilst we were not able to identify any specific disease phenotype, one Post COVID placenta had a breakdown of the microvilli and another had several large sub-apical vacuoles in the syncytiotrophoblast which could be similar to the virion containing membrane-bound cisternal spaces described by Sisman et al. (39); however, no virion-like particles were seen within. None of the placentas had any obvious pathology.

We were able to include women in the Post COVID group of this study who had COVID-19 infection during their second or third trimester of pregnancy. Including these women along with women who tested positive in the third trimester, allowed for us to investigate whether maternal infection earlier in pregnancy resulted in morphological changes in the placentas, as this may be the point in gestation in which any insult to the placenta will have the largest effect. However, we did not see any specific pathology in the placentas of the women who had SARS-CoV-2 infection in the second or third trimester, possibly due to reduced severity of disease symptoms.

One of the limitations of this study is the uncertainty about whether the mothers in the control group had COVID-19 during their pregnancy, as antibody testing has not been implemented in the UK. In a recent survey of UK households, from December 2020 to July 2021, between 39 and 50% of people testing positive for COVID-19 were asymptomatic (40), therefore, there is the possibility that some of the women may have had asymptomatic COVID-19 during their pregnancy. Finally, no women included in the study has severe maternal disease, i.e., were not admitted to an intensive care unit due to COVID-19 infection, and there were no adverse outcomes such as stillbirth. Inclusions of cases with the most severe symptoms and signs may best allow for the correlation between maternal infection and placental dysfunction to be determined, as these infections may have had more of an impact on the placenta, for example as a result of severe maternal hypoxia.

4.3. Conclusions

In our cohort of women, with otherwise healthy pregnancy outcomes, there was no specific placental pathology associated with maternal SARS-CoV-2 infection during pregnancy. The observed increase in the number of placental macrophages warrants further investigation to see if these cells are responding to any possible maternal hypoxia affecting the placenta (which increases cytokine release from placental tissue). Analysis of cytokines in both maternal and cord blood, and placental samples would provide information as to the inflammatory status following maternal SARS-CoV-2 infection. Further analysis of placental samples, such as the examination of any differences in cell proliferation and apoptosis, would allow for any subtle changes to be found. Placentitis may be a phenotype only observed in pregnancies with a poor pregnancy outcome, but it does not appear to be present following mild maternal SARS-CoV-2 infection.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

Research ethical approval was given (18/NW/0451). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MS conceived the study, performed immuno/histochemical staining, image analysis, statistical analysis, and manuscript preparation. AG helped perform immunohistochemical staining and image analysis. EA helped perform immunohistochemical staining. CJ prepared semithin sections of placentas submitted for TEM and provided analysis of EM images. GB performed histopathological analysis of placentas. AH conceived the study, provided guidance for laboratory work and manuscript preparation. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fviro.2022.813312/full#supplementary-material

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Interleukin-10 Delays Viral Clearance in the Placenta and Uterus of Mice With Acute Lymphocytic Choriomeningitis Virus Infection During Pregnancy

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Pregnant mice infected with Lymphocytic Choriomeningitis Virus (Armstrong) (LCMV-Arm) experience high viral loads in the placenta and uterine tissue by 5–8 days post-infection, a time when the virus is nearly completely cleared from the spleen and blood. Interleukin 10 (IL-10) plays a crucial role in T cell responses associated with systemic viral clearance. Using the LCMV-arm model of infection, we examined first, whether IL-10 is involved in viral clearance in the placenta and uterine tissue and secondly, the potential mechanisms underlying this phenomenon. C57BL/6 (WT) and mice deficient in IL-10 (IL-10 KO) were infected with LCMV-Arm on day 10 of pregnancy. Placenta and uterine tissue, collected 2 and 8 days later, were analyzed using real time RT-PCR, plaque assays for viral load, and flow cytometry. In WT mice placenta and uterine tissue expression of IL-10 was elevated with LCMV-Arm infection. Fetus resorption was elevated in WT on days 2 and 8 post-infection as compared to IL-10 KO, and by day 19 of gestation delivery was greater. Viral loads in the placenta and uterine tissue were resolved early in IL-10 KO mice, but persistent in tissues of WT mice. Levels of NRF2 and FAS were equivalent, but BCL2L11 was higher in IL-10 KO uterus. IL-6, Interferon-β (IFN-β), CCL2, and IL-17 levels were also equivalent. IL-10 KO tissues tended toward higher expression of interferon-γ (IFN-γ) and had significantly lower expression of Transforming growth factor beta (TGF-β). The proportion of placenta and uterine tissue CD8 T cells expressing the activation markers $CD44^{hi}$ and PD1 were elevated in IL-10 KO mice. These data suggest that high IL-10 expression at the fetal-maternal interface following LCMV-Arm infection prevents clearance of viral load by impairing CD8 T cell activation and poses a significant threat to successful pregnancy outcome. The ability to modulate IL-10 expression at the maternal-fetal interface may help overcome negative pregnancy outcomes arising during acute LCMV and other viral infections in humans.

Keywords: CD8 T cells, cytokines, pregnancy, lymphocytic choriomeningitis (LCMV), mouse

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INTRODUCTION

Emerging infections with such agents as Zika virus and Coronavirus, and more common agents such as Influenza and Cytomegalovirus continue to serve as reminders about our deficient understanding of maternal immunity. This is especially true with regard to the maternal fetal interface. In spite of potentially relevant conflicting data [reviewed elsewhere (1)], there still exist models of maternal tolerance which rely on mechanisms of inherent suppression of maternal immunity. These mechanisms are thought to act systemically or at the maternal-fetal interface, and at the tissue or cellular level. The study of infections during pregnancy offers an opportunity to examine and determine the relevance of some of these mechanisms. In addition, it presents a means to uncover possible subtle elements and regulatory circuits in the maternal immune system. These may support fetal tolerance, but at the same time protect fetus and mother against overwhelming disease. Accumulating evidence suggests that pregnancy drives the expansion of unique cellular phenotypes particularly at the maternal fetal interface (2–4) which may specifically express mechanisms which can both support immune surveillance and minimize harmful anti-fetal tissue responses (5, 6).

Lymphocytic choriomeningitis virus (LCMV) is an ambisense single stranded RNA virus belonging to the family *Arenaviridae,* which can cause human disease (7, 8). Generally, infection occurs through contact with aerosolized mouse feces and urine, causing a self-limited febrile illness (9). However, the virus can be transmitted vertically in mice (10) and humans (11). In humans, transmission vertically results in congenital neurologic abnormalities (12, 13) while death is an outcome if viral transmission occurs *via* transplantation of infected organs (14, 15) in immune suppressed patients. A recent estimate is that ∼4% of pregnant women may be seropositive with LCMV specific IgG (16).

Laboratory strains of this virus have long been used to study mechanistic aspects of the elicited immune response in mice. Infection with the LCMV-Armstrong strain results in an acute infection with a peak systemic viral load about 3 days after inoculation. This is followed by a rise in CD8 virus-specific effector T cells that peaks between 5 and 8 days post-infection (17) and which contributes to the resulting clearance of virus. By day 15 of infection, the CD8 T cell population consolidates and forms a memory T cell pool (18). In contrast, infection with Clone 13 leads to persistent infection and is associated with dysregulation of T cell function (19).

We have previously observed that infection with the LCMV-Arm on day 8–10 of pregnancy results in systemic (spleen, peripheral blood) immunity, including specific CD8 T cell expansion, interferon-γ (IFN-γ) production, cytotoxicity and viral clearance that is equivalent to that found in non-pregnant mice infected at the same time. However, by 8 days after infection, when the systemic circulation is cleared of virus, the placenta and uterus remain infected (20) until birth.

Interleukin-10 (IL-10) is an important driver of "anti-inflammatory" and regulatory immune functioning in subsets of T cells (21), B cells (22) "alternative" macrophages (23), and dendritic cells (24). It is a complex moderator of autoimmunity (25) and anti-tumor immunity (26). Particularly, the activity of IL-10 is thought to be a driver of pathogen persistence and chronicity in parasitic (27) and viral infections (28–31), including infection with LCMV Clone 13 in non-pregnant mice. This activity is thought to be related to its role in inhibiting T cell production of IFN-γ and other immunomodulatory mechanisms (28, 31, 32). IL-10 may also have pleiotropic functions as it may control angiogenesis (33) vascular function (34) and apoptosis (35). In pregnancy, IL-10 is expressed by trophoblast (36) and decidua (37). It is also thought to be important in early pregnancy development, as relative deficiency is a contributor to early pregnancy loss in both mouse models (38, 39) and in human recurrent early pregnancy loss (40). In addition, mid-gestation deficiency in IL-10 has been linked to the inflammatory response leading to lipopolysaccharide-induced preterm birth in mouse models (41–43). However, early higher expression of IL-10 (44) or a group of factors that include IL-10 (45) in humans identifies women who subsequently have preterm birth, and is elevated in chronic placental infection [e.g., malaria (46)] in humans. This suggests a complex relationship between viral infection, immunity, IL-10 and homeostasis in pregnancy-related tissues. To begin to understand this relationship, we returned to our finding of viral persistence in mice infected with LCMV-Arm, which does not cause systemic viral persistence, but does cause prolonged infection in the placenta with mid-gestation infection (20). We hypothesized that IL-10 modulates the local immune response to LCMV-Arm. We report that deficiency in IL-10 led to a significantly lower viral load in the placenta and uterine tissue 8 days post mid-gestation infection. This was correlated with alterations of cytokine expression in these tissues, and an increase in activated CD8 T cells, but a decrease in the extent of fetal resorption. These data point to a complex interaction between maternal immunity, acute LCMV infection and the fetal-placental unit, and support an important role for IL-10 in aberrant pregnancy outcome following acute infection with LCMV.

MATERIALS AND METHODS

Mice and Infection

Adult male and female C57BL/6 (B6, or "Wild Type" WT stock # 00664) and IL-10 KO mice of the same genetic background (Stock # 002251) were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were maintained under specific pathogen-free conditions and used in procedures approved by the Institutional Animal Care and Use Committee at the University of Vermont and in accordance with The Association for Assessment and Accreditation of Laboratory Animal Care. Females were mated with same-strain males and the males were removed 24 h later which was taken as ∼day 0 of pregnancy. On day 10 of gestation, pregnant B6 and IL-10 KO females were infected with LCMV-Arm (2×10^5 PFU/mouse) by intraperitoneal (IP) injection in 100 ul PBS or left uninfected and unmanipulated (20). At days 2 and 8 post-infection (i.e., ∼days 12 and 18 of gestation) mice were euthanized by CO_2 inhalation. Fresh samples from the spleen,

uterine tissue (wall and decidua), placenta and uterine draining lymph nodes were harvested and either used immediately for flow cytometry or stored in RPMI (Sigma, St. Louis, MO) or frozen at −80°C until further use for either plaque assay or RNA expression. Analysis elaborated here include one representative, healthy-appearing (e.g., not necrotic) placental sample or one uterine sample (2–3 implantation sites and underlying nonplacental, e.g., decidua/endometrium/myometrium, tissue plus intervening non-implantation uterus) per mouse. All infection-related experiments were carried out in an ABSL2+ facility at the University of Vermont.

Flow Cytometry

The spleen, uterine tissue, placenta and uterine draining lymph nodes of pregnant B6 and IL-10 KO mice were placed in "Ghost special" medium [Iscove's Modified Dubeco's Medium, IMDM, without bicarbonate (Gibco, Carlsbad, CA)], supplemented with 1% fetal bovine serum (FBS; Gibco). From spleen and uterine draining nodes, single cell suspensions were generated through mechanical dissociation using 70 um nylon mesh. Uterine tissue and placenta were washed once in Ghost medium, cut into small pieces and subjected to enzymatic digestion by incubation in Hanks Balanced Salt Solution (HBSS, Gibco/Invitrogen, Grand Island, NY) containing 200 U/ml hyaluronidase (Sigma), 0.2 mg/ml DNAse I (Sigma) and 28 U/ml Liberase Blendzyme 3 (Roche, Indianapolis, IN) for 20 min in a 37°C warm water bath. After digestion, samples were pressed through 70-μm mesh. The resulting cell suspension was washed with phosphate buffered saline (27) (Mediatech, Manassas, VA) supplemented with bovine serum albumin (0.1%; PBS-BSA; Sigma). Approximately 1×10^6 cells were incubated in a 1:50 dilution of 2.4G2 (BD Biosciences, San Jose, CA) in order to block non-specific antibody staining due to Fc receptors, and then incubated for 1 h at room temperature in a specific antibody mix, washed twice with PBS-BSA and then fixed with PBS-BSA+1% paraformaldehyde (Fisher Scientific, Fair Lawn, New Jersey). The antibodies used in these studies included CD45.2 (clone 104, PerCP-Cy™ 5.5), PD1(Clone J43 PE), from e-Bioscience (San Diego, CA); CD8 (Clone 5H10-PE-Texas Red), Invitrogen, and CD44 (clone IM7 APC BD-Biosciences, San Jose, CA). Samples were processed with a LSRII flow cytometer (BD Biosciences) and the results of ∼10K cells were analyzed using FLOWJO software (Version 8.8, Tree Star, Inc, Ashland, OR). The gating scheme used was previously described (47).

Plaque Assay

Placenta and uterine tissue were dissected, weighed and kept in 200 μl of RPMI (Sigma) supplemented with 1% FBS at −80°C until they were homogenized using a Mini-BeadBeater setup (BioSpec, Bartlesville, OK). Plaque assays were then performed on homogenates as previously described (20). Briefly, serial dilutions of virus-infected homogenates were placed on monolayers of VeroE6 cells and incubated for 1 h at 37°C. A warm mix of 1× M199 media (Sigma) and 0.5% agarose was overlaid and allowed to solidify for 20 mins. Plates were then incubated for another 6 days, fixed with 25% paraformaldehyde (Fisher Scientific) and stained with 0.1% crystal violet. Excess stain was washed with water and then the plaques were visually counted. Data are expressed as PFU (number of plaques $\times 10^6$) / gm of tissue used in the assay).

QRT-PCR

Placenta (one representative healthy-appearing per mouse) and samples of uterine tissue (2–3 implantation sites with intervening tissue) were dissected, immediately frozen and stored at −80° C until ready for processing. Total RNA was extracted from 0.5 to 1 mg of tissue using the PrepEase RNA spin kit from USB [The iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA)] was used to synthesize cDNA from 250 ng of RNA template using a mix of random hexamers and oligo dTs. From each sample 1μl cDNA was used to amplify the genes of interest. QRT-PCR was performed on an ABI Prism 7000 (Applied biosystems-CA) using Power Sybrgreen master mix. Each sample was run in triplicate and the CTs were averaged. The following primers (forward and reverse, each in 5′-3′ orientation) were used for amplification:

Beta-2 microglobulin (β2m) ATGCTATCCAGAAAAC CCCTCAAA and CAGTTCAGTATGTTCGG CTTCCC; **Fas,** AACAAAGTCCCAGAAATCGCCTATG and TCCTGTCTCCTTTTCCAGCACTT; **Forkhead box protein P3 (Foxp3)**, AATGGGTGTCCAGGGAGC and TGGCAGTGCTTGAGAAACTC; **Transforming growth factor beta (Tgf-β)**, CGCAACAACGCCATCTATGAG and TGCTCCACACTTGATTTTAATCTCTGC; **Interferon gamma (Ifn-γ),** CCTCATGGCTGTTTCTGGCTGTTA and CATTGAATGCTTGGCGCTGGACC; **Ifn-γ receptor**, CAGGTAAAGGTGTATTCGGGTTCC and CCAGGCAGATACATCAGGATACATAAT; **Interleukin-10 (IL-10),** TTACTGACTGGCATGAGGATCA and GAAAGAAAGTCTTCACCTGGCTGA; **IL-17,** GACTCTCCACCGCAATGAAGACC and CCCACACCCACCAGCATCT; **IL-6,** AGAAAGACAAAGCCAGAGTCCTTCAG and GTCCTTAGCCACTCCTTCTGTGACT; **Bcl-2-like protein 11 (Bcl2L11)** GACGGAAGATAAAGCGTAACAGTTGT and TCCATACGACAGTCTCAGGAGGAA; **Nuclear factor (Erythroid-derived 2)-like Nrf2** ATGATGGACTTGGAGTTGCCA and GCTCATAGTCCTTCTGTCGC; **Interferon beta (Ifn-β)**TGTCCTCAACTGCTCTCCAC and CCTGCAACCACCACTCATTC; **CCL2 (monocyte chemoattractant protein-1, MCP-1),** TGATCCCAATGAGTAGGCTGGAG and ATGTCTGGACCCATTCCTTCTTG. Relative expression was determined using the ΔΔCT method with an uninfected uterine sample as a comparator, and the results of PCR with **β2m** as a "loading control."

Statistics

Data was graphed, visualized for trends and analyzed using GraphPad Prism 7 (GraphPad Software, San Diego, CA). Data points are displayed individually with lines delineating means or the tops of columns delineating medians. Data passing at least one of three normality tests were compared using parametric

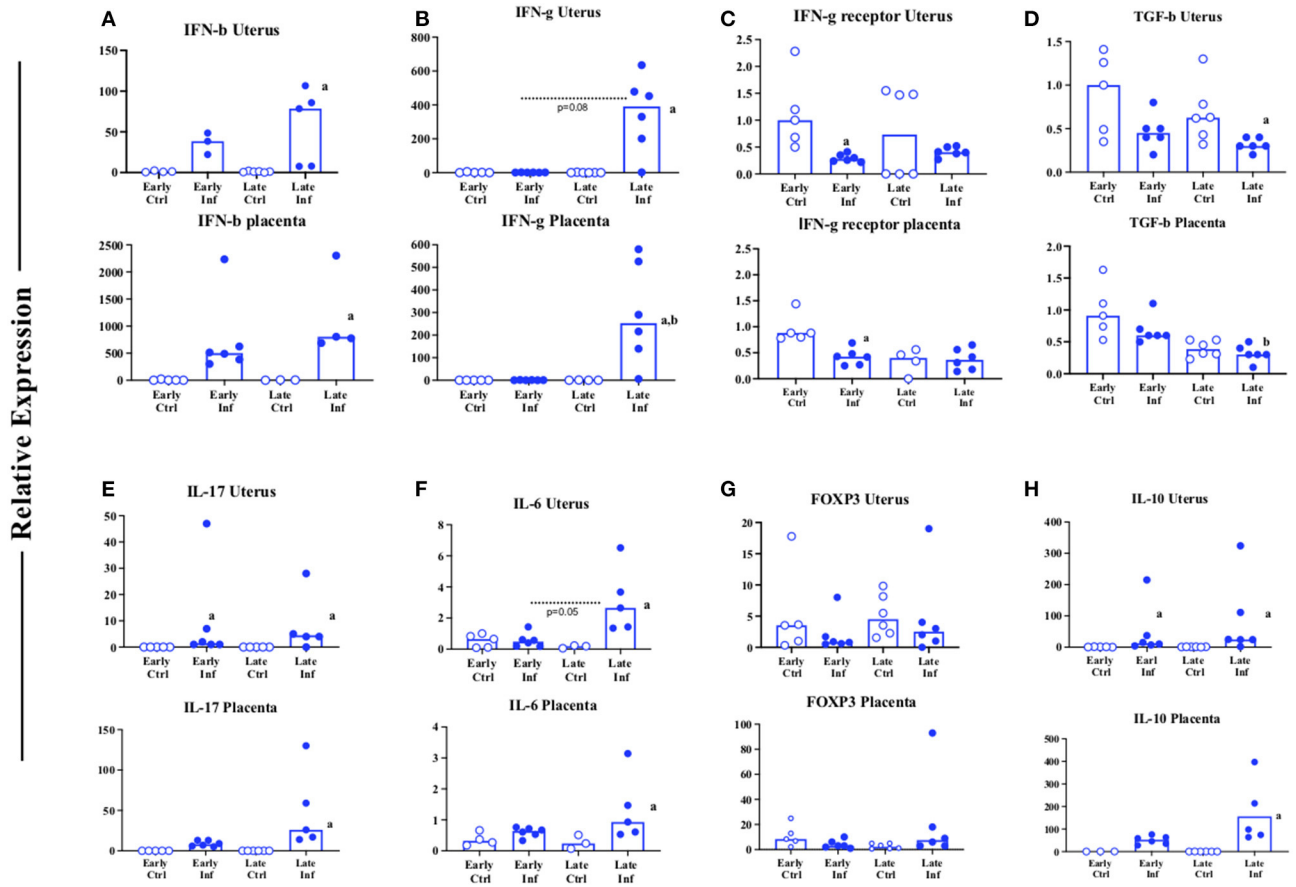

FIGURE 1 | Cytokine profile of uterine and placental tissues in normal mice infected with LCMV during pregnancy. C57BL/6 (B6) mice were infected (closed symbols) or not infected (open symbols) ip with 2×10^5 pfu of LCMV Armstrong on day 10 of pregnancy. **(A–H)** Values for the uterus (top) or the placenta (bottom) of the samples collected. ∼Day 12 (early), or ∼18 (late) of pregnancy the mice were euthanized, and the tissues indicated were tested by QT-PCR for expression. Y axis: relative expression using the ΔΔCT method compared to sample from an unmanipulated animal. X axis: time grouping. Each symbol represents an individual sample from an individual (distinct) mother ($n = 4$–6 mothers). Inf denotes samples from an infected mouse while Ctrl denotes samples from an unmanipulated mouse. Data was analyzed using Kruskal Wallis test with Dunn's correction for multiple comparisons testing. Letters: a denotes significance (e.g., $p < 0.05$) when comparing infected to control samples obtained at the same gestational age; b denotes significance between samples obtained early or late post infection. Trends ($p < 0.1$) are noted numerically.

analysis. Two groups were compared with the t test while more-than-two group analysis used ANOVA with post-test correction for multiple-comparison testing. For data not passing normality, two group comparisons utilized the Mann-Whitney test, while more than two groups utilized Kruskal Wallis testing, and adjusted using the Dunn's multiple comparisons test. Significance was set at $p < 0.05$, although some trends are noted.

RESULTS

Previous studies of LCMV infection in the mouse placenta and decidua showed that 8 days after infection with an intraperitoneal dose of LCMV (20), the systemic circulation developed an increase in peripheral blood and spleen LCMV-specific CD8 cells and activated CD4 cells as occurs in non-pregnant mice. However, while the spleen is cleared of LCMV by 8 days post infection, the decidua (uterus) and placenta is not. One possible explanation for this difference is enhanced viral uptake, as subsequent studies revealed the increased expression of a potential receptor for the virus in mid gestation placenta (48). However, one potential rational for prolonged viral infection in the relevant tissues are the adaptive and innate immune responses generated in those tissues, as deficiency in tissue specific immunity is a potential contributor to viral persistence in other models of infection. To begin to assess the local immune and inflammatory response to LCMV infection, we examined the expression of a variety of cytokines relevant to innate and adaptive immunity in uterine and placental tissues of C57BL/6 (Wild Type, WT) mice. Adult mice were mated to same-strain males and infected on ∼day 10 of pregnancy, since previous studies suggested a very high rate of resorption with infection earlier in gestation (20). **Figure 1** shows a comparison to the tissues in uninfected mice. Two timepoints are examined. "Early" refers to 2 days post infection, ∼ 12 days of gestation, while

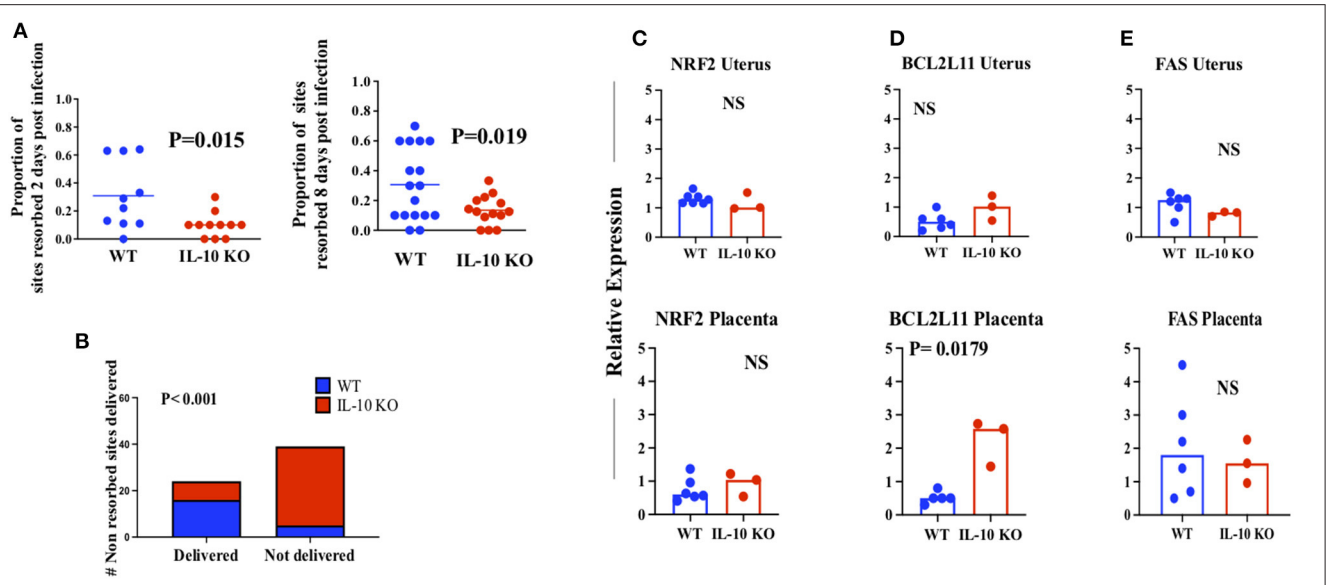

FIGURE 2 | Effect of LCMV infection on pregnancy outcome in WT and IL-10 KO mice. **(A)** Proportion of implantation sites resorbed (necrotic) 2 days (left, $n = 10$ WT and 11 IL-10 KO mothers euthanized) and 8 days (right, $n = 17$ WT and 14 IL-10 KO) post infection. Each symbol represents a mother euthanized. **(B)** Number of non-resorbed sites delivered. Uteri ($n = 5$ WT and 7 IL-10 KO) were opened on day 19 of gestation and the presence of a pup was counted as a "non-delivered" site. Lines, sample means. *p* values, analysis of significance by the t test. **(C–E)** RNA expression by QT-PCR of the indicated molecules in the indicated tissues on day ∼18 of gestation. Bars, sample medians. *p* values, analysis of significance by the Mann Whitney test. Blue symbols, WT; Red symbols IL-10 KO. NS, not significant at the $p < 0.05$ level.

"late" refers to 8 days post infection, ∼18 days of gestation. While expression of molecules such as IFNγ-R (**Figure 1C**) and **FOXp3** (**Figure 1G**) are not significantly changed over time and infection, three other patterns of RNA expression are discernable. In one pattern (e.g., **Figure 1B,** IFN-γ) expression does not appear to be different from uninfected tissues until late in infection. In another (e.g., **Figure 1D**, TGFβ) infection over time is associated with decreased expression. In a third, (e.g., **Figure 1E**, IL-17, uterus) expression is elevated soon after infection and continues to be elevated late in infection, raising the speculation of pleiotropic functions in the context of infection.

One cytokine with this pattern raised this same speculation: IL-10 (**Figure 1H**). Because of our finding, and the existing evidence in the literature, we examined pregnancy outcome in infected WT mice and in mice deficient in Interleukin-10 (IL-10 KO, **Figure 2**). Because we suspected a high level of resorption in the IL-10 KO mice based on a mouse model of early pregnancy loss (38), we examined the uteri of WT and IL-10 KO mice 2 and 8 days after infection. When we reviewed the proportion of resorption sites with necrotic fetal-placental units consistent with resorption, WT mice had a significantly greater proportion at both 2 (**Figure 2A**, left) and 8 days (**Figure 2A**, right) after infection as compared to IL-10 KO mice who experienced a level roughly similar to that found in uninfected WT mice (49). Because IL-10 deficient mice are more susceptible to preterm birth in response to toll like receptor activation with agents such as LPS (42), we expected that deficiency in IL-10 in the face of LCMV infection would lead to earlier delivery. Observations suggest that mice on this genetic (e.g., C57BL/6) background have a gestational length of ∼19.5 days (50). Because of this we undertook an investigation the number of non-resorbed implantation sites did or did not have an attached fetus (non-delivered vs. delivered **Figure 2B**). Unexpectedly, analysis of the calculated proportions suggested that proportionately more WT pups of infected mothers had delivered by day 19 of gestation (**Figure 2B**). Parturition is the outcome of a complex developmental process [reviewed in (51)] that likely is influenced by infection. To begin to probe the potential factors that might mediate oxidative stress (which could also lead to pathways such as senescence), cell death, and tissue dysregulation in infected WT and IL-10 infected tissues at day ∼18 of gestation, we examined tissue RNA expression of NRF2 (52), BIM/BCL2L11 (53, 54) and FAS/CD95 (35, 54) (**Figure 2**). Although uterine NRF2 is decreased by infection in WT mice (**Supplementary Figure 1**), we observed that both NRF (**Figure 2C**), and FAS (**Figure 2E**) were similarly expressed in tissues of IL-10 KO mice as compared to WT. In addition, RNA expression of the pro-apoptotic mitochondrial molecule BCL2L11 was increased only in the placenta in IL-10 KO mice (**Figure 2D**).

Differences in reproductive outcome in WT and IL-10 KO mice could be due to viral load over time after infection in reproductive tissues, even if virus is cleared from systemic lymphoid tissues (20) (**Supplementary Figure 2**). We thus examined uterus and placenta of WT and IL-10 KO mice using a plaque assay (**Figure 3**). We infected mice on ∼day 10 of gestation and harvested tissues 2 (early) and 8 days later. We found that viral loads appeared equivalent and low early post

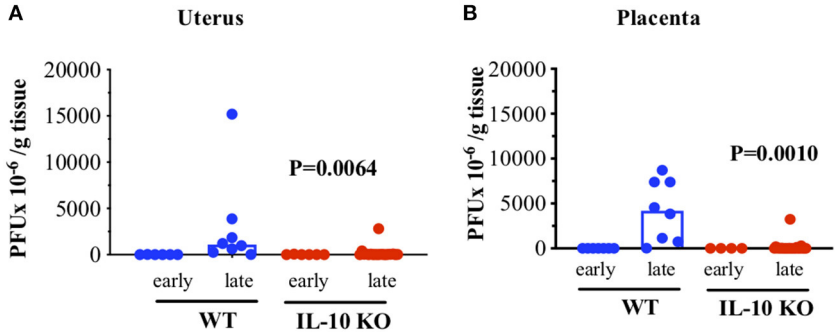

FIGURE 3 | Viral load in WT versus IL-10 KO mice infected with LCMV. WT (C57BL/6) females or IL-10 KO females were mated to same-strain males and on day ∼10 of gestation were infected as in **Figure 1** and euthanized on days ∼12 and ∼18 of gestation. Placentas were homogenized and then tested for viral load by plaque assay. **(A)** Uterine tissues (n = ∼10–20/strain/time). **(B)** Placental tissues (n = ∼10–20/strain/time). Y axis: PFU × 10^{-6} virus per gram tissue as assessed by plaque assay. X axis: early (2 days post infection or ∼12 of gestation) or late (8 days post infection or day ∼18 of pregnancy). Each symbol is a unique tissue representing a distinct mother. Blue, WT tissues; Red, IL-10 KO tissues. Bars indicate median for the sample group. p values generated by Kruskal Wallis testing with Dunn's multiple comparisons test to compare WT vs IL-10 KO late values. Significance set at the $p < 0.05$ level.

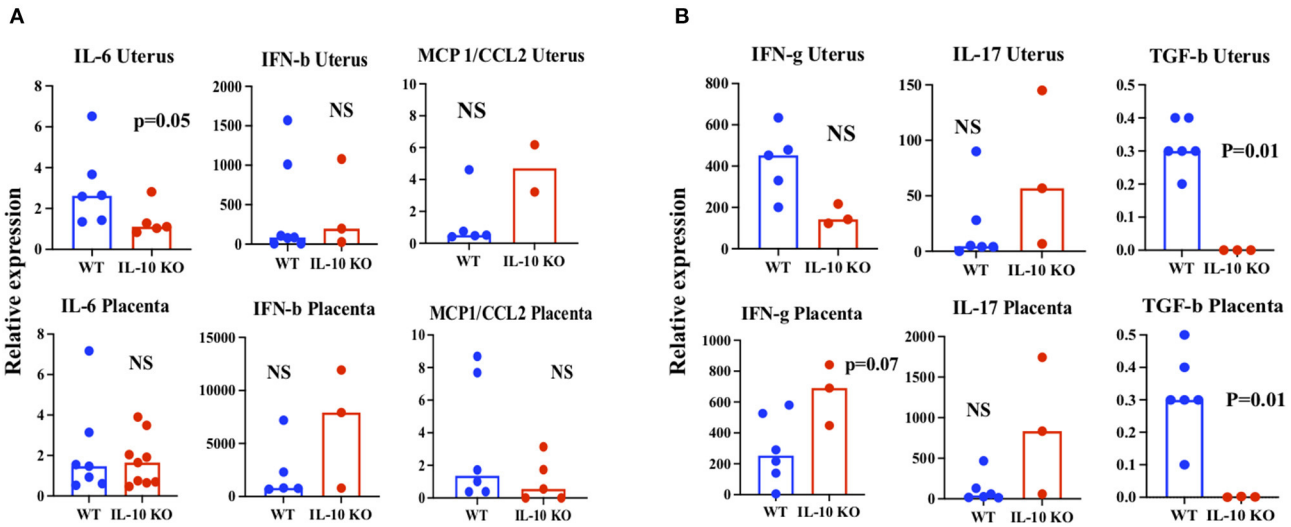

FIGURE 4 | RNA expression of cytokines in WT vs. IL-10 KO tissues. Tissues from WT (blue) or IL-10 KO (red) mice who were infected on ∼day 10 of pregnancy and harvested 8 days later were harvested and RNA extracted to determine expression by QT-PCR as in **Figure 1**. Each symbol denotes a single tissue from a unique mother. **(A)** "Innate immunity" cytokines IL-6 (left), Interferon-β (middle) and CCL2 (right). **(B)** "Adaptive immunity" cytokines Interferon–γ, IL-17, Transforming growth factor-β. Upper panels, uterus. Lower panels, placenta. Bars—medians. p values analysis of significance utilized the Mann Whitney test. NS, not significant at the $p < 0.05$ level. Trends ($p < 0.1$) are noted.

infection in both uterus (**Figure 3A**) and placenta (**Figure 3B**) from WT and IL-10 KO mice. However, late post infection, the viral load was statistically greater in both tissues from WT as compared to IL-10 KO mice.

A potential connection between late pregnancy loss (or shortened gestation) and viral load could be related to innate immunity, especially as this connection has been documented in other infections (55). In contrast, the driver of our observations could be related to adaptive immunity. To begin to examine this potential dichotomy, we examined the expression of molecules related to innate vs. adaptive immunity in tissues from WT or IL-10 KO mice. Tissue samples from WT and IL-10 KO mice taken 8 days after infection e.g., day ∼18 of gestation were harvested and examined for RNA expression of IL-6 (56, 57), IFN-β (58), and CCL2 (59) which are part of the innate response to viral infection (**Figure 4A**). Further, we examined IFN-γ (60), IL-17 (61) and TGF-β (60, 62) (**Figure 4B**) which are considered important for adaptive immunity. At this time point, there was no difference in expression of innate immune mediators (**Figure 4A**). However, we observed a trend toward increase in IFN-γ in the placenta of

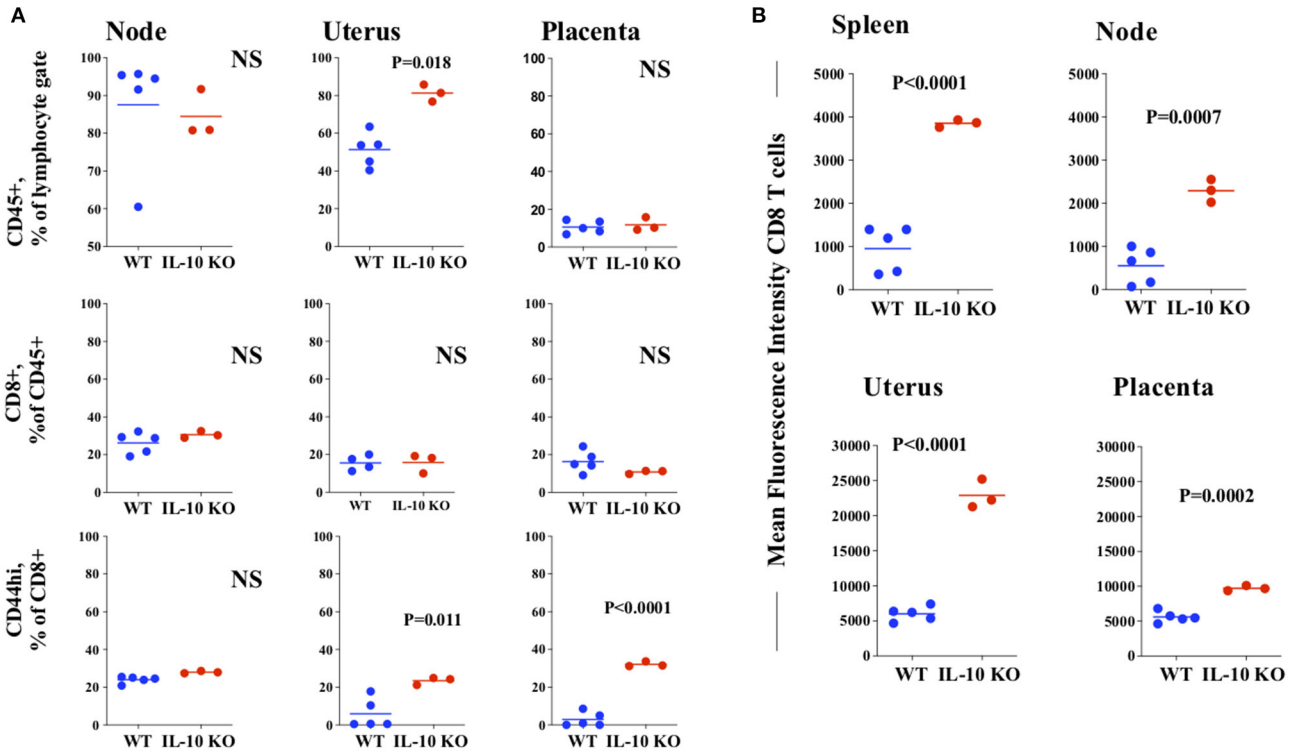

FIGURE 5 | Flow cytometric analysis of tissues from infected normal and IL-10 deficient mice. Single cells suspensions were obtained from tissues harvested from LCMV-infected pregnant WT (blue symbols) and IL-10 KO (red symbols) mice 8 days after infection on ~18 of gestation. Each symbol represents a single tissue from a unique mother. These were stained with antibodies to CD45, CD8, CD44 and PD1 **(A)** Top panel, percentage of lymphoid gate (by side and forward scatter) cells positive for CD45; Middle panel, percentage of CD45+ cells observed to be CD8+; Bottom panel, percentage of CD8+ cells highly expressing CD44 consistent with activation. **(B)** Expression of PD1 on CD8+ cells in each of the tissues assayed. Lines, means. *p* values indicate analysis by *t* test. NS, not significant at the $p < 0.05$ level, Node: uterine draining lymph node.

IL-10 KO mice and lower TGFβ in both tissues of IL-10 KO as compared to WT mice (**Figure 4B**).

This finding allowed speculation that the local CD8 T cell response might be different in the tissues of WT and IL-10 KO mice late in infection. To test this, we harvested tissues for examination by flow cytometry. We first analyzed single cell suspensions of tissues to estimate the proportion of relevant cells (**Figure 5A** and see **Supplementary Figure 4A**). CD45+ cells were increased in proportion in IL-10 KO uterus as compared to WT (**Figure 5A**, top row, middle panel), while the proportion of CD8+ cells was equivalent regardless of tissue (**Figure 5A**, middle row). However, the proportion of CD8+cells that were positive for the activation marker CD44 was higher in both the uterus and placenta of IL-10 KO mice as compared to WT (**Figure 5A**, bottom row, middle and right panels and see **Supplementary Figure 4B**, left panel). This suggested that either there was increased trafficking and or local activation of CD8+ cells in IL-10-infected vs. WT- infected utero-placental tissues (**Figure 5A**).

The molecule programmed death-1, PD1/CD279 (22, 63) is expressed on T cells and is associated with regulation of T cell homeostasis, activation and tolerance. Expression is upregulated on activation (64) and expression is associated with modulation of the response to viral infection. LCMV infection with less virulent strains leads to transient upregulation of PD1 and retained functional capacity, while chronic infection with more virulent strains leads to retained PD1 expression as part of the "exhausted" T cell phenotype (65). Based on previous findings of infection with a more virulent strain (28), we expected to see a decreased level of PD1 on CD8 T cells from IL-10 KO mice. Although the size of the systemic CD8 T cell pool is similar in infected WT and IL-10 deficient mice (**Figure 5A**, middle row, left panel), population-based PD1 expression was increased in IL-10 KO CD8 T cells of both the spleen and uterine draining lymph nodes (**Figure 5B**, top row, and see **Supplementary Figure 4B**, right panel) as compared to WT. In addition, the expression of PD1 in both uterine and placental CD8 T cells was elevated in IL-10 deficient tissues compared to WT (**Figure 5B**, bottom row). Taken together, this data suggests tissue specific regulation of the CD8 T cell pool in the face of viral infection.

DISCUSSION

These studies are driven by an earlier finding that infection with LCMV leads to persistent infection of the uterus and placenta,

but not the fetus, despite the fact that the strain used is deemed less virulent and does not usually lead to persistent infection in adult animals (66). We were interested in the role of IL-10 because of its potential role in modulation of both innate and adaptive immunity.

The key findings presented here are the decreased viral load late in infection in IL-10 KO as compared to WT mice, and that IL-10 deficiency in the face of infection leads to comparatively *less* pregnancy loss and potentially prolonged gestation. Based on previous data in IL-10 deficient mice, and the increased expression of proapoptotic proteins such as BCL2L11 (**Figure 2C**) the later finding is somewhat counterintuitive. The trend for increased expression of genes such as IL-6 (**Figure 4A**) is however consistent with developmental dysregulation (57) in the WT pregnancies examined. Although representative of distinct pregnancies, the relatively small sample size in our expression studies demands cautious interpretation. Despite the likelihood that more significant differences might be revealed with a much larger, potentially challenging number of mice, we were able to detect differences consistent with the idea that our findings may be related to differential activity of tissue-localized CD8 T cells.

In studies of neonatal male mice, intracerebral infection with LCMV-Arm, the strain used in these studies, leads to persistent infection as adults, with viral nucleic acid present in several tissues (67). However, in adult mice, infection with LCMV-Arm leads to systemic tissue clearance which occurs in $\sim$ 8 days. This clearance is associated with long-term LCMV- specific immunity and protection against more virulent strains. Key mechanisms of viral persistence in neonatally infected mice include central tolerance, as the virus is persistent in the thymus as well as other tissues and adoptive transfer of T cells from immune mice leads to viral clearance (68).

Viral persistence can be achieved in adult mice through administration of certain clones of LCMV (e.g., clone 13) which, interestingly enough, were isolated from the spleen of mice infected neonatally (66). Viral persistence was initially related to lack of functional cytoxic T cells. Recently it has been observed that strains of LCMV which cause either limited or chronic LCMV infection systemically, alter male urinary scent proteins (69). The effect on urinary scent proteins appears to last longer than it takes for systemic clearance, and this leads to the speculation that the reproductive tract may harbor "non-virulent" virus for a longer than expected (as compared to systemic circulation) time in males as well as pregnant females. This may support extended examination of different viral strains and LCMV specific immunity in the non-pregnant reproductive track of females (70).

The fact that injection of LCMV-Arm may generate different clones of varying immunity (66) raises the possibility that uteroplacental tissues may generate sub-strains with enhanced tissue specific binding or tropism (48). This may lead to clones which cause persistent infection and would thus be termed "virulent." Further studies are needed to examine the potential presence of altered virus genetic elements, such as those relevant to the L protein (71) in placental tissues.

The existing data may support a model whereby there are three different biologies of LCMV infection during pregnancy. First, infection (more likely early) in pregnancy tends to embryonic loss (20), observed in our studies as "resorption" (**Figure 2**). Second, infection leads to utero-placental prolonged viral presence at a time during enhanced expression of a putative viral receptor (48). This may lead to labor complications [e.g., dystocia (20)] and increased perinatal mortality. The third, perhaps with infection in late gestation exists along the same continuum as infection in the brain within the first day of life. During which the placental status is not clear, but the fetus becomes chronically infected due to infection of the immature thymus. These three "biologies" might be related to inherent elements of anatomy (72), viral tropism (48) and mechanisms of host defense and innate immunity which may differ by strain [e.g., SWR/J and HA/ICR (10) vs. C57BL/6 (our studies)]. This should be examined in future investigations.

Since IL-10 is expressed in trophoblast, one could expect that it plays a developmental role as do cytokines such as IFN-γ (73) as well as a protective immune regulatory role in pregnancy. Indeed, this is how we interpreted early expression of IL-10 in women who would go on to have preterm birth (44). We posited that these women experienced some dysregulatory insult for which IL-10 expression was enhanced in a compensatory manner. Because of the proposed role of IL-10 in "anti-inflammatory responses" IL-10 has been examined in models of recurrent pregnancy loss and preterm birth. In models of recurrent pregnancy loss, utero-placental deficiency in IL-10 leads to increased loss (38). Moreover, in models of Toll-like receptor mediated preterm birth, deficiency is related to increase in innate immune activation [e.g., NK cells (41, 74)] and a more profound phenotype. However, in the context of LCMV infection, where we would expect that deficiency would lead to enhanced inflammation, we did not observe increased expression of inflammatory cytokines (**Figure 4A**), an early-expressed interferon **(Figures 1A, 4A**) or a modulator of innate immune cell trafficking (**Figure 4A**; **Supplementary Figure 3**). Further, we did not see overall decreased "resiliency" in uteroplacental tissues of IL-10-KO vs. WT tissues (**Figure 2**) as pregnancy outcome was improved, and there was no difference in RNA expression of molecules such as NRF2 which should be defense against oxidative stress and FAS which should mediate apoptosis. Our only finding was increased RNA expression of the mitochondrial pro-apoptotic molecule BCL2L11. This may support examination of mitochondrial function and overall metabolism in the context of LCMV infection.

Cell death and tissue destruction in LCMV infection is said to be related to T cell activation and effector function since the virus is considered to be non-lytic (75). However, the correlation between viral load and poor pregnancy outcome we observed could be explained by subtle (e.g., nonlytic),

toxic effects of the virus on utero-placental tissues or innate mechanisms not examined. If this would be the case, then viral clearance related to immunity would be expected to be protective. This is consistent with our findings. The ability for pregnant animals to clear systemic LCMV, generate anti LCMV memory, and protect against virulent strains (20) tends to mitigate against both central (e.g., deletion in thymus) and systemic (e.g., global, non-thymic) tolerance as a cause for viral persistence in utero-placental tissues. Moreover, it appears in these studies and those previously published that CD8 T cells can traffic to uteroplacental tissues in response to LCMV infection, although this may be due to dysregulation of mechanisms which limit trafficking to this site (76, 77) in the uninfected case.

Existing data suggests that not only chronic infection, but also homeostatic expansion in response to niche, cytokines such as IL-7, or environmental antigen (78) can lead to T cells with a molecular and phenotypic signature similar to "exhaustion" (78), but that the majority of these cells are cleared *via* FAS/FAS ligand interactions. We have observed that maternal T cells may undergo homeostatic expansion, possibly in response to fetal antigen (54, 79). This is associated with the increased expression of molecules such as Il-7 receptor and, in the uterus, expression of PD1, granzyme and downregulation of CD8 (49, 54). This suggests a "quasi exhausted" phenotype which can be relieved systemically (80) and possibly locally through inflammation. The factors driving this phenotype may explain the relative persistence of virus in uteroplacental as opposed to systemic tissues.

Chronic LCMV infection is associated with increased IL-10 production in antigen presenting cells and decreased T cell function. Further, antibody mediated blockade of the IL-10 receptor restores T cell function and enhances viral clearance (28). Consistent with this, in our studies, deficiency in IL-10 is associated with decreased viral load in utero-placental tissues and with a calculated increase in the proportion of CD8 T cells (uterus, **Figure 5A**). This correlated with increased expression of the bona fide activation marker CD44 (81) on uterine and placental CD8 T cells (**Figure 5A**). We were intrigued by the increased CD8 T cell expression of PD1 in both systemic and local tissues of infected IL-10 KO as compared to WT (**Figure 5B**), when we expected this molecule to be downregulated in IL-10 KO cells. We were further somewhat surprised by the contrast with CD44, which occurred at a relatively higher level in CD8 T cells of only local IL-10 KO tissues (**Figure 5A**). We attribute this to the short time-frame of these experiments, as PD1 may be elevated for a time after antigen is cleared and during the revival of exhausted cells [reviewed in (22)]. In addition, we could speculate that genetic deficiency in IL-10 may developmentally alter (upregulate) PD1 even in the uninfected state. PD1 is actually a complex modulator of immunity (82) *via* interaction with a number of ligands at least one of which, PD-L1 is upregulated by IL-10 (22). PD1 signaling is also closely linked to T cell metabolism (83). As such, the highly metabolically active state of the placenta may influence the cellular expression and function of PD1 and its ligands.

Our data, taken in this context, is consistent with a model for a unique phenotype in uterine/placental tissues compared to systemic CD8 T cells (3, 84), which should in the future be analyzed with respect to exact anatomic site (85) and fetal sex (43). From our point of view (1, 86), these data suggest that in local tissues this may not be a phenotype of inherent suppression or limitation. This state may be one of heightened early activation, which may lead to a state which is like exhaustion, but which can be "cured" by tissue-derived factors when needed to clear viral infections. The concept of a unique intrauterine CD8 T cell phenotype- one that exhibits elements of the "exhaustion-that-can-be-relieved" phenotype is supported by studies in other animal models (87–89) and in humans (90–92). Our hope is that consideration of this concept can be utilized to develop therapeutic interventions to prevent congenital infection.

DATA AVAILABILITY STATEMENT

Requests to access the datasets should be directed to Elizabeth A. Bonney, elizabeth.bonney@med.uvm.edu.

ETHICS STATEMENT

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

AUTHOR CONTRIBUTIONS

EB prepared manuscript. All authors participated in experiments and data analysis. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fviro.2022.829991/full#supplementary-material

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Downregulation of Transcriptional Activity, Increased Inflammation, and Damage in the Placenta Following *in utero* Zika Virus Infection Is Associated With Adverse Pregnancy Outcomes

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Zika virus (ZIKV) infection during pregnancy causes serious adverse outcomes to the developing fetus, including fetal loss and birth defects known as congenital Zika syndrome (CZS). The mechanism by which ZIKV infection causes these adverse outcomes, and specifically the interplay between the maternal immune response and ZIKV replication has yet to be fully elucidated. Using an immunocompetent mouse model of transplacental ZIKV transmission and adverse pregnancy outcomes, we have previously shown that Asian lineage ZIKV disrupts placental morphology and induces elevated secretion of IL-1β. In the current manuscript, we characterized placental damage and inflammation during *in utero* African lineage ZIKV infection. Within 48 h after ZIKV infection at embryonic day 10, viral RNA was detected in placentas and fetuses from ZIKA infected dams, which corresponded with placental damage and reduced fetal viability as compared with mock infected dams. Dams infected with ZIKV had reduced proportions of trophoblasts and endothelial cells and disrupted placental morphology compared to mock infected dams. While placental IL-1β was increased in the placenta, but not the spleen, within 3 h post infection, this was not caused by activation of the NLRP3 inflammasome. Using bulk mRNAseq from placentas of ZIKV and mock infected dams, ZIKV infection caused profound downregulation of the transcriptional activity of genes that may underly tissue morphology, neurological development, metabolism, cell signaling and inflammation, illustrating that *in utero* ZIKV infections causes disruption of pathways associated with CZS in our model.

Keywords: flavivirus, inflammation, IL-1β, neurological development, trophoblasts

INTRODUCTION

Pregnant women and their developing fetuses are at high risk for severe outcomes from a variety of viral, bacterial, and parasitic infections including the "TORCH" pathogens (*Toxoplasma gondii*, other, rubella virus, cytomegalovirus, herpes simplex virus), which are grouped because of their propensity to induce congenital disease (1, 2). Viral infections, specifically, often cause adverse consequences during pregnancy for the mother, fetus, or both. Pregnant people are at higher risk than nonpregnant people for severe disease from many viruses, including Influenza A viruses and Hepatitis E virus, which may be due to immunological changes associated with pregnancy (1, 3–5). Viruses also can infect the placenta, fetus, or both to cause adverse consequences, such as loss of pregnancy, preterm birth, birth defects, and growth and neurodevelopmental delays in the fetus and newborn (2, 4).

Zika virus (ZIKV) is a single-stranded positive sense RNA virus in the family *Flaviviridae* that received international and scientific attention in 2015–2016, when the virus spread throughout the Americas causing microcephaly and other congenital malformations, such as problems with hearing, vision, and mobility, in the babies of infected pregnant people (6, 7). The causal connection between ZIKV infection during pregnancy and subsequent birth defects, which are collectively referred to as Congenital Zika Syndrome (CZS), was confirmed with the detection of ZIKV RNA in amniotic fluid of pregnant women carrying children with microcephaly and the isolation of ZIKV from the brain of an infant who died after birth (6, 8). Notably, multiple systematic reviews and meta-analyses performed after the end of the epidemic confirmed the association between ZIKV infection during pregnancy and CZS (9–12).

Despite the causal link between prenatal ZIKV infection and adverse pregnancy and neonatal outcomes, the mechanism by which ZIKV infection contributes to CZS remains unclear. To date, most studies suggest that viral replication in the fetal brain as well as immunopathology induced by maternal inflammation cause adverse outcomes (13, 14). Animal models of ZIKV infection during pregnancy [reviewed in (14)] provide insight into mechanisms of ZIKV pathogenesis. Mouse models are commonly utilized for studies of viral pathogenesis (15), and their short gestation period and large litter sizes provide a benefit for studies during pregnancy (16).

Since the 2016 ZIKV epidemic, we have investigated the pathogenesis of ZIKV infection during pregnancy after intrauterine infection in immunocompetent mice. Collectively, we have shown ZIKV infection during pregnancy causes placental pathology, reduced fetal viability, congenital malformations, and reduced cortical thickness that is induced by maternal inflammation rather than direct virus infection (17, 18). We have identified placental secretion of IL-1β as a cause of inflammation and placental dysfunction (18). Elevated IL-1β signaling during pregnancy damages the placenta by disrupting placental architecture, including distortion of the labyrinth layer and reduction in trophoblast invasion, which may contribute to adverse neonatal health consequences (18, 19). Canonically, IL-1β activation and secretion is dependent on activation of the NLRP3 [nucleotide-binding domain leucine-rich repeat (NLR) and pyrin domain containing receptor 3] inflammasome (20), which is active in placental cells of humans and mice (21–23).

As the organ of the maternal-fetal interface, the placenta is necessary for appropriate fetal development, protection against insults (e.g., viruses), provision of nutrients, oxygen, and waste exchange, and enabling maternal-fetal endocrine cross-talk (2, 16, 24, 25). Microbes associated with congenital disease, including ZIKV, likely have mechanisms to overcome placental defenses, resulting in damage to the placenta and disrupted fetal development [reviewed in (2)]. In the current study, we sought to investigate the mechanisms of placental damage during ZIKV infection, by characterizing the cells and pathways that underly ZIKV-induced placental damage and adverse fetal outcomes.

MATERIALS AND METHODS

Viruses and Cells

Zika virus (ZIKV) strain IB H 30656 (Nigeria, 1968) was purchased from the American Type Culture Collection (ATCC, # VR-1839). The Paraíba (Brazil, 2015) strain was kindly provided by Stephen Whitehead of the National Institute of Allergy and Infectious Diseases. All procedures for handling ZIKV were approved by the Institutional Biosafety Committee. Stocks of ZIKV strains were generated by infecting Vero E6 cells at an MOI of 0.001 50% tissue culture infectious doses (TCID50) per cell in DMEM (Sigma #D5796) supplemented with 2.5% Fetal Bovine Serum (FBS, Gibco #26140079), 1 mM sodium pyruvate (Sigma #S8636), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco #15149-122), and 2 mM l-glutamine (Gibco #25030-081). Approximately 96 h after infection, the supernatant fluids were collected, clarified by centrifugation (900 g for 10 min), and stored in aliquots at −80°C. ASC expressing RAW 264.7 (RAW-ASC) cells (InvivoGen #raw-asc) were grown in DMEM (Sigma #D5796) supplemented with 10% heat-inactivated FBS (Gibco #26140079), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco #26140079), 100 μg/ml Normocin™ (InvivoGen #ant-nr-1), and 10 μg/ml blasticidin (InvivoGen #ant-bl-05). RAW-ASC cells were plated on six well plates, primed with Pam3CSK4 (InvivoGen #tlrl-pms) at a concentration of 100 ng/ml for 3 h and then stimulated with 10 μM of Nigericin (InvivoGen #tlrl-nig) for 6 h as a positive control for inflammasome component westerns.

Experimental Mice

Timed-pregnant adult (2–3 months of age) CD-1 IGS mice were purchased from Charles River Laboratories (strain code 022). Animals arrived at embryonic day 9 (E9) and were housed in pairs in a specific-pathogen-free animal BSL2 facility at Johns Hopkins University with *ad libitum* access to food and water. Mice were acclimated for 24 h prior to experiments (26) and procedures for animal experiments were performed consistently at the same time of day.

ZIKV Infections

At E10, mice were anesthetized continuously with isoflurane mixed with oxygen and underwent mini-laparotomy in the

lower abdomen for ZIKV injections. Animals received either 10^6 TCID50 units of ZIKV in 100 μl or 100 μl DMEM alone as previously described (18, 26). Briefly, the inoculum was divided equally into four injections delivered into the uterine myometrium, opposite the placenta and between the gestational sacs of the first five fetuses closest to the cervix of one uterine horn. The contralateral uterine horn was not manipulated. All placental experiments were performed with placentas from the horn that was either ZIKV or mock inoculated. Routine closure was performed after injections and dams were returned to cages for recovery. Investigators were not blinded to the treatment group allocation.

Tissue Collection

Mice were euthanized by CO_2 exposure followed by cardiac exsanguination at either 3, 6, or 48 h post infection (hpi), depending on the experiment. At the time of euthanasia, the total number of viable and nonviable fetuses was quantified for each pregnant dam. Fetal viability was determined as the percentage of fetuses within the inoculated uterine horn for ZIKV- and mock-infected dams that were viable. Small or discolored fetuses or the absence of a fetus at an implantation site were counted as nonviable. Maternal spleen as well as the uterine horn, placentas, and fetuses of the inoculated horn were collected with tissue either flash frozen in a dry ice–isopropanol bath or fixed in 4% paraformaldehyde (Fisher Scientific # AAJ19943K2). All animal studies were conducted under animal BSL2 containment.

Total RNA Extraction and qRT-PCR

Tissues were weighed and homogenized in 1 ml of QIAzol Lysis Reagent (QIAGEN #1023537) using Lysing Matrix D tubes (MP Biomedicals #6913100) in a MP Fast-prep 24 5G instrument. Total RNA was extracted from tissue samples using the RNeasy Lipid Tissue Mini Kit (QIAGEN #74804), according to the manufacturer's instructions. ZIKV RNA copies were determined by one-step qRT-PCR reaction using the QuantiTect Probe RT-PCR Kit (QIAGEN #20443) according to the manufacturer's protocol. The real-time PCR primers and probe for ZIKV RNA detection were the ZIKV 1162c set described previously (27), and the primer sequences and concentrations were as follows: Fwd (100 μM), 5′-CCGCTGCCCAACACAAG-3′; Rev (100 μM), 5′-CCACTAACGTTCTTTTGCAGACAT-3′; probe (25 μM), 5′-/56-FAM/AGCCTACCT/ZEN/TGACAAGC AATCAGACACTCAA/3IABkFQ/-3′ (Integrated DNA Technologies). ZIKV RNA copies were determined relative to a standard curve produced using serial 10-fold dilutions of ZIKV RNA isolated from ZIKV stocks with a known infectious virus titer.

Hematoxylin and Eosin Staining and Immunohistochemistry

At 48 hpi (E12), mice were euthanized and placentas were fixed overnight at 4°C in 4% PFA. The following day, placentas were washed five times with PBS and immersed in 30% sucrose until saturation. Using a Leica CM1950 cryostat, the specimens were cut at 20-μm thickness and mounted on positively charged slides (Fisher Scientific #12-550-15). Routine hematoxylin and eosin (H&E) staining was performed to evaluate the morphological change of the placentas. For immunohistochemical staining, antigen retrieval was performed by boiling in citrate buffer (pH 6, Vector Lab, H-3300-250) for 20 min. Slides were washed with PBS, which was followed by permeabilization in PBS solution containing 0.05% Triton X-100 and 10% normal goat serum (Invitrogen #50197Z) for 30 min. Placentas were incubated with mouse anti-flavivirus group antigen (1:100 ThermoFisher #MA1-7397), rabbit anti-vimentin (1:200, Abcam # ab92547), or rabbit anti-cytokeratin (1:200, Dako #Z0622) overnight at 4°C. The next day, sections were rinsed with PBS and then incubated with donkey anti-mouse (ThermoFisher #R37115) or donkey anti-rabbit (ThermoFisher #R37119) fluorescent secondary antibodies (ThermoFisher #R37115) diluted 1:500 for 3 h at room temperature. DAPI (Roche #10236276001) was applied for counterstaining, followed by mounting with Fluoromount-G (eBioscience #00-4958-02). Cell density of vimentin and cytokeratin positive cell quantification was performed using Image J (1.47v). For each placenta, six random images in labyrinth at the middle level (thickest) of placenta were taken, and the average fluorescent area calculated for that placenta. One placenta per dam was used and 3–4 dams per group were analyzed.

TUNEL Staining

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) was performed with Click-iT™ Plus TUNEL Assay (ThermoFisher #C10617) per the manufacturer protocol. Placental slides were fixed with 4% paraformaldehyde in PBS for 15 min at 37°C, rinsed with PBS, and permeabilized with proteinase K solution for 15 min at RT followed by staining with TUNEL working solution for 60 min at 37°C. Specimens were mounted on glass slides using Fluoromount-G (eBioscience #00-4958-02). For each placenta, 10 random images in labyrinth at the middle level (thickest) of placenta were taken, and the average percentage TUNEL+ (TUNEL+ fluorescent signal divided by the DAPI fluorescent signal) given for that placenta. One placenta per dam was used and 3–4 dams per group were analyzed.

In situ Hybridization

In situ hybridization was performed using RNAscope Multiplex Fluorescent Reagent Kit v2 Assay (ACDBio #323136). Following the protocol provided by the manufacture, RNAscope® Probe-V-ZIKV (ACDBio #467771) was applied to detect the viral signal in fetuses. All the images were viewed using a Zeiss Axioplan 2 microscope (Jena, Germany). Images were taken using a Zeiss AxioCam MRM.

IL-1β ELISA

Flash frozen placentas were weighed and homogenized in a 1:10 weight per volume of PBS using Lysing Matrix D tubes (MP Biomedicals #6913100) in a MP Fast-prep 24 5G instrument. Placental homogenates were stored at −80 °C until analysis. IL1β in placental homogenates was measured by ELISA according to the manufacturer's protocol (Abcam #100704).

Western Blot

Flash frozen placentas were weighed and homogenized in 1× Cell Lysis Buffer (Cell Signaling Technology #9803) with 1× Protease Inhibitor cocktail (Sigma-Aldrich #P8340) and 10 mM sodium fluoride (Fisher Scientific #S299 100) (20 μl lysis buffer per mg tissue). Protein lysates were stored at −80°C until analysis. The protein concentration of each lysate was measured using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific #23225). For each sample, 25 μg of protein was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on NuPAGE 4–12% Bis-Tris gels (Thermo Fisher Scientific #NP0329). The gel was blotted onto Immobilon-FL PVDF Membrane (Millipore #IPFL00010) and the membranes were blocked using a 1:1 mixture of 1× PBS/Tween-20 solution (Sigma-Aldrich #P3563) and Intercept blocking buffer (LI-COR Biosciences #927-70001) for 30 min at room temperature. The membranes were treated with primary antibody diluted in blocking solution overnight at 4°C on a rocker. The membranes were washed with PBS-Tween thrice (10 min per wash) and incubated in secondary antibody solutions for 60 min at room temperature on a rocker. The membranes were washed thrice (10 min per wash) in PBS-Tween and imaged on a ProteinSimple FluorChem Q imager. Individual bands were quantified using Image Studio software (LI-COR Biosciences; version 3.1.4). The signal from each band was normalized against the GAPDH signal as a loading control and graphed as arbitrary units. Primary antibodies included: rabbit anti-phospho-NF-κB p65 (Ser536) (Cell Signaling #3033), mouse anti-NFkB p65 (Thermo Fisher Scientific #33-9900), rabbit anti-NLRP3 (Thermo Fisher Scientific #MA5-32255), rat anti-caspase 1 (Thermo Fisher Scientific #14-9832-82), and mouse anti-GAPDH (Abcam #ab8245). Secondary antibodies included: goat anti-mouse Alexa Fluor 488 (Thermo Fisher Scientific #A11001), donkey anti-rabbit Alexa Fluor Plus 647 (Thermo Fisher Scientific #A32795), and goat anti-rat Alex Fluor 647 (Thermo Fisher Scientific #A21247).

mRNAseq and Analysis

Quantitation of Total RNA was performed with the Qubit BR RNA Assay kit and Qubit Flex Fluorometer (Invitrogen/ThermoFisher), and quality assessment performed by RNA ScreenTape analysis on an Agilent TapeStation 2200. Barcoded libraries for mRNA-Seq were prepared from 100 ng Total RNA using the Tecan Universal Plus mRNA Seq Library kit with NuQuant, according to manufacturer's recommended protocol. Quality of libraries was assessed by High Sensitivity DNA Lab Chips on an Agilent BioAnalyzer 2100. Quantitation was performed with NuQuant reagent, and confirmed by Qubit High Sensitivity DNA assay, on Qubit 4 and Qubit Flex Fluorometers (Invitrogen/ThermoFisher). Libraries were diluted, and equimolar pools prepared, according to manufacturer's protocol for appropriate sequencer. An Illumina iSeq Sequencer with iSeq100 i1 reagent V2 300 cycle kit was used for final quality assessment of the library pool. For deep mRNA sequencing, a 200 cycle (2 × 100 bp) Illumina NovaSeq SP run was performed at Johns Hopkins Genomics, Genetic Resources Core Facility, RRID:SCR_018669.

iSeq and NovaSeq data files were uploaded to the Partek Server and analysis with Partek Flow NGS software, with RNA Toolkit, was performed as follows: pre-alignment QA/QC; trimming of reads; alignment to mm 39 Reference Index using STAR 2.7.8a; post- alignment QA/QC; quantification of gene counts to annotation model (Partek E/M, Ensembl Transcript Release 103); filter and normalization of gene counts; and, identification and comparison of differentially expressed genes with GSA (gene specific analysis). From resulting gene lists, clustering and biological interpretation was performed. Ingenuity Pathways Analysis software (Qiagen) was used to identify molecular networks of relevance, and the pathways and biological processes most significantly related in the data sets.

All sequence files and sample information have been deposited at NCBI Sequence Read Archive, NCBI BioProject: BioProject: accession number PRJNA797437.

Statistical Analysis

Fetal viability data were analyzed with a χ^2 test. ZIKV RNA copies, TUNEL staining, IHC quantification, and Western Blot data were analyzed with unpaired two-tailed *t*-tests. IL-1β ELISA data were analyzed with two-way ANOVA with Bonferroni's *post hoc* for multiple comparisons. Data are presented as mean ± SEM. Mean differences were considered statistically significant at $p < 0.05$. Statistical analyses were performed using GraphPad Prism v9.2 (GraphPad Software).

RESULTS

In utero Exposure to ZIKV Causes Placental Infection, Vertical Transmission, and Fetal Loss

Intrauterine infection of immunocompetent pregnant mice at E10 or E14 with either African or Asian lineage strains of ZIKV causes fetal infection and demise (18, 26). The use of a mouse-adapted ZIKV strain was hypothesized to cause a more robust phenotype (28). Therefore, the historic IB H 30,656 Nigeria 1968 (Nigeria ZIKV) strain, which has been passaged 21 times in suckling mice (29) was used. Consistent with previous studies using clinical isolates of South American ZIKV (18, 26), intrauterine infection at E10 with Nigeria ZIKV reduced fetal viability at 48 h post infection (hpi) compared with mock inoculated dams (**Figure 1A**).

ZIKV RNA copies were quantified at 48 hpi in placentas and spleens of dams infected with ZIKV at E10 (**Figure 1B**). ZIKV RNA was highly detectable in placentas and spleens of infected animals at 48 hpi (**Figure 1B**). ZIKV protein also was robustly detected in the labyrinth of placentas (**Figure 1C**, representative image) suggesting that infection may disrupt gas and nutrient exchange between mother and fetus.

Since Nigeria ZIKV has already been shown to productively infect fetuses, a 2015 Brazil clinical isolate (Brazil ZIKV) was used to infect pregnant dams at E10, with fetuses collected 48 hpi to measure the presence ZIKV RNA in the whole body by ISH. Consistent with previous studies using only quantitative PCR of fetal heads or bodies (21, 26, 28, 30), ZIKV RNA was

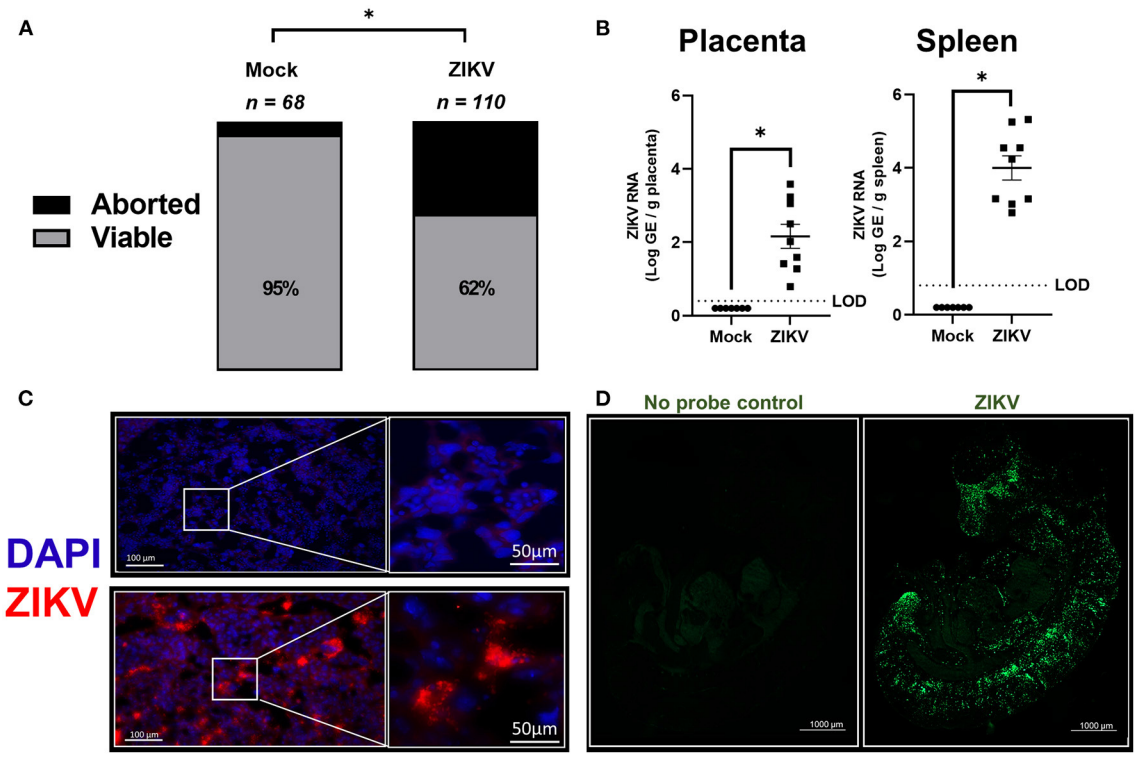

FIGURE 1 | *In utero* exposure to ZIKV causes placental infection, vertical transmission, and fetal loss. At embryonic day (E) 10, pregnant mice underwent intrauterine injection of 10^6 $TCID_{50}$ units of Nigeria ZIKV, Brazil ZIKV, or media (mock). **(A)** At 48 h post infection (hpi), dams were euthanized, and fetal viability was determined as the percentage of fetuses within the inoculated uterine horn (n = total number of fetuses from 11 to 16 dams per group from two independent replicates). **(B)** Nigeria ZIKV RNA copies were quantified from placenta or spleen collected 48 hpi. Data represent mean ± standard error of the mean from two independent replications (n = 7–9/group). **(C)** Placentas collected 48 hpi from either mock (upper panel) or Nigeria ZIKV (lower panel) infected dams were paraformaldehyde fixed and immunostained for ZIKV (red) and 4′6-diamidino-2-phenylindole (DAPI, blue) to label nuclei. Representative images taken at 20× magnification (left) and further zoomed 5.5 fold (right) are shown. **(D)** ZIKV RNA (green) was measured in fetuses of Brazil ZIKV infected dams by *in situ* hybridization (ISH) at 48 hpi. Samples were also imaged without RNA probe incubation as a control (left) to remove background and a representative image at 5× magnification is shown for fetuses of ZIKV infected dams (right). Significant differences (*$p < 0.05$) were determined by χ^2 test **(A)** or unpaired two tailed t-test **(B)**. Limit of detection (LOD) indicated with a dashed line. Scale bar: 100 or 50 µm (IHC, **C**), 1,000 µm (ISH, **D**).

predominantly localized to the developing fetal head and spinal cord (**Figure 1D**). Taken together with previously published studies (18, 26), these data illustrate that both African and Asian strains of ZIKV traverse the placenta and vertically infect fetuses with a tropism for the central nervous system, which is consistent with CZS.

ZIKV Infection Induces Placental Damage, Cell Death, and Disruption of Trophoblast and Endothelial Cell Layers

To further characterize the phenotypic placental damage caused by *in utero* ZIKV infection, placental morphology was analyzed (**Figure 2A**). Placentas of Nigeria ZIKV, collected at 48 hpi, showed significant damage, characterized by hemorrhage and mixing of maternal and fetal blood in the labyrinth as compared with placentas from mock infected dams (**Figure 2A**, highlighted in the lower panels), suggesting the loss of barrier function of trophoblast–endothelial cell layers. To define whether placental tissue damage included cell death, TUNEL staining in the labyrinth of placentas was performed, with the percentage of TUNEL positive cells quantified and reported as a proportion of DAPI+ cells (**Figure 2B**). The percentage of TUNEL+ cells in placentas of ZIKV infected dams was nearly double that of placentas of mock infected dams (**Figure 2B**, 3.3% in mock and 6.3% in ZIKV, $p = 0.0186$), indicating that ZIKV infection during pregnancy induces cell death in the placenta.

To further characterize the damage to the placenta, changes to the trophoblast-endothelial cell barrier, which separates maternal and fetal blood in the labyrinth of the murine placenta (16, 25), was evaluated. Using cytokeratin (trophoblasts, **Figure 3A**) and vimentin (endothelial cells, **Figure 3B**) staining of placentas collected at 48 hpi, there was a significant loss of trophoblasts in placentas of ZIKV infected dams as indicated by a 51.5% reduction ($p = 0.0458$) in cytokeratin positive-area compared with mock placentas (**Figure 3A**, representative image and quantification). Similarly, disruption of the endothelial cell layer was observed, which could be seen morphologically by the loss of the streak-like vessel integrity seen in placentas of mock inoculated dams (**Figure 3B**, representative image).

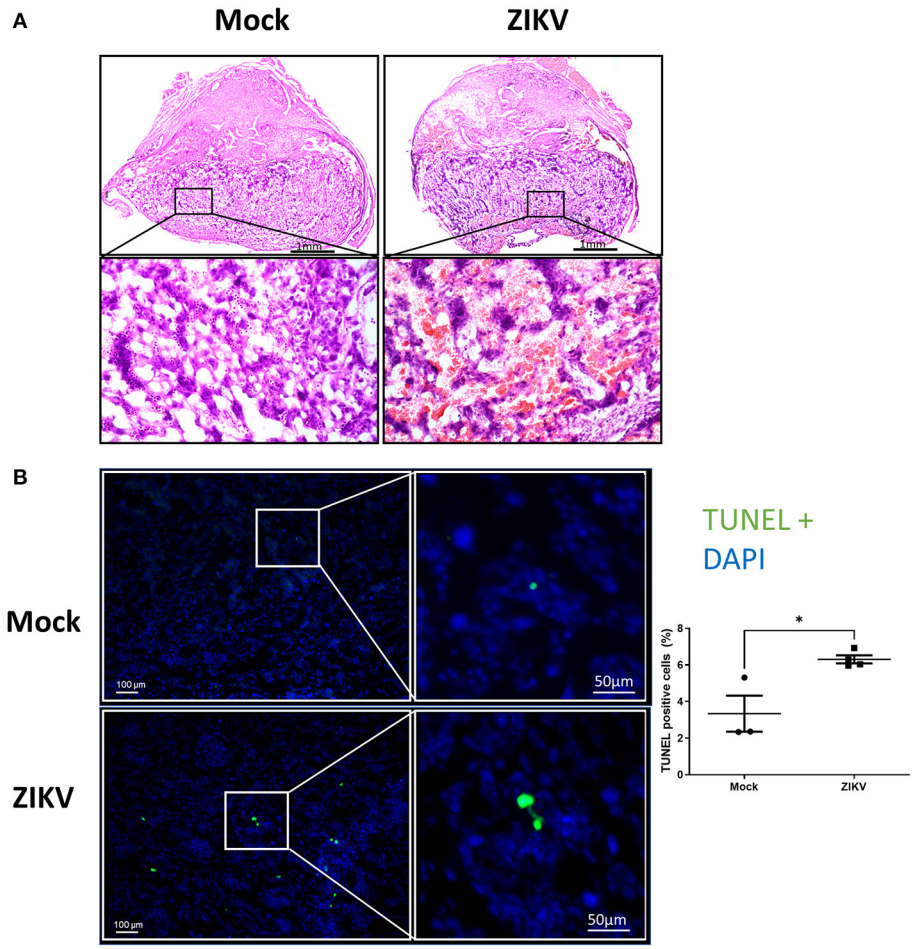

FIGURE 2 | ZIKV infection induces placental damage and cell death. At E10, pregnant mice underwent intrauterine injection of 10^6 $TCID_{50}$ units of Nigeria ZIKV or media (mock). Dams were euthanized at 48 hpi and placentas were paraformaldehyde-fixed. **(A)** Representative H and E images were taken at 5× magnification (upper panels) and 20× magnification (lower panels). **(B)** Representative TUNEL+ (green) stained placentas with DAPI to label nuclei taken at 100× magnification (left) and quantification of TUNEL+ cells as a percentage of DAPI+ cells. Data represent mean ± standard error of the mean (n = 3–4 dams/group, each dot indicates 1 placenta and is the mean quantification of 10 fields of view). Significant differences (*$p < 0.05$) were determined by unpaired two tailed t-test. Scale bar: 1 mm (HandE, **A**), 100 or 50 µm (TUNEL, **B**).

ZIKV infection caused a 34% decrease in vimentin-positive area compared to mock infected placentas (**Figure 3B**). Together, these data suggest that *in utero* ZIKV infection damages the placenta, including the placental labyrinth zone and disrupts both the trophoblast and endothelial cell layers of the maternal-fetal barrier. Disruption of the morphology of the labyrinth zone can compromise oxygen, nutrient, and waste exchange and impact fetal growth and viability (24, 25).

Increased Placental IL-1β in the Absence of NLRP3 Inflammasome Upregulation

Previous studies have shown that IL-1β signaling during pregnancy induces placental damage and disruption of the placental labyrinth (19). To test the hypothesis that IL-1β underlies placental damage following infection with African lineage mouse adapted ZIKV, pregnant dams were infected at E10 with Nigeria ZIKV and placentas and spleens were collected at either 3, 6, or 48 hpi to measure IL-1β in tissue homogenates (**Figures 4A,B**). In placentas from ZIKV infected dams, IL-1β was increased at 3 hpi to levels consistent with systemic IL-1β treatment (19), and returned to baseline levels that were equivalent to placentas from mock inoculated dams by 6 hpi (**Figure 4A**) and 48 hpi (data not shown). No significant change in IL-1β concentrations in the spleen was observed with ZIKV infection (**Figure 4B**), suggesting that the increased placental IL-1β does not originate from systemic circulation.

IL-1β expression and release is canonically dependent upon the activation of inflammasomes, including the NLRP3 inflammasome (20), and both murine and human placental cells have the capacity to activate the NLRP3 inflammasome to release IL-1β (22, 23). Induction of NF-κB is a key component of the priming step of inflammasome activation (31). We hypothesized that the NLRP3 inflammasome would be activated by ZIKV infection of the placenta and might underlie elevated placental

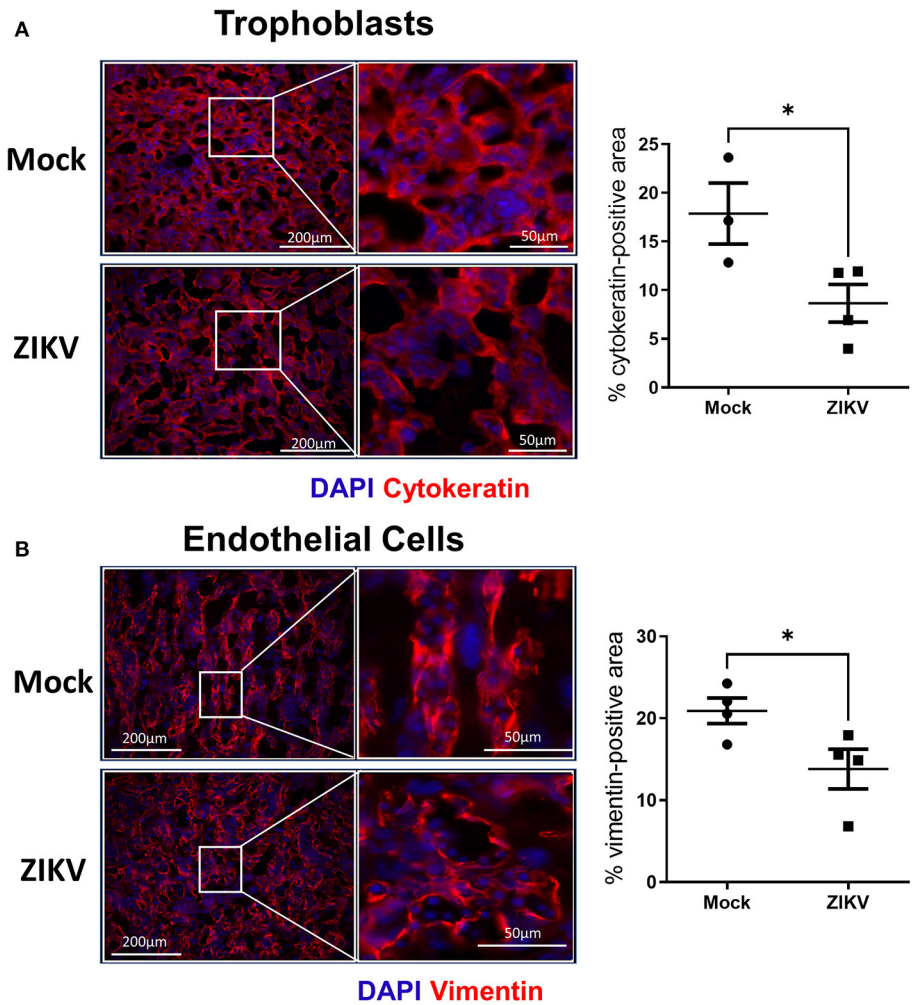

FIGURE 3 | ZIKV infection disrupts the trophoblast and endothelial layer of the labyrinth zone. At E10, pregnant mice underwent intrauterine injection of 10^6 $TCID_{50}$ units of Nigeria ZIKV or media (mock). Dams were euthanized at 48 hpi, and placentas were paraformaldehyde-fixed and immunostained for cytokeratin (**A**, red) to mark trophoblasts or vimentin (**B**, red) to mark endothelial cells and DAPI (blue) to label nuclei. Representative images taken at 20× magnification (left) and further zoomed (3.7- and 2.4- fold for trophoblasts and endothelial cells, respectively, right) are shown. Quantification of the percentage positive area for each marker is shown. Data represent mean ± standard error of the mean (n = 3–4 dams/group, each dot indicates 1 placenta and is the mean quantification of 6 fields of view). Significant differences (*$p < 0.05$) were determined by unpaired two tailed t-test. Scale bar: 200 µm.

secretion of IL-1β. Using Nigeria ZIKV, pregnant dams were infected at E10 and euthanized at 3 hpi during peak placental IL-1β secretion and at 6 hpi, after IL-1β returned to baseline. Protein expression of components of the NLRP3 inflammasome cascade, including phosphorylated (at Ser536) and total-NF-κB p65, NLRP3 and caspase 1 (**Figures 4C,D** for blots, **Figures 4E,F** for quantification) were quantified in placental homogenates, relative to GAPDH (**Figures 4E,F**).

Protein expression of pNF-κB p65 in the placenta did not differ between ZIKV and mock infected dams at either timepoint (**Figures 4C,E** for 3 hpi and **Figures 4D,F** for 6 hpi). Placental NLRP3 expression in ZIKV compared to mock infected dams also did not differ at either timepoint (**Figures 4C,E** for 3 hpi and **Figures 4D,F** for 6 hpi). Together with the expression of pNF-κB p65, this is evidence that intrauterine ZIKV infection is not inducing NLRP3 inflammasome priming in the placenta at 3 or 6 hpi. Cleavage of pro-caspase 1 occurs upon assembly of inflammasomes, including the NLRP3 inflammasome, activating caspase 1 to process downstream products including IL-1beta, IL18, and Gasdermin D (32, 33). Measurement of cleaved caspase 1 is used as an indicator of inflammasome activation (19, 34, 35). Protein expression of the 20 kDa cleaved caspase 1 subunit was below the level of detection in placentas of both mock and ZIKV infected dams, while pro-caspase 1 was detectable and did not differ between ZIKV and mock infected dams (**Figures 4C,E** for 3 hpi and **Figures 4D,F** for 6 hpi). To ensure that the antibody utilized for measuring cleaved caspase-1 was functional, caspase-1 was measured in ASC expressing RAW 264.7 (RAW-ASC) cells, a mouse macrophage cell line capable of activating the NLRP3 inflammasome and illustrated robust expression (**Figure 4G**).

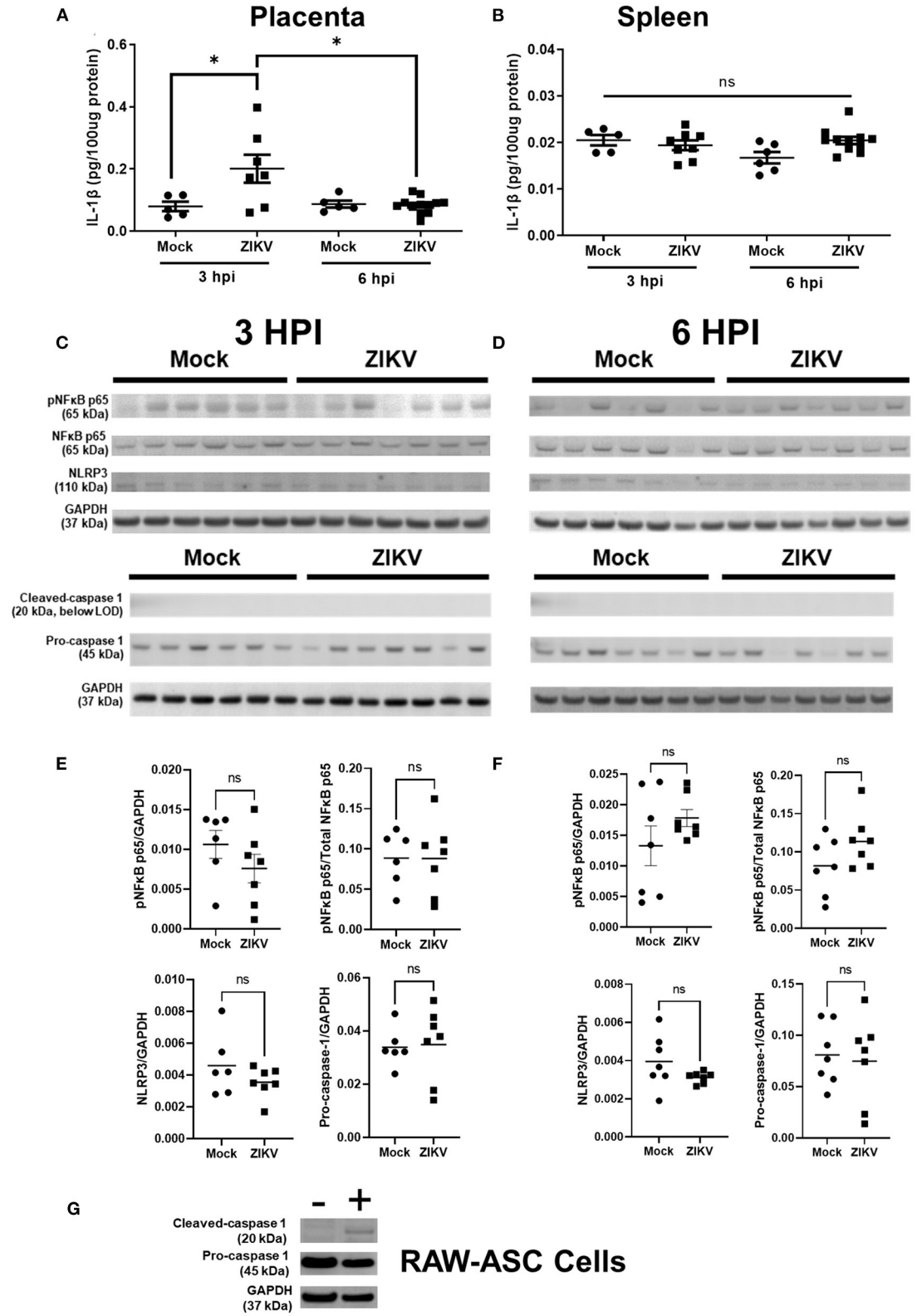

FIGURE 4 | Intrauterine ZIKV infection transiently increases the IL-1β concentration but does not induce NLRP3 inflammasome upregulation or activation in placenta. At E10, pregnant mice underwent intrauterine injection of 10^6 $TCID_{50}$ units of Nigeria ZIKV or media (mock). Placentas **(A,C–F)** and spleens **(B)** were collected at 3 hpi

(Continued)

FIGURE 4 | (C,E) or 6 hpi **(D,F)**, homogenized, and analyzed by ELISA **(A,B)** or Western blot for phosphorylated (p)NFκB p65, total NFκB p65, and NLRP3 or cleaved-caspase 1 (20 kDa cleavage fragment) and pro-caspase 1. GAPDH is shown as the loading control. Placentas from two independent experiments were analyzed together to avoid gel to gel variability **(C,D)**. Quantified protein expression **(E,F)** of pNFκB p65, NLRP3, and pro-caspase 1 in the placenta is shown as the fluorescence signal for each protein normalized to GAPDH. Phosphorylation of NFκB p65 is also shown as a proportion of total NFκB p65 per placenta. As a positive control for caspase-1 expression, RAW-ASC cells were primed and stimulated and cell lysate collected for Western blot analysis **(G)** Data represent mean ± standard error of the mean [n =5–12/group (ELISA), 6–7/group (WB)]. Significant differences were determined (*$p < 0.05$; ns, not significant) were determined by unpaired two tailed *t*-test (WB) or two-way ANOVA with Bonferroni *post hoc* test (ELISA).

Taken together, these data indicate that the NLPR3 inflammasome is not activated in the placenta in response to intrauterine Nigeria ZIKV infection.

ZIKV Infection Downregulates Transcriptional Activity in the Placenta, Including Genes Associated With Cellular Function and Development

To transcriptionally evaluate the role of ZIKV infection and its contribution to the observed placental damage and disruption of substrate exchange in the placental labyrinth, we performed bulk mRNA sequencing of placentas from Nigeria ZIKV and mock inoculated dams collected 48 hpi. Utilizing standard cutoffs of a p-value ≤ 0.05 and fold change $-2<$ or >2, there were 928 differentially expressed genes between placentas of ZIKV and mock inoculated dams. Of these 928 genes, the majority (794) were downregulated in response to ZIKV infection (**Figure 5A**), revealing that ZIKV induces broad downregulation of host gene expression in the placenta during infection. To further characterize the effects of ZIKV infection on placental function, expression results of the 928 differentially expressed genes were imported into QIAGEN Ingenuity Pathway Analysis (IPA) to evaluate the interactions and associations of genes up or downregulated in the placenta during ZIKV infection (**Figures 5B–F**). The top five networks of diseases and functions containing genes differentially expressed in the placenta during ZIKV infection were "Cellular Development, Gene Expression, Tissue Morphology (**Figure 5B**)", "Cell Signaling, Cell-To-Cell Signaling and Interaction, Nucleic Acid Metabolism (**Figure 5C**)", "Cell-To-Cell Signaling and Interaction, Molecular Transport, Small Molecule Biochemistry (**Figure 5D**)", "Endocrine System Disorders, Gastrointestinal Disease, Immunological Disease (**Figure 5E**)", and "Digestive System Development and Function, Neurological Disease, Ophthalmic Disease (**Figure 5F**)". Broadly, this network analysis further showcases the downregulation of host genes in the placenta by ZIKV infection (**Figures 5B–F**, green indicates significant downregulation). Genes associated with tissue morphology (**Figure 5B**) were downregulated by ZIKV infection between 2.056- and 1827.577-fold (fold change of specific genes indicated by shade) supporting the role of ZIKV in the morphological changes seen in the labyrinth of the placenta (**Figures 2A**, **3B**). Genes associated with key cellular functions, including gene expression (**Figure 5B**), cellular signaling (**Figures 5C,D**), nucleic acid metabolism (**Figure 5C**), and molecular transport (**Figure 5D**) were downregulated between 2.004- and 947.01-fold by ZIKV infection, potentially causing cellular stress and the tissue damage observed histologically in placental slices (**Figures 2**, **3**). Expression of genes associated with endocrine, gastrointestinal, immunological, neurological, and ophthalmic development and diseases were downregulated between 2.021- and 1348.931-fold by ZIKV infection (**Figures 5E,F**), consistent with evidence associating placental neurodevelopment gene expression with child neurobehavior (36, 37). Placental and fetal development is a tightly regulated process (16, 38), and downregulated gene expression changes induced in the placenta by ZIKV infection may have long-term consequences on the fetus.

DISCUSSION

Placental inflammation, dysfunction, and damage are major contributing factors to adverse perinatal outcomes during maternal infections (39–43). Intrauterine infection of dams with diverse ZIKVs cause productive infection of the placentas and fetuses, disrupt the placental architecture, and reduce fetal viability. Increased secretion of IL-1β, but not other proinflammatory cytokines, in the placenta causes adverse fetal outcomes in our model (18). In this study, we sought to further characterize the placental damage observed after intrauterine ZIKV infection, both transcriptionally as well as at cellular and tissue levels. Disruption of the maternal-fetal barrier, including cell death and significant reductions in both trophoblasts and endothelial cells within the labyrinth zone, were observed. ZIKV also induced dramatic downregulation of genes in the placenta, including those associated with cellular functions and tissue morphology, which may provide a potential transcriptional mechanism of the observed damage to both the placenta and fetal tissue.

Phylogenetic analyses indicate two major lineages of ZIKV, the Asian lineage and the African lineage, with viruses of the 2015–2017 epidemic clustering within the Asian lineage (44, 45). While lineage-specific mutations exist (45, 46), the contributions of these mutations to pathogenesis and development of CZS remains unresolved. Most studies indicate that African lineage strains are more virulent than Asian lineage strains *in vivo* (47–49). For example, African lineage viruses replicate to higher titers in fetal organs than do Asian lineage viruses in a porcine model (49). The molecular mechanisms that define these lineage-dependent differences remain unclear. In our model of intrauterine ZIKV infection of pregnant mice, both Asian (Brazil, 2015; Puerto Rico, 2015) and African (Nigeria, 1968) lineage viruses replicate in the

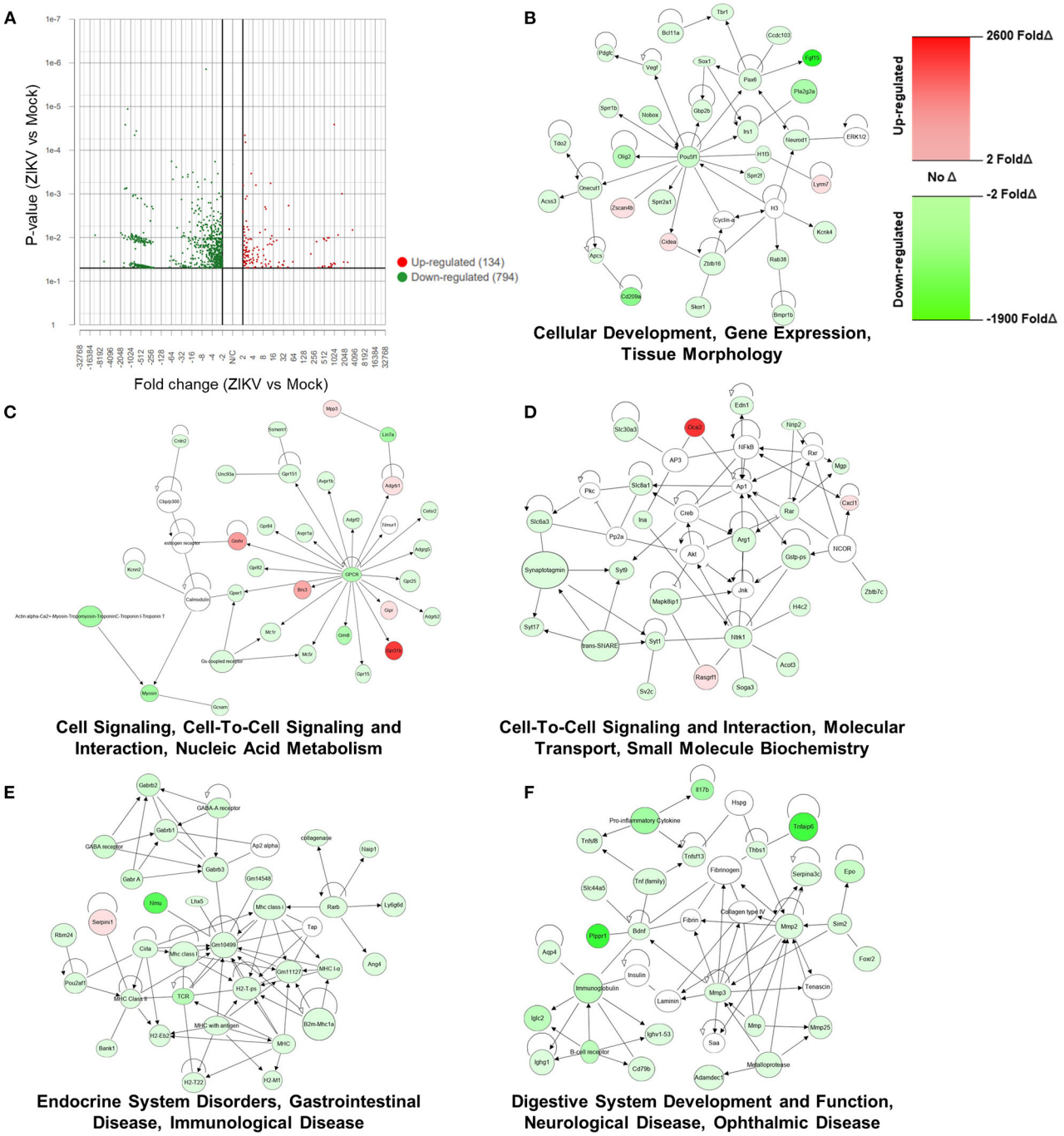

FIGURE 5 | ZIKV infection downregulates host mRNA expression in the placenta, including genes associated with cellular function and development. At E10, pregnant mice underwent intrauterine injection of 10^6 $TCID_{50}$ units of Nigeria ZIKV or media (mock). Dams were euthanized at 48 hpi, placentas collected, and RNA extracted. mRNA libraries for NGS were prepared with Tecan Genomics Universal Plus mRNA Seq kit and sequenced with a 200 cycle (2 × 100 bp) SP run on an Illumina NovaSeq 6,000 and analyzed in Partek® Flow® **(A)** and QIAGEN Ingenuity Pathway Analysis **(B–F)**. A Volcano Plot showing the 928 genes that were differentially expressed between ZIKV and mock placentas using cut-offs of p-value ≤ 0.05 and fold change $-2<$ or >2. Red indicates upregulation and green indicates downregulation; individual dots indicate individual genes **(A)**. The top five networks associated with the 928 differentially expressed genes, were identified using Ingenuity Pathway Analysis. Red indicates significant upregulation and green indicates downregulation with the shade indicating the magnitude of the fold change **(B)**.

placenta, induce placental damage, upregulate placental IL-1β secretion, and reduce fetal viability (18, 26). Additionally, both Asian and African strains vertically transmit, with ZIKV detectable in fetal tissues, including the brain and spinal cord, and cause cortical thinning, congenital malformations, and behavioral deficits in offspring (18, 26). Our data add to a growing body of literature suggesting that both Asian and African lineage viruses have the capacity to cause adverse perinatal outcomes.

We have shown previously that adverse perinatal outcomes, including disruption of the placental architecture, congenital malformations, and reduced fetal viability are caused by transient elevation of placental IL-1β, which is reversed by coadministration of an IL-1 receptor antagonist (18). Elevated IL-1β secretion occurs after intrauterine infection with either Asian [Brazil, 2015; (18)] or African (Nigeria, 1968) lineage viruses. Further, studies of intrauterine inflammation in the absence of replicating virus infection through intrauterine injection of lipopolysaccharide further reveal a role for IL-1β as a key mediator of perinatal brain injury and disrupted offspring neurodevelopment (19, 50–53). Consistent with these observations, treatment of dams with recombinant IL-1β at E14-E17 of pregnancy results distortion of the placental labyrinth structure, decreased numbers of mononuclear trophoblast giant cells, and reduced proportions of endothelial cells as compared to placentas from control dams, with fetal brains exhibiting evidence of reduced cortical neuronal morphology (19). Collectively, these data suggest that IL-1β is a significant mediator of placental dysfunction and adverse perinatal outcomes. The molecular mechanism of increased placental IL-1β secretion, however, has not been reported. Based on results from the current study, the NLRP3 inflammasome was not activated in response to ZIKV infection in the placenta. Evidence exists, however, for IL-1β activation and secretion *via* non-canonical pathways (54–57), which should be considered in future studies.

During gestation, the placenta is the site of nutrient, oxygen, and waste exchange between the mother and fetus. In mice, this occurs specifically in the placental labyrinth, a structure where maternal and fetal blood are separated by three layers of trophoblasts and one layer of endothelial cells (16, 24, 25, 58). Evidence from diverse models indicates that deficits in maternal-fetal substrate exchange cause adverse fetal outcomes, including intrauterine growth restrictions and abortion (38, 59–62). We and others have previously observed placental damage after ZIKV infection (18, 26, 30), a finding also seen in human term placentas (63). In the current study, we have further defined this damage as a significant loss of trophoblasts and endothelial cells within the maternal-fetal barrier, and identified transcriptional changes associated with the observed placental damage. Others have focused on transcriptionally profiling cells infected with ZIKV *in vitro* [reviewed in (64)] or have used human placental tissue at-term, well after ZIKV infection has been controlled (65, 66). In contrast, we utilized placentas collected during peak ZIKV replication (26) and found robust downregulation of genes, including those involved in cellular function and tissue modeling. As placental and fetal (including fetal neurodevelopment) is a highly regulated process (16, 24, 25, 38), these transcriptional changes may have long-lasting effects on the offspring, in addition to underpinning the observed placental damage.

Infection with viruses, including influenza A viruses, Cytomegalovirus, and Hepatitis E virus, during pregnancy also result in maternal inflammation, transplacental and fetal infections, or both (1, 2, 13). There is a growing need to elucidate the role of virus replication vs. maternal inflammation as mediators of adverse perinatal outcomes from infections, including SARS-CoV2. Several studies illustrate SARS-CoV2 infection during pregnancy contributes to negative maternal and fetal outcomes, such as preterm birth, although presence of vertical transmission is debated (67–69). Animal models of viral infections during pregnancy will be integral for identifying the molecular mechanisms of adverse pregnancy outcomes.

DATA AVAILABILITY STATEMENT

mRNAseq data have been deposited at NCBI Sequence Read Archive, NCBI BioProject accession number PRJNA797437. Other data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Johns Hopkins Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

SK, IB, PC, JL, MS, AP, HN, and ACh conceptualized and designed the experiments. PC, JL, MS, HN, ACh, and IB performed animal experiments. PC, MS, HN, and AP grew and quantified viruses. PC, JL, ACh, AD, AJ, and ACa performed assays. PC, MS, AD, and ACa statistically analyzed and graphed data. PC and SK wrote the manuscript with input from all authors. All authors read and provided edits to drafts and approved the final submission.

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REVIEW
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Vertical Zika Virus Transmission at the Maternal-Fetal Interface

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Zika virus (ZIKV) is spread by mosquito bites or *via* sexual or vertical transmission. ZIKV-infected adults are generally asymptomatic, but can display mild symptoms including fever, joint pain, rash and conjunctivitis. However, during pregnancy, vertical ZIKV transmission can cause placental dysfunction and elicit severe fetal defects, including microcephaly, retinopathy, fetal growth restriction and/or stillbirth. Since no FDA-approved vaccine or anti-viral agents are currently available, ZIKV infection poses a global maternal-fetal health challenge. The maternal-fetal interface consists of maternal decidual and immune cells as well as fetal-derived trophoblasts. Compared to other cell types at the maternal-fetal interface, syncytiotrophoblasts, which form the outer layer of floating villi, are less-permissive to ZIKV, thereby preventing ZIKV transmission to the underlying cytotrophoblasts and/or other cells such as Hofbauer cells or fetal endothelium in the villi. However, anchoring villi are tightly attached to the decidua and their cytotrophoblastic cell columns are ZIKV-permissive, suggesting this location as the most likely site of ZIKV vertical transmission. Thus, at the maternal-fetal interface, maternal decidual cells likely serve as a reservoir of ZIKV persistence since they: 1) overexpress viral entry molecules compared to trophoblasts; 2) are highly permissive to ZIKV infection in a gestational age-dependent manner (more easily infected earlier in gestation); 3) augment ZIKV infection of weakly permissive primary cytotrophoblast cultures; and 4) display local maternal-immune tolerance, which prolongs ZIKV survival to facilitate fetal transmission. This review focuses on molecular mechanisms underlying ZIKV infection of cells at the human maternal-fetal interface, thus highlighting how decidual cells enhance propagation of ZIKV in extravillous cytotrophoblasts and why development of agents that eliminate ZIKV persistence in reproductive tissues before pregnancy is crucial to prevent perinatal ZIKV transmission.

Keywords: Zika virus, decidual cells, trophoblasts, maternal-fetal interface, vertical transmission

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INTRODUCTION

Throughout pregnancy, the placenta is a specialized tissue that acts as a physical and immunological barrier against invading pathogens to protect the developing fetus from infectious agents. Unlike most viruses, which cannot cross the placental barrier, Zika virus (ZIKV), varicella zoster virus, rubella, and cytomegalovirus are transmitted from the mother to the fetus by infecting various

placental cell types (1–3). Serving as major antecedents of infection-related global morbidity and mortality during pregnancy, these pathogens cause congenital anomalies and placental dysfunction resulting in adverse pregnancy outcomes such as preterm birth or fetal growth restriction and/or miscarriage (1–8). Therefore, pregnant women represent a vulnerable population for viral infections since pregnancy confers a unique immune status that facilitates maternal tolerance of the semi-allogenic fetus and enables viral infections (9, 10). Better understanding of the role and mechanism(s) responsible for viral infections during pregnancy has become increasingly relevant because of the risk of current pandemic.

ZIKV utilizes vertical route to cross the placenta and reach fetal neuronal tissues causing severe fetal defects (11, 12). Vertical transmission was confirmed by detection of ZIKV RNA in the cerebral tissues of the aborted fetuses and placental tissues as well as the increased numbers of neonatal neurodevelopmental defects during the ZIKV outbreak (13, 14). Identification of ZIKV-infected cell types as well as better understanding of the cellular and molecular mechanisms utilized by the ZIKV to cross the placenta are particularly important in preventing viral transmission to the fetus as well as ameliorating fetal prognosis and adverse pregnancy outcomes. Therefore, improved understanding ZIKV pathogenesis during pregnancy may guide the design and/or development of therapeutic agents against ZIKV.

This review focuses comprehensively on underlying molecular mechanisms of vertical ZIKV transmission at the maternal-fetal interface and highlights the trimester-dependent role of decidual stromal cells in promoting ZIKV replication as well as describes the potential use of Food and Drug Administration (FDA) approved drugs against ZIKV infection, thereby preventing perinatal ZIKV transmission.

ARCHITECTURE OF THE MATERNAL-FETAL INTERFACE

The placenta is a unique, multifunctional organ that supplies oxygen and nutrients to the fetus to promote its development and functions as a physical barrier that protects the fetus against infections (9, 10). In humans, following implantation, collaboration between trophoblasts and extraembryonic mesodermal cells permits formation of the placenta's floating and anchoring villi (15–17). Subsequently, the anchoring villi attach to the decidua basalis (17–19). **Figure 1A** represents schematized cells in the human maternal-fetal interface, which is composed of the maternally derived decidua and fetal-derived placenta. Both cytotrophoblasts (CTBs) and syncytiotrophoblasts (STBs) originate from the trophectoderm layer of the blastocyst (19, 20). CTBs are highly proliferative mononuclear cells that are attached to a basal membrane within the placental villi (**Figure 1A**). Villous

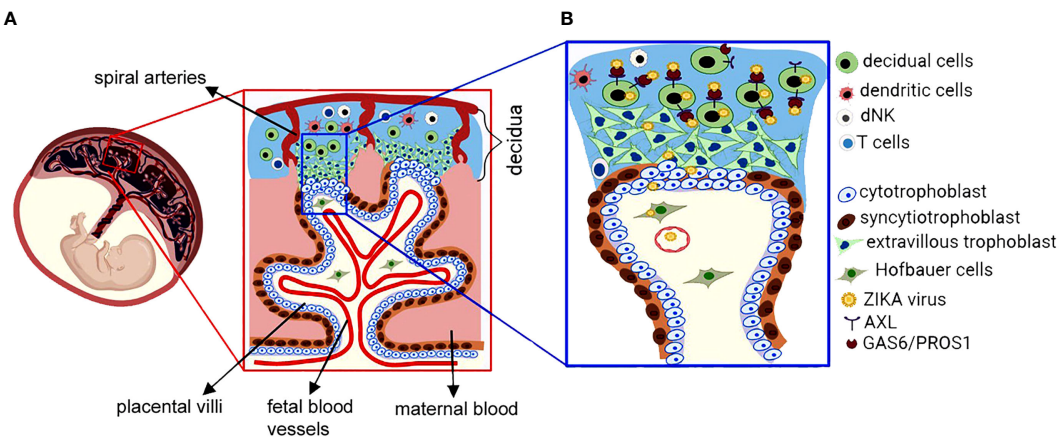

FIGURE 1 | Schematic presentation of the maternal-fetal interface and ZIKA virus infection. **(A)** The fetus-derived placental villi, which are bathed in maternal blood within intervillous space are composed of floating villi and anchoring villi that invade the maternal decidua. The maternal decidua consists of decidual cells that differentiate from uterine endometrial stromal cells, maternal blood vessels and maternal immune cells consisting of decidual natural killer (NK), dendritic cells (DC) and T cells. The placental villi are composed of fetal blood vessels, villous fibroblasts and Hofbauer cells in the villous core as well as proliferative cytotrophoblasts and multinucleated syncytiotrophoblasts, which cover the surface of the placental villous and direct contact to maternal blood. Extravillous trophoblasts are differentiated from cytotrophoblasts and invade the maternal decidua and maternal spiral arteries. Substantial crosstalk exists between maternal decidual cells and fetal trophoblasts that are essential for a successful pregnancy. **(B)** Representative presentation of decidual cell-mediated vertical transmission of the ZIKA virus. The enlarged inset represents the potential route of ZIKA virus (ZIKV) transmission from decidual cells to fetal cells. At the maternal-fetal interface, decidual cells exhibit higher viral entry/attachment factors AXL, GAS6 and PROS1 and they are more permissive to direct ZIKV infection in a gestational age-dependent manner compared to extravillous trophoblast or cytotrophoblast cells. Thus, ZIKV infects and spreads *via* decidual cells to fetal trophoblasts or Hofbauer cells or fetal endothelial cells. Therefore, decidual cells act as reservoirs for trimester-dependent placental transmission of ZIKV, thereby accounting for the higher ZIKV-infection susceptibility and more severe fetal sequelae observed in early *versus* late pregnancy.

CTBs are progenitor cells for STBs, which are multinucleated, terminally differentiated cells formed by fusion of CTBs. STBs cover the outer cell layer of placental floating villi and are in direct contact with the maternal circulation (**Figure 1A**) (3). Thus, STBs serve as the initial site of defense against pathogen(s) attempting to cross the placental barrier. In addition to their defensive role, STBs facilitate maternal-fetal oxygen exchange and nutrient transport and produce several growth factors and hormones that are critical for fetal development (3, 21). In the anchoring villi (**Figure 1A**), proliferation of CTBs forms extravillous trophoblasts (EVTs) of the cytotrophoblastic cell column, which differentiate into interstitial and endovascular CTBs that invade the maternal decidua and spiral arteries, respectively, thereby facilitating the spiral artery remodeling to markedly increase utero-placental blood flow required for fetal survival and growth (21–23).

In humans, the maternally derived decidua originates from the endometrium and promotes immunological tolerance of the semi-allogenic fetus as well as host defense against pathogens (24–28). The decidua is comprised of decidual stromal cells, glandular epithelial cells, maternal blood vessels and an immune cell population dominated by decidual natural killer (NK) cells and macrophages, with smaller percentages of dendritic cells and T lymphocytes (**Figure 1A**) (29, 30). Decidual cells are large, round, polyploid, epithelial-like cells derived from progesterone-induced decidualization of endometrial fibroblast-like stromal cells and are required for the establishment and maintenance of a normal pregnancy. Impaired decidualization is strongly associated with recurrent pregnancy loss or placental accreta or maternal hemorrhage confirming the importance of decidual cells in maintaining pregnancy (31–33). In the decidua, maternal immune cells play a crucial role in defending against infection with their numbers and subtypes changing dynamically during pregnancy. As the dominant lymphocyte population in the decidua, decidual NK cells participate in trophoblast invasion and spiral artery remodeling attaining maximum numbers during the first trimester then declining near term (34). Moreover, decidual NK cells play critical roles in promoting anti-viral innate immunity as well as placental development by producing several soluble factors (34–38). Conversely, lower T cell numbers are present in the first *versus* third trimester (39). In addition to decidual NK cells, decidual $CD8^+$ T cells play a key role in balancing the paradoxical requirement for induction of maternal–fetal tolerance and anti-viral immunity (40).

EPIDEMIC OF ZIKV INFECTION

ZIKV is a member of *Flaviviridae* that includes Dengue, West Nile, Japanese encephalitis, and yellow fever viruses and is primarily spread by the bite of the infected female *Aedes* mosquito (41, 42). The ZIKV was first isolated from a rhesus monkey in the Zika forest of Uganda in 1947 (43, 44). The first ZIKV infection outside of Africa occurred in Indonesia in 1981 (45), and then spread across the South Pacific islands in 2007, reaching Brazil in 2015 (43, 46). In 2016, a major ZIKV outbreak occurred in Central and South America and became a worldwide public health problem (46–48). ZIKV infections in adults are generally asymptomatic, but they can display mild symptoms including fever, joint pain, rash, and conjunctivitis (49, 50). However, during the 2015-16 epidemic in South America, ZIKV became a global health threat because of the dramatic increase in accompanying developmental defects *e.g.*, microcephaly, ocular changes and retinopathy in up to 20% of children of affected mothers as well as a remarkable increase in adults with Guillain-Barré syndrome (46–48). The Centers for Disease Control and Prevention reported 42,750 symptomatic ZIKV cases in the U.S. and territories including 7,407 ZIKV-infected pregnant women delivering 283 live infants with ZIKV-associated birth defects and 17 pregnancy losses; among 1450 babies born to ZIKV infected mothers, 6% had ZIKV-associated birth defects such as small head size, eye damage *etc.*, whereas 9% developed postpartum nervous system problems *e.g.*, seizures, swallowing, movement problems, or developmental delays (51). In one large Brazilian series, Brasil et al. (11) reported that the rate of fetal death in ZIKV-infected pregnancies was 7% and overall adverse outcomes were 46% *vs.* 11.5% among newborns from ZIKV-noninfected women. Additionally, the recent findings from population-based birth defects surveillance data reported a four-fold increase in the prevalence of birth defects related to ZIKV infection during pregnancy (12). These observations suggest delayed postnatal sequalae and the need for long-term monitoring of children that were exposed to ZIKV *in utero* and born with a normal head circumference (52). Although no additional ZIKV infections have recently been reported in the Americas, ZIKV infections and outbreaks still occur in India (53) and China (54), indicating that ZIKV continues to pose a maternal-fetal health challenge.

ZIKV GENOME ORGANIZATION

The ZIKV genome consists of a single-strand positive-sense (ss) RNA with approximately 10.7 kilobases in length that contains a single open reading frame flanked with ~100 nucleotides at 5' and ~400 nucleotides at 3' untranslated region (UTR) (41, 55). As displayed in **Figure 2A**, the open reading frame of the ZIKV genome encodes three structural proteins (the capsid, precursor membrane, and envelope) and seven non-structural (NS) proteins (NS1, 2A, 2B, 3, 4A, 4B and 5). The encoded single polyprotein is subsequently cleaved by both viral and host proteases to yield structural and NS proteins. The structural proteins play a role in viral attachment, entry, assembly, and pathogenicity, whereas the NS proteins are involved in viral replication, polyprotein processing and attenuating host antiviral responses (55–58). The viral promoter is located at 5' UTR with a cap site, and the terminal end of the 3' UTR contains a conserved dinucleotide (CU) instead of polyadenylation (59–61). The mature capsid (C) protein protects the viral genome and is produced by cleavage of the immature C-protein by the viral protease. The membrane (M) protein, which is located on the viral surface, is generated by cleavage of precursor (pr) M protein by a host furin protease (62). The envelope (E) protein is a major

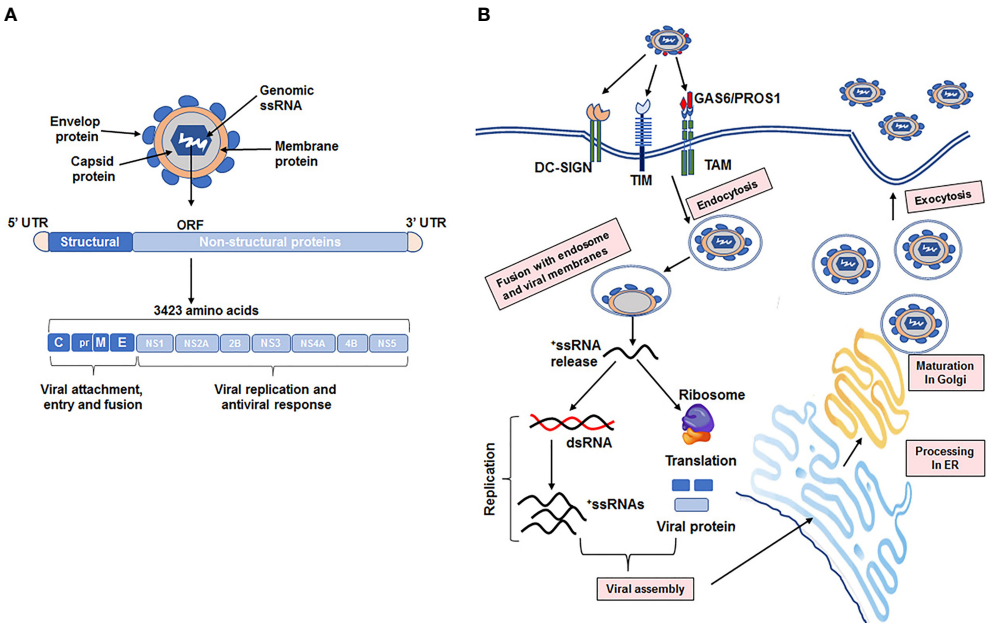

FIGURE 2 | Detailed structure of the ZIKA virus genome, replication, and maturation into host cells. **(A)** The ZIKA virus (ZIKV) genome is single stranded positive sense RNA, which is approximately 10.7 kilobase in length and contains a single open reading frame (ORF) with two flanking untranslated regions on the capped 5′-end and the 3′-end lacking a polyA-tail. ORF encodes a single polyprotein comprised of 3423 amino acids, which is post-transcriptionally cleaved by viral and host cell proteases into 3 structural proteins (the capsid (C), precursor membrane (prM), and envelope E) and 7 non-structural proteins (NS; NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5). Structural proteins mediate viral attachment, entry, and fusion into host cells, whereas non-structural proteins are involved in viral replication and the host antiviral response. **(B)** Schematic presentation of ZIKV entry, replication, and maturation in host cells. The initial step in ZIKV infection is viral attachment *via* the viral envelop (E protein and phosphatidylserine; PS) to receptors (DC-SIGN, TIM, and TAM family members) on the host cell membrane. Subsequently, this binding mediates internalization into host cells *via* clathrin-mediated endocytosis. Then, the viral RNA is released into the host cytoplasm following fusion of viral and host endosomal membranes. The released RNA is immediately bound onto host ribosomes and translated into a polyprotein that is cleaved by viral and host cell proteases to form mature structural and nonstructural proteins. Viral replication is mediated by viral RNA-dependent RNA polymerase and methyltransferase on the surface of the endoplasmic reticulum (ER), resulting in a double stranded (ds) RNA. Transcription and replication of the dsRNA result in the formation of new viral mRNAs and ssRNA genomes, respectively. Following viral assembly of capsid proteins at the ER, the virion is transported to the Golgi apparatus where virion prM protein is cleaved to form the mature M protein. Finally, the host cell releases the mature virus by exocytosis.

viral surface protein that mediates viral entry by binding to host cell surface receptors (63, 64). Among NS proteins, central enzymatic activities are primarily encoded by NS3 and NS5 proteins. NS3 protein displays serine protease, RNA triphosphatase, and helicase activities, whereas NS5 possesses methyltransferase and RNA-dependent RNA polymerase (RdRP) activities (65, 66). The methyltransferase activity is required for methylation of the 5'-end caping of viral genomic RNA. Thus, viral RNA cannot be distinguished from host RNAs and consequently is translated into viral proteins by host ribosomes. RNA-dependent RNA polymerase generates a negative sense RNA using the viral positive sense ssRNA as a template, and thus is essential for viral genome replication and transcription (41, 67).

As depicted in **Figure 2B**, the initial step in ZIKV infection is viral attachment to host cell surface receptors followed by endocytosis, enabling the virus to release ssRNA into the host cell cytoplasm (63, 65, 66). Subsequently, the ssRNA acts as a genome template that is translated into a viral polyprotein, which functions as a viral genome replicator by recruiting ssRNA to viral replication complexes. ZIKV replication occurs on the surface of the endoplasmic reticulum, resulting in a double stranded RNA genome synthesized from the genomic ssRNA by viral RdRP (68–70). The resultant double stranded RNA genome is then either transcribed to viral mRNAs for viral translation or replicated to produce new ssRNAs. Assembly of immature viral proteins and genome occurs in the endoplasmic reticulum, then transported to the Golgi apparatus for viral prM protein cleavage, which then fuse to form competent mature virions. These mature virions are then released by exocytosis (69, 70).

ZIKV ENTRY RECEPTORS

Like other viruses, ZIKV can infect and replicate in various cell types by attaching to several surface proteins that facilitate viral binding and entry into host cells (71). Since ZIKV displays a wide range of cellular tropisms, identification of ZIKV entry factor(s) is/are crucial to delineate details of ZIKV tropisms and pathogenesis. Several cellular receptors that contribute to ZIKV infection have been identified. These include C-type lectins *aka* dendritic cell-specific intercellular adhesion molecule-3-

grabbing nonintegrin (DC-SIGN) and phosphatidylserine (PS) receptors (71), which serve as entry co-factors for ZIKV, include members of the T-cell immunoglobulin (TIM) (72) and the TYRO3, AXL, MERK (TAM) family (73, 74). TIM-family members (TIM1-4) bind viral PS in the viral membrane, whereas TAM members bind PS indirectly, through the soluble intermediates growth arrest-specific 6 (GAS6) and protein S1 (PROS1) that act as a bridge for ZIKV-TAM receptor binding, which induces viral endocytosis (**Figure 2B**) (73, 74). Among these putative ZIKV receptors, AXL is the most studied receptor for ZIKV infection. AXL is a transmembrane receptor that contains an extracellular and intracellular tyrosine kinase domain. The AXL extracellular domain acts a ZIKV entry factor by binding to its ligands GAS6 and PROS1, whereas the tyrosine kinase domain of AXL is involved in mediating an innate immune response as well as other biological processes including cell proliferation, differentiation, and survival (75, 76). Previous studies demonstrated that AXL-overexpressing human fetal neuronal stem and glial cells, astrocytes, endothelial and microglial cells are highly susceptible to ZIKV infection (74, 77), implicating the role of AXL in ZIKV-induced neuropathology. Later studies showed that the reduced AXL levels elicited by small interfering RNA or neutralizing antibodies dramatically diminished ZIKV infection in dermal fibroblast or glial cell cultures, thereby supporting the crucial role of AXL in ZIKV infection (74, 78). In contrast to *in vitro* studies, *in situ* results using *Axl*-deficient mice (79, 80) indicated that Axl may not be required for ZIKV infection in mice, suggesting that Axl expression and/or Axl-mediated signaling pathway(s) in mice are different than in human primary cells.

ZIKV TRANSMISSION

ZIKV is primarily spread to humans by the bite of infected female *Aedes aegypti* and *albopictus* mosquitoes (43, 81, 82), thereby, accepting the skin as the initial host target. Infected mosquitoes transmit ZIKV into the epidermis or directly into the circulation (78, 83, 84). Thus, epidermal cells serve as the initial site of ZIKV infection since epidermal keratinocytes, dermal fibroblasts, and immature dendritic cells are reported to be permissive to ZIKV infection (78).

Sexual transmission from male-to-female or *vice-versa* is also common and is responsible for about 32–54% of ZIKV infections (85, 86). Although the highest transmission occurs from male-to-female, female-to-male and male-to-male transmission are also reported (87–91). Detection of ZIKV RNA in semen (92), vaginal and cervical secretions (93, 94) supports the contribution of sexual ZIKV transmission in the increased numbers of infected people in high-risk areas as well as travelers to areas with risk of ZIKV. In the male reproductive tract, ZIKV infects testicular somatic and germ cells such as Sertoli cells, spermatozoa *etc.* (92, 95). In a mouse model, ZIKV persistence was reported in testicular tissues up to four weeks post-infection resulting in testicular inflammation, atrophy, and infertility (96, 97). Moreover, in humans, ZIKV RNA was detected in semen for 6 months (92) and in semen used for assisted reproductive technology up to 112 days (95), indicating that infected men serve as a potential long-term reservoir for sexual transmission.

Similarly, prolonged ZIKV shedding was initially reported in vaginal secretions up to 14 days after ZIKV infection (94). However, a later investigation by Reyes et al. reported prolonged viral shedding in vaginal secretions up to 6 months (98). This difference in ZIKV persistence in vaginal tissues is likely associated with the menstrual cycle phase during ZIKV infection. In support of this suggestion, vaginal ZIKV inoculation during diestrus in AG129 mice causes longer ZIKV survival (~10 days post-infection) and greater lethality compared with inoculation during estrus (~3 days post-infection) (99), indicating that ZIKV shedding is affected by estrous cycle stages. Additionally, vaginal inoculation of pregnant wild-type mice resulted in fetal brain infection and FGR (100), suggesting that ZIKV infection *via* sexual transmission route can amplify the severity of vertical transmission. ZIKV RNA was also detected in ovaries and uterus in animal models (101, 102), as well as in human oocytes (103). *In vitro* studies indicate that human endometrial stromal cells (HESCs) obtained from cycling endometrium are highly permissive to ZIKV infection (104, 105), and that viral replication is increased during decidualization (105). Taken together, these studies indicate that ZIKV persistence in both male and female reproductive tissues plays an important role in the management of ZIKV infection, since both male and female reproductive tissues serve as sanctuary and reservoir for prolonged ZIKV survival (72, 89). Thus, eradication of ZIKV persistence in the reproductive tissues is expected to reduce the risk of perinatal transmission yielding invaluable public health benefits.

Vertical transmission of ZIKV from the infected mother to the fetus causes severe fetal outcomes *aka* congenital ZIKA syndrome, which includes microcephaly, seizures, hypertonia, and other neurological problems as well as ocular and skeletal anomalies *etc.* (106–108),. During pregnancy, maternal ZIKV infection elicits placental dysfunction resulting in adverse pregnancy outcomes such as FGR, miscarriage and/or stillbirths. Araujo et al. (109) reported that 83% of neonates exposed to prenatal ZIKV infection were small for gestational age in Brazil during the 2015 epidemic, whereas in the United States, the rate of small for gestational age was reported to be 11.2% in women with antenatal ZIKV infection (110). Another study from Brazil reported that the rate of overall adverse outcomes was 46%, which included 7% of fetal death in ZIKV-infected pregnancies (11). Recently, Mercado-Reyes et al. (7) reported the pregnancy outcomes among pregnant women with ZIKV symptoms in Columbia between 2016-2018. Among the 1180 pregnancies, adverse pregnancy outcomes were found to be 22.4%, which included 1.4% pregnancy losses, 9.7% PTBs, 6.9% low birth weights and 4.6% small for gestational age. All these studies indicated that following placental transmission, ZIKV reaches the fetal brain where it causes severe detrimental defects by interrupting proliferation, migration, and differentiation as well as inducing apoptosis of neuronal progenitor cells (111–115). Recent studies demonstrated that ZIKV can infect a broad

range of cells in the human placenta including cytotrophoblasts, endothelial cells, fibroblasts, amniotic epithelial, and/or Hofbauer cells (71, 115–118) as well as maternal decidual cells (104) and cause severe histopathological changes (119, 120). Initially, STBs were assumed to be the site of ZIKV entry. However, STBs are reported to resist ZIKV attachment and replication (121), therefore they are less-permissive to ZIKV infection, suggesting that maternal-fetal ZIKV transmission occurs at other placental site(s). The ZIKV resistance in STBs may be related to low expression of ZIKV attachment molecules such as TAM receptors (104) and/or high expression of interferon (IFN)-induced antiviral genes *RIG-1*, *IFIH1*, *ISG15*, *etc.* (121), as well as high production of type III IFNs, specifically IFNλ1, which protects host cells against ZIKV infection (122). Moreover, recently, Miranda et al. reported (123) the reduced expression of tight junction proteins, particularly claudin-4, and increased paracellular permeability of STBs obtained from ZIKV infected women, displayed an alternative mechanism of ZIKV transmission to other placental cells. On the other hand, compared to STBs, CTBs are ZIKV-permissive, suggesting that ZIKV replication in CTBs mediates its transmission to the fetus. However, ZIKV infection of CTBs in the floating villi is highly unlikely since the outer layer of the villi formed by STBs is in direct contact with maternal blood, thereby preventing ZIKV infection of CTBs in the floating villi. Thus, the anchoring villi are the most likely site of ZIKV infection of CTBs. Guzeloglu-Kayisli et al. (104) provided strong evidence that decidual cell-derived factors facilitate placental transmission by amplifying ZIKV replication in CTBs, the primary cell type in anchoring villi, which attach directly to the decidua. Collectively, these findings indicate that ZIKV persistence in immune privileged sites such as testis, brain and placenta potentiate sexual and vertical ZIKV transmission (77, 87, 89, 124). Of note, transmission of ZIKV *via* blood transfusion has also been documented, especially in Brazil (125, 126).

ZIKV INFECTION OF DECIDUAL CELLS IS THE PRIMARY SITE OF VERTICAL TRANSMISSION

Fetal dissemination of any infectious agent requires transmission through the placenta attached to the immunologically active uterine decidua or *via* hematogenous transmission (1–3). As mentioned above, STBs are less permissive to ZIKV infection. Thus, maternal-fetal ZIKV transmission must occur at other potential placental site(s) at the maternal-fetal interface, suggesting that decidual cells or EVTs or both are the most likely site(s) of ZIKV transmission to the fetus. *Ex vivo* and *in vitro* decidual cell cultures demonstrated that decidual cells act as both a reservoir and source of ZIKV transmission to adjacent anchoring villi at the maternal-fetal interface, thereby representing a primary vertical transmission site (**Figure 1B**) (104, 118, 127). Guzeloglu-Kayisli et al. (104) reported that human decidual cell cultures are more permissive to ZIKV infection, replication, and viral release than human primary cultured CTBs. Similarly, cultured human decidual cells express significantly higher levels of viral entry and bridging molecules *AXL, GAS6* and *PROS1* (**Figure 1B**) than primary cultures of either CTBs or STBs. In addition, compared to cultured CTBs and STBs, decidual cells differentially express a set of genes that are involved in viral replication and/or infection (104). Similarly, Tabata et al. (116) found high AXL expression in decidual cells and EVTs obtained from second trimester placentas, but low levels of AXL expression in villous CTBs obtained from second and term trimester placentas, suggesting that AXL expression varies depending on the gestational age and placental cell types. This study also reported ZIKV infection in decidual cells, CTBs, EVTs, and Hofbauer cells, supporting ZIKV transmission from decidual cells to chronic villi and fetal circulation as the primary vertical transmission route (116). Moreover, Richard et al. (128) demonstrated that fetal endothelial cells are permissive to ZIKV infection and display higher AXL and GAS6 levels, indicating that the ZIKV infects fetal endothelial cells by using these entry molecules to cross the placental barrier. Similarly, Zheng et al. (129) reported higher AXL levels in decidual stromal cells and perivascular cells and lower levels in decidual dendritic cells and macrophages at the maternal-fetal interface using Single-Cell RNA sequencing. Additionally, Guzeloglu-Kayisli et al. (104) found that ZIKV replication and release is amplified in primary CTB cultures treated with ZIKV-infected decidual cell supernatants compared to direct ZIKV infection of these cells, indicating that decidual cells not only serve as a ZIKV reservoir, but also facilitate ZIKV infection of CTBs (**Figure 1B**). Furthermore, Wesblum et al. found that ZIKV induces expression of distinct innate immune response genes, particularly those related to anti-viral interferon signaling in decidua *vs.* chronic villi explant cultures, suggesting that this antiviral response paradoxically promotes a rapid and robust replication of ZIKV in decidual cells (127).

GESTATIONAL AGE DEPENDENCE IN ZIKV INFECTION OF DECIDUAL CELLS

The risk of vertical transmission exists throughout pregnancy, whereas the greatest risk of severe fetal abnormalities is strongly associated with ZIKV infection in the 1st and 2nd trimester (46–48). Consistent with this clinical information, higher ZIKV replication is detected in decidual cells obtained from first trimester placentas compared to decidual cells from term placentas (104). However, the biological mechanism(s) utilized by ZIKV to cross to the placenta and the cause of the inverse relationship between the gestational age of ZIKV infection and its severity remain unclear. As potential mechanism(s) responsible for gestational age dependent ZIKV infection, we reported that: 1) decidual cells isolated from first trimester placentas exhibit higher viral entry/attachment molecules *AXL*, *GAS6*, and *PROS1* than term decidual cells, indicating a higher risk of ZIKV infection in early pregnancy and greater subsequent detrimental ZIKV effects on the fetus (104); 2) decidual cells obtained from term placentas display a strong anti-viral response

to ZIKV infection that correlates with lower ZIKV replication in term *vs.* first trimester decidual cell cultures (104); and 3) mid-gestation decidua expresses higher IFNα and IFNλ levels compared with early decidua, indicating an inverse correlation between IFN levels and ZIKV susceptibility during gestation (127). In addition to these mechanisms, dynamic changes in the immune cell populations at the maternal-fetal interface during pregnancy could alter ZIKV susceptivity (130, 131). For example, decidual NK cell numbers reach a maximum during the first trimester but decline near term. Conversely, lower T cell number are present in the first trimester than at term (39).

POTENTIAL THERAPEUTIC OPTIONS AGAINST ZIKV INFECTION

Unfortunately, no approved effective treatment or vaccine currently exist against ZIKV infection. Since recent ZIKV outbreaks resulted in significant adverse health effects, several investigations focused on development of drugs and vaccines to treat or prevent *in utero* ZIKV transmission. As examples, a plasmid-based DNA vaccine (132, 133) or modified recombinant measles virus vaccine backbone (NCT02996890) or mRNA-based vaccine (134) or inactivated whole ZIKV vaccine (135) candidates have been developed and tested to prevent ZIKV infection. In several vaccine studies, viral prM and E proteins have been intensively targeted since both prM and E in the ZIKV surface are accepted as the primary antigenic target (136). Moreover, many monoclonal antibodies targeting ZIKV proteins have been described in detail (137, 138). However, the main concern related to therapeutic potential for clinical applications is viral escape due to the high mutation rates of ZIKV genome.

To provide a potential treatment against the detrimental effects of ZIKV infection, several different strategies have been employed aimed at blocking viral replication or inhibiting viral protein synthesis. Several FDA-approved drugs have been investigated for their potential rapid response to the ZIKV outbreak since their mechanisms and safety as well as their pharmacokinetic and pharmacodynamic profiles of these drugs are well documented. Predictably, screening these drugs for their effectiveness against ZIKV infection should rapidly advance their approval for clinical use than newly identified drugs. Some FDA-approved candidate drugs against ZIKV infection are given in **Table 1**. As examples, Sofosbuvir, an FDA-approved drug used for the treatment of chronic hepatitis C virus infection, was investigated as a potential inhibitor of ZIKV RNA-dependent RNA polymerase (145). Although Sofosbuvir inhibited ZIKV infection in human hepatocellular carcinoma (Huh-7) and placental choriocarcinoma cells, no inhibitory effect was observed in Vero or A549 cells (139, 140), indicating cell type dependent anti-ZIKV activity. Recently, Mesci et al. (141) reported *in vitro* and *in vivo* protective effects of Sofosbuvir against ZIKV by demonstrating inhibition of ZIKV replication and ZIKV-induced apoptosis in human neuronal progenitor cell cultures as well as reduction of ZIKV titers in the serum of Sofosbuvir administered pregnant SCID immunodeficient mice.

Nitazoxanide was investigated as another potential treatment against ZIKV by inhibiting the viral protease complexes NS2B-NS3 that play essential roles during viral polyprotein processing (146). Nitazoxanide is a potent antiparasitic drug used to treat anaerobic bacterial and protozoal infections and possesses broad spectrum activity against many viruses (147). The FDA approved Nitazoxanide for treatment of diarrhea and enteritis in adults and in children ≥12-months (148–151). Nitazoxanide ingested with food is absorbed from the gastrointestinal tract and hydrolyzed in plasma to form its active metabolite, tizoxanide with serum levels attaining up to 10 μg/mL (152). Clinical trials revealed its efficacy against rotavirus and norovirus gastroenteritis in children and adults (153, 154). Moreover, Nitazoxanide therapy against influenza is currently a phase III clinical trial (NCT02612922). Its broad range of anti-viral activity (147, 154–157) suggests that Nitazoxanide induces a cell-specific effect rather than a viral-specific effect. However, the mechanism(s) mediating antiviral activity of Nitazoxanide and/or tizoxanide is/are not completely elucidated. The anti-viral activities of Nitazoxanide and its bioactive metabolite tizoxanide against ZIKV were first tested in Vero and A549 cells. Both agents significantly inhibited ZIKV infection in these cell types (158). Subsequently, Li et al. (146) demonstrated that Nitazoxanide inhibited ZIKV infection by decreasing viral replication and viral protein expression in human placental epithelial cells, human neuronal progenitor cells and human pluripotent stem cell line. Thereafter, De Souza et al. (142) found that Nitazoxanide reduced ZIKV viral loads up to 2 logs in primary cultured chorionic cells obtained from human term placentas and in a human cervical epithelial cell line. Recently, Guzeloglu-Kayisli et al. (104) evaluated the anti-ZIKV activity of tizoxanide in primary cultures of HESCs obtained from cycling

TABLE 1 | Summary of candidate anti-ZIKV drugs and their mechanisms.

Drugs	Known mechanism	Anti-Zika activity tested cell types	Reference
Sofosbuvir	RNA polymerase inhibitor	. human hepatocellular carcinoma (Huh-7)	(139, 140)
		. human neuronal progenitor cell cultures	(141)
Nitazoxanide	protease complexes inhibitor	. chorionic cells and cervical epithelial cell line	(142)
		. human endometrial cells, decidual cells and cytotrophoblasts	(104)
Atovaquone	RNA synthesis inhibitor	. JEG3, chronic villous	(143)
Efavirenz	Nucleoside inhibitor	. human neuroblastoma cells, astrocytes, Vero	(144)
Tipranavir	Protease inhibitor	. human neuroblastoma cells, astrocytes, Vero	(144)
Dasabuvir	RNA polymerase inhibitor	. human neuroblastoma cells, astrocytes, Vero	(144)

endometrium, decidual cells obtained from first trimester and term placentas, as well as in primary cultures of CTBs from term placentas and found that tizoxanide significantly reduces ZIKV replication in all these cell types. Cao et al. (158) demonstrated that pre-treatment with either Nitazoxanide or tizoxanide failed to inhibit ZIKV replication in Vero cell line, but adding either drug post-infection exerted an anti-ZIKV effect, suggesting that both drugs inhibit infection after viral attachment. Taken together, these results provide solid evidence supporting the potential use of Nitazoxanide or tizoxanide to prevent ZIKV infection and associated fetal abnormalities.

Additionally, Yamamoto et al. (159) screened a library of 1017 FDA-approved drugs targeting ZIKV E protein and identified Atovaquone as an effective drug against ZIKV infection in both mammalian Vero and mosquito-derived C6/36 cells *in vitro*. Atovaquone is a well-known anti-malaria and anti-parasitic drug (143) and is a coenzyme Q analogue that inhibits the mitochondrial cytochrome complex III and pyrimidine synthesis (160). The anti-viral effect of Atovaquone against ZIKV infection was tested in JEG3 trophoblast cells as well as *ex vivo* chorionic villous explants, suggesting that Atovaquone may protect placental transmission of ZIKV and could be a potential candidate against ZIKV during pregnancy (160). Similarly, Stefanik et al. also (144) identified potential anti-ZIKV candidates by screening FDA-approved drugs that interact with ZIKV NS3 and NS5 proteins and found that only three drugs: Efavirenz, an antiretroviral drug against HIV, Tipranavir, a HIV protease inhibitor, and Dasabuvir, a RNA polymerase inhibitor against Hepatitis C virus, inhibited ZIKV titers in Vero cells as well as in primary human brain cortical astrocytes and a neuroblastoma cell line (144).

CONCLUSION REMARKS

This review discusses in detail the putative mechanism(s) responsible for ZIKV infection at the maternal-fetal interface. Specifically, it reveals the role of immunologically active decidual cells, which are highly permissive to ZIKV infection and likely act as both a reservoir and source of ZIKV transmission to adjacent anchoring villi at the maternal-fetal interface. Moreover, the trimester-dependent responses of decidual cells to ZIKV infection could elucidate the clinically important questions such as why pregnant women are highly susceptible to ZIKV infection and why the subsequent effects are more detrimental in the first trimester than in late pregnancy. Finally, this review discusses the anti-ZIKV effects of FDA-approved candidate drugs that were demonstrated to inhibit ZIKV replication and dissemination. Accordingly, these drugs represent potential therapeutic candidate(s) that block perinatal ZIKV transmission, thereby averting its harmful effects on the fetus.

In conclusion, both current and previous pandemics demonstrated that viral infections pose a major risk during pregnancy because of their detrimental effects on the fetus and adverse pregnancy outcomes. Therefore, determination of viral tropisms and host factors at the maternal-fetal interface are crucial to improve understanding the mechanism(s) and/or route (s) employed by emerging viruses. Predictably, prevention of viral infections during pregnancy will be more rapidly accomplished by screening of anti-viral effects of FDA-approved drugs that were previously verified as to their modes of action, safety, and pharmacokinetic and pharmacodynamic profiles.

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The authors OG-K, UK, FS, and CL made substantial contributions to this work and approved the final version of the manuscript.

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REVIEW
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Modeling the Human Placenta to Investigate Viral Infections During Pregnancy

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Throughout gestation, the placenta is vital for proper development of the fetus. Disruptions in trophoblast, the main functional cell type of the placenta, stress the pregnancy, with potential adverse outcomes for both mother and baby. While the placenta typically functions as an effective pathogen barrier to protect the fetus, there are scenarios in which viral infections either cross the placenta or disturb its function. Here, we briefly review placental morphologic and functional changes across pregnancy and how these relate to routes for and protection from pathogens. We then explore the potential advantages and limitations of the current primary trophoblast models (primary cultures/explants, cell lines, trophoblast stem cells and trophoblast organoids) and stem cell-derived trophoblast models (naïve and primed embryonic stem cells [ESCs], and blastoids) and discuss these in the context of what is already known about (1) how viruses cross the placenta and the mechanisms that are used for its protection and (2) how these protective mechanisms change across gestation.

Keywords: trophoblast, placenta, virus, maternal infections, models, stem cells, organoids

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INTRODUCTION

The success of human pregnancy relies on a healthy and functional placenta. The placenta is responsible for gas, nutrient, and waste exchange while concurrently functioning to protect the fetus from dangerous substances or microorganisms originating in the mother. While the placenta protects against many pathogens, there are multiple examples of maternal infections that cause adverse pregnancy outcomes or congenital deficits in the fetus. The most well-known are complications associated with the "TORCH" pathogens, which include ***T****oxoplasma gondii*, **o**ther [including Zika virus (ZIKV; *Flaviviridae*; *Flavivirus*) and human immunodeficiency virus (HIV; *Retroviridae*; *Lentivirus*)], **r**ubella virus (RuV; *Matonaviridae*; *Rubivirus*), human **c**ytomegalovirus (HCMV; *Herpesviridae*; *Cytomegalovirus*), and **h**erpes simplex virus 1 and 2 (HSV-1, HSV-2; *Herpesviridae*; *Simplexvirus*) (1). Researchers are currently investigating whether severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2; *Coronaviridae*; *Betacoronavirus*) can cross the placenta as well. Given the current pandemic and threats of future viral outbreaks, it is important to understand how the placenta can succeed or fail in protecting the fetus from maternal infections. In this review, we focus on how viruses can cross the placenta throughout gestation and envision how

researchers can utilize newly derived human placental models to further our understanding of these processes.

PLACENTAL STRUCTURES ACROSS GESTATION AND ROUTES OF PATHOGEN TRANSMISSION

The placenta originates from the trophectoderm, the outermost layer of the human blastocyst (**Figure 1A**). Initial implantation of the embryo into the maternal endometrium (decidua) begins with the formation of a highly invasive primary syncytiotrophoblast (SCT) layer migrating ahead of a zone of proliferating cytotrophoblast (**Figure 1B**). This early, pre-villous placenta has been defined as primitive trophoblast. During this stage of pregnancy, crossing the SCT is the only placental route for vertical (mother-to-fetus) transmission of viruses to the fetus. Little is known about the human primitive placenta and what is known stems from rare, archived hysterectomy samples and non-human primate research (2–4). At this early time in its development, the placenta is essentially nourished entirely by glandular secretions in the maternal decidua (5). While studying this peri-implantation phase in humans is nearly impossible, murine models suggest that the placenta can enable vertical virus transmission during this time window, i.e., prior to mature hemochorial placental formation (6–8). A human cohort study estimated congenital HCMV infection occurred at a rate of 45% after maternal periconceptional infection (defined as one week before and five weeks after the last menstrual period) providing further evidence of placental susceptibility during this time window (9).

As pregnancy progresses, the proliferative cytotrophoblast cells eventually break through the primitive syncytium and begin forming the primary chorionic villous structures. Villous cytotrophoblast cells (VCT) fuse to form a continuous layer of non-invasive multi-nucleated SCT (**Figure 1C**). At the distal ends of some of these villi (anchoring villi), the SCT layer is absent and instead, the VCT form cytotrophoblast cell columns (CCC) that anchor the placenta to the basal plate. In early pregnancy, this external layer of cytotrophoblast cells is known as the cytotrophoblastic shell, which spreads laterally to form a continuous layer between the anchoring villi and the decidua basalis (10, 11). The cytotrophoblast cells within the cell columns subsequently differentiate into extravillous trophoblast cells (EVT), which invade deeply into the decidua (**Figure 1C**). Initially, these EVT also form plugs in the maternal spiral arteries to prevent blood flow to the developing villous structures. The CCC and EVT are in contact with cells residing in the maternal decidua and serve as a potential route for viral transmission to the fetus *via* the villous placenta prior to onset of maternal blood flow into the intervillous space.

Around the 10th week of pregnancy, the EVT plugs dissipate and the EVT continue a process that remodels the spiral arteries by replacing the endothelial layer with endovascular trophoblast (EndoVT) to allow relatively unfettered maternal blood supply to the intervillous space (12) (**Figure 1D**). Once the plugs are dissolved, the SCT layer is bathed directly in maternal blood. This provides an additional route for viral particles present in maternal blood to infect the growing villous placenta (across the SCT layer). To infect a fetus, the virus must then traverse the underlying layer of VCT to enter the villous core that contains fetal blood vessels, fibroblasts, and fetal macrophages known as Hofbauer cells. These Hofbauer cells have the potential to

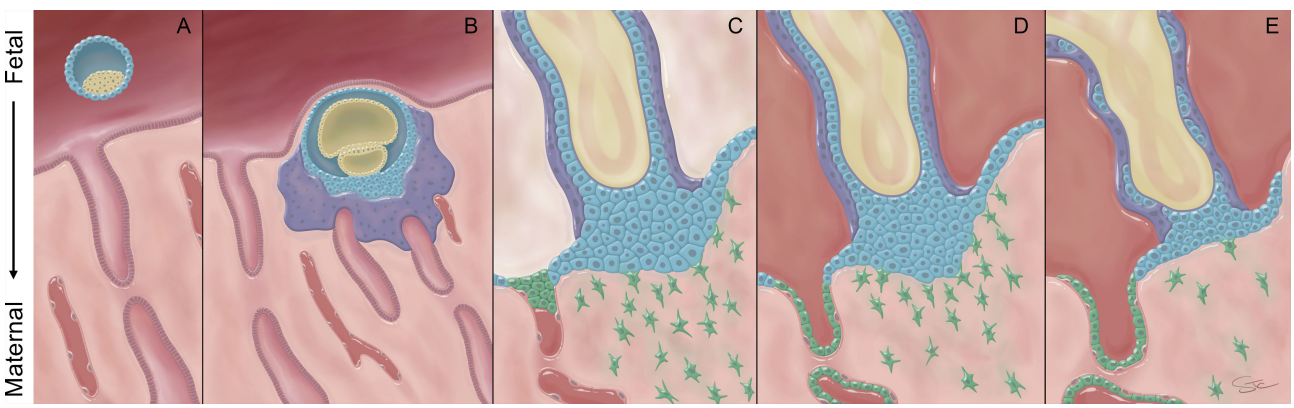

FIGURE 1 | Morphology of the human placenta across gestation. **(A)** Just prior to implantation, the blastocyst is comprised of an inner cell mass (yellow) that is surrounded by a single layer of trophoblast, termed the trophectoderm (blue). **(B)** Once the embryo contacts the maternal decidua, the trophectoderm forms a highly invasive primitive syncytium (purple). This multi-nucleated syncytium contacts maternal glands that provide histotroph to nourish the growing embryo. Beneath the primitive syncytium, the villous cytotrophoblast cells (VCT; blue) proliferate and eventually break through the primitive syncytium to generate tree-like primary and, ultimately, more mature villous structures. **(C)** Villi that reach and anchor to the maternal decidua are the source of the cytotrophoblast cells that will form the cytotrophoblastic shell (blue). Extravillous cytotrophoblast cells (EVT, green) invade into the decidua and interact with maternal cells and plug the maternal spiral arteries to prevent onset of maternal blood flow into the space between the placental villi, called the intervillous space. **(D)** Near the end of the first trimester, the EVT plugs dissolve and the EVT continue to remodel the spiral arteries to establish robust maternal blood flow into the intervillous space. **(E)** By the end of pregnancy, the VCT layer becomes sparse and the cytotrophoblast shell discontinuous.

provide an additional layer of defense through their response to Toll-like receptor stimulation (13, 14). Studies have indicated that Hofbauer cells are susceptible to viral infections, including TORCH pathogens, HIV (15) and ZIKV (16, 17), but respond differentially to these infections.

As the primary villi mature and pregnancy progresses, the number of migratory and spindle-like EVT decreases and the cell columns become more compact (18, 19) (**Figure 1D**). In addition, the VCT become sparse and infrequent underneath the continuous layer of SCT lining the villi (20, 21) (**Figure 1E**). While the focus of this review is on the placenta's role in vertical transmission, the maternal decidua also contributes to infection risk and maternal response to infection during pregnancy (22). The fetal membranes, the decidua parietalis and the decidua basalis all have distinct immune cell phenotypes and may play roles in vertical transmission that differ based on the pathogen involved (23, 24). For instance, the best studied of these, the decidua basalis, contains multiple types of immune cells, including natural killer cells, dendritic cells, macrophages and T cells (24) indicating a robust potential to respond to viral insults. In contrast, there is also evidence that the decidual tissue can host active infections of TORCH pathogens, like HCMV and ZIKV, implying a role as a reservoir for infection during pregnancy (25–28). The balance among these decidual responses, and those of "para-placental" routes involving the parietal decidua and fetal membranes, likely differ by pathogen and level and timing of infectious load and, like the placenta, contribute to the ultimate risk for vertical transmission.

PLACENTAL PROTECTION FROM AND SUSCEPTIBILITY TO INFECTION

The SCT serves as the primary trophoblast barrier between fetal and maternal blood. The structure of the syncytial layer in and of itself serves as a protective boundary. The multinucleated and continuous layer of SCT lacks cell junctions, which are often hijacked by viral particles as entry points in other tissues (1). The dense actin structure underneath the brush boarder of the SCT surface may also play a role in preventing pathogen invasion and attachment (29, 30).

The chromosome 19 miRNA cluster (C19MC) has been demonstrated to have antiviral properties, specifically at term (31, 32). This miRNA cluster is located on a maternally imprinted chromosome and is predominantly expressed by trophoblast. However, less is known about the functional role and potential of C19MC miRNAs to provide viral protection during early gestation. One study assessed release of C19MC miRNAs into maternal circulation following successful *in vitro* fertilization (IVF) procedures (33). The investigators in this study were able to detect C19MCs in maternal blood as early as 2 weeks after blastocyst implantation, with a substantial increase during the first trimester of the resultant pregnancy. It is hard to deduce whether this initial low level of C19MC miRNA expression is due to the small size of the embryo (and thus the placenta) at this earliest stage of pregnancy or if there are indeed changes in C19MC miRNA expression across gestation that outpace placental growth and villous maturation. With the establishment of new first-trimester trophoblast models, discussed in the following sections, (34, 35), it is now possible for researchers to assess the role of these C19miRNAs in early gestation.

Generally speaking, trophoblast constitutively produces interferons (IFNs) which may aid in placental protection from viral insults (36–38). In mice, loss of type I IFN signaling leads to increased placental viral load upon exposure to murine herpesvirus-68 and increased vertical transmission to the fetus (39). Most viruses have developed a variety of strategies to avert immune recognition and allow for unencumbered propagation within the infected cell with subsequent transmission. For instance, many flaviviruses are capable of antagonizing type I IFN responses (40, 41). ZIKV NS5, the viral RNA-dependent RNA polymerase which is the most conserved protein amongst flaviviruses, binds to and subsequently targets signal transducer and activator of transcription 2 (STAT2) for degradation (42). STAT proteins are part of the signaling cascade that converts cytokine signals into immune cell responses, including proliferation and differentiation, to fight viral invasion (43). This same mechanism is conserved for another flavivirus, dengue virus (DENV), although here STAT2 degradation relies on successful binding of ubiquitin protein ligase E3 component N-recognin 4 (UBR) to the NS5 protein (44). NS5 has also been shown to bind cellular heat shock protein 90 leading to instability and subsequent degradation of janus kinase (JAK), another member of the signaling pathways involved in antiviral responses, (45). Type III interferon responses also rely on JAK/STAT signalling cascades and therefore may be similarly antagonized by flaviviruses (46). However, we expect that this possibility of antagonism would be reduced in trophoblast cells, considering that their expression and release of type III IFNs is constitutive and can therefore be independent of viral insults (47, 48).

Transport of maternal IgG across the SCT layer of the placenta occurs through the neonatal Fc receptor (FcRn) (49). The Fc region of IgG only binds to FcRn at acidic pHs and therefore must be taken up by endosomes within the SCT. The mechanism underlying the crossing of the stroma or fetal endothelium by IgG after SCT uptake, however, remains elusive (50). In humans, onset of transfer occurs around 13 weeks of gestation and continually rises during pregnancy (51). Maternal transfer of IgG can offer passive immunity to the developing fetus. This has been demonstrated in infants born to mothers who were vaccinated against Influenza virus (*Orthomyxoviridae*) and tetanus-diphtheria-pertussis (Tdap) during pregnancy (52–54). Recently, researchers discovered that in the third trimester neonates can acquire SARS-CoV-2 antibodies *via* placenta transfer (55). However, there is the potential for a virus to hijack FcRn to facilitate its own entry and vertical transmission across the SCT layer. Both HIV and HCMV have been shown to utilize this IgG-virion transcytosis mechanism to gain access to the placenta (56, 57).

The adverse fetal outcomes first reported among pregnant women in South America who were exposed to Asian strains of ZIKV were the first strong evidence for vertical transmission of this virus (58–60), which had caused previous outbreaks in Africa and Asia without reported fetal effects. Antibody-dependent enhancement (ADE) (61) is just one of the suggested mechanisms for this change in transmission dynamics. ADE occurs when a prior virus infection generates (cross-reacting/heterotypic) antibodies that are poorly neutralizing against a subsequent infection by a separate but similar virus (62). Instead, these antibodies can lead to increased pathogenicity for the subsequent infection. Sequence alignments indicate that various ZIKV isolates share approximately 99% amino acid sequence identity and that similar levels of homology can be found between the four DENV serotypes (DENV1-4, 98-99% amino acid sequence identity) (63). Of all *Flavivirus* species, DENV1-4 and ZIKV share the most similar amino acid sequences. This has led researchers to investigate whether the cross-reacting antibodies produced following a primary infection with DENV might influence disease outcome upon subsequent infection with ZIKV. One case report has suggested ADE in a converse combination of flavivirus infections. Here, prior ZIKV infection enhanced infection by DENV1, which led to severe and fatal consequences (64).

ADE of ZIKV transmission has been demonstrated in mice previously infected with DENV, suggesting that DENV-specific antibodies are able to increase the incidence of ZIKV vertical transmission and associated fetal microcephaly (65, 66). This process likely involves FcRn and transcytosis of ZIKV attached to a non-neutralizing, cross-reactive, DENV-recognizing antibody (65, 67, 68). The human populations exposed to ZIKV during the outbreaks in South America in 2015/2016 also exhibited high levels of DENV immunity (i.e., in DENV-endemic regions) (69), suggesting that ADE caused by subsequent ZIKV infection in DENV-exposed women might be involved. There are, however, conflicting observations. One study using type I interferon receptor-deficient mice indicated that anti-DENV monoclonal antibodies (mAbs) may act to neutralize ZIKV and thereby protect the fetus of a ZIKV-infected mother from vertical transmission of the virus (70). Another group reported that DENV immunity provided enhancement of ZIKV infection *in vitro* (as observed in a K562 human erythroleukemic cell line, an otherwise nonpermissive cell line but one that bears an Fcγ receptor) but not *in vivo* (i.e., AG129 mice), suggesting that the variation in results may be an artifact of the exploited *in vitro* model systems (71). Differences in stimulated antibody titers and variations in the pathogenicity of specific viral strains could also be responsible for these discrepant outcomes.

MODELING PLACENTAL INFECTIONS *IN VITRO*

Understanding the potential and the limitations of existing and emerging *in vitro* model systems for early human placental development is of the upmost importance (72–74). Recent advancements in available human trophoblast models provide a platform to further investigate how viruses infect the placenta and to explore the possible consequences of such infection on placental function and fetal health. Animal models do provide some insight into placental susceptibility to infections; however, all have significant structural and functional differences that must be considered (75, 76). For this reason, we have chosen to only discuss human models here. For those interested in further exploration, we suggest extensive reviews of these models by others (77–79). While many studies have elucidated human trophoblast susceptibility or resistance to different viruses, the savvy reader must carefully account for discrepancies among the gestational ages represented by the selected trophoblast models, the specific trophoblast lineages assessed by these models, and the plausibility of vertical transmission *via* the route that is being mimicked *in vitro*.

Primary Cultures: Explants and Cells

Primary trophoblast cells and placental explant cultures have been used to demonstrate placental susceptibility to several viruses associated with adverse pregnancy outcomes (**Figure 2A**). Primary cultures are restricted by their relatively short lifespan, the limited range of gestational ages from which they can be obtained and the relative frequency of sample availability. Primary cultures from term pregnancies rapidly differentiate to SCT and lack a robust migratory set of EVTs (19, 80). This essentially limits the infectious disease researcher to the study of SCT susceptibility to pathogens, including viruses, at term. First trimester explants can capture all subtypes of trophoblast but are inaccessible to many researchers. Further, sample availability can be infrequent, making replication of studies challenging when using primary first trimester samples.

Trophoblast lineage susceptibly to a multitude of viruses has been demonstrated using first-trimester placental explants. For example, it was shown that the specific site of HCMV replication resides in first-trimester VCT and that the overlaying SCT is relatively resistant to HCMV infection (81, 82). SCT express the Epidermal Growth Factor Receptor (EGFR) but lack integrin coreceptors, both of which are critical for efficient HCMV attachment and subsequent cell entry and intracellular replication (83). Using first trimester explants, integrin co-receptor expression in VCT led to HCMV entry and replication, whereas SCT and proximal cytotrophoblast columns, which do not exhibit such co-receptor expression, supported viral attachment but not cell entry or replication. Interestingly, HCMV may also interfere with syncytialization, as primary human term cytotrophoblast differentiation was suppressed following HCMV infection (84). Evidence regarding placental susceptibility to HCMV in early versus late pregnancy is conflicting, but it is generally observed that SCT are infected less frequently and do not allow for productive viral replication when compared with VCT (81, 82, 85, 86). These discrepancies may be in part due to the use of the high-passage HCMV strain's, AD169 and Toledo, in most prior studies (87). Glycoprotein mutations, gained through viral propagation in fibroblast cells, are now characterized in the HCMV strains,

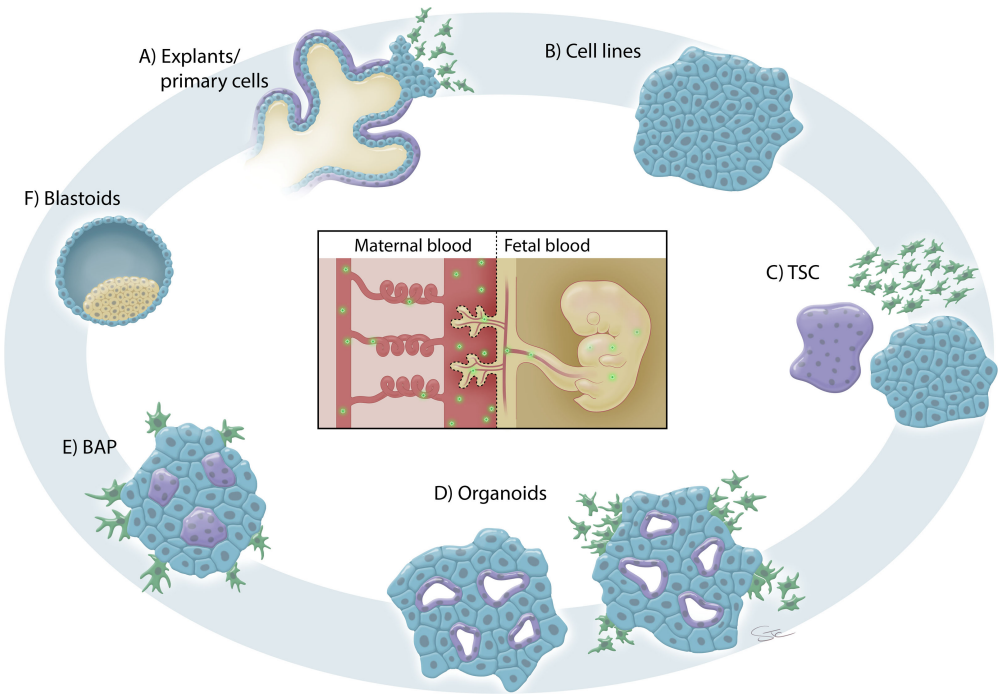

FIGURE 2 | *In vitro* trophoblast models for placental infection studies. **(A)** Primary cells and explant cultures from the first, second and third trimesters of human pregnancy have been used to study placental susceptibility to infection. These explant cultures contain all the trophoblast lineages (VCT, blue; SCT, purple; EVT; green) albeit at different proportions depending on the gestational age of the sampled tissues. **(B)** Various immortalized and cancer-derived trophoblast cell lines have also been used to study placental susceptibility to infection. The cartoon is simplified to demonstrate that typically only a single cell type is represented in these models. **(C)** Trophoblast stem cells (TSC) provide a new model for the study of lineage-specific effects of viral infections during pregnancy since all trophoblast lineages can be induced. TSC can be generated from embryonic/induced pluripotent stem cells or isolated from blastocyst outgrowths and first trimester placental tissue. **(D)** Trophoblast organoids can be generated from first trimester placentas. Organoids more closely mimic the villous structures of the placenta than two dimensional models and can be induced to differentiate into EVT. Trophoblast organoids can be utilized to study vertical transmission of virus as the layers of the placental villi are present in three-dimensions (albeit "inside-out"). **(E)** Stem cell-derived trophoblast (BAP) have been used to study placental susceptibility to several viral infections. This model most closely represents early placentation and contains a heterogenous population of trophoblast cells. **(F)** Blastoids, generated from embryonic/induced pluripotent stem cells, provide a new model of early human embryo structures. Blastoids could be used to investigate placental susceptibility to infection during the peri-implantation phase of pregnancy.

AD169 (88–90) and Toledo (91), and contribute to the loss of tropism for epithelial and endothelial cells. One study found that SCT from first and third-trimester placentas can be infected with HCMV although the third-trimester samples required higher viral doses (85). Similar to HCMV, experiments using primary cells and explants from first and second trimester placental samples indicated that ZIKV infects VCT, proximal cell columns and EVT but SCT were once again spared (17, 92). There are conflicting observations concerning the susceptibility of human term SCT to ZIKV infection (28, 47, 93–95). Given the limitations of primary cell cultures, these discrepancies could be related to variation in cell culture methods and/or infection protocol variations and differences in the specific ZIKV-strains and viral titers used among studies. Human first, second, and third trimester placental explant cultures have also been used to demonstrate the role of DENV-related ADE in ZIKV vertical transmission (67, 96, 97). Exploitation of anti-DENV antibodies by ZIKV to aid transport across the placental barrier could explain how the latter could cross what is typically an otherwise non-permissive barrier for many viruses during the third trimester of human pregnancy (47). This same enhancement was not evident with sera from yellow fever virus (YFV; *Flaviviridae*; *Flavivirus*) or chikungunya virus (*Togaviridae*; *Alphavirus*) infected subjects (96). Notably, YFV is not as closely related to ZIKV as is DENV.

Primary cultures (of term SCT) have been used to investigate the packaging and release of C19MC miRNAs into exosomes. These exosomes have been demonstrated to confer resistance against numerous viral insults to recipient cells (31, 47, 98–100). These same studies also demonstrated that SCT were relatively resistant to infections caused by picornaviruses (Coxsackie B virus and Poliovirus), rhabdoviruses (Vesicular stomatitis virus), herpesviruses (HCMV, HSV-1), and flaviviruses (ZIKV, DENV) (31, 47). While primary human term SCT are relatively resistant to ZIKV, inhibition of JAK signaling increased viral replication in these cells (101). This inhibition of JAK-STAT signalling, i.e., interferon signalling, is likely necessary for productive infection as these term SCT are known to constitutively mount an interferon response (47, 102). SCT isolated and cultured from first-trimester placentas expresses the innate immune recognition receptor, toll-

like receptor 3 and mounts an antiviral IFNβ response upon activation of this receptor (38). Mid-to-late gestation SCT constitutively releases type III IFNλ (47, 48), which has been reported to prevent viral transmission at skin and mucosal surfaces (103). It is notable that infection of primary human term SCT was minimal and only led to production of what the authors describe as "thin coated" virions when these term SCT were exposed to a clinical isolate of ZIKV (PRVABC59 strain from Puerto Rico, 2015), suggesting release of immature, infection-deficient viral particles (101). In contrast, upon interruption of JAK-STAT signaling, ZIKV infection of these same cells produced mature, "thick coated", and likely more virulent virions. Interestingly, in human macrophages, ZIKV infection does not antagonize STAT2 phosphorylation and this leads to restricted viral replication (104).

Cell Lines

Trophoblast-like cell lines have been frequently used to study placental infections. Unlike primary cultures, these cells are permissive to genetic manipulations and provide a better tool for mechanistic studies. These cell lines can be grouped into two main types: lines derived from choriocarcinomas (BeWo, JAR, JEG-3) and lines derived from first trimester placental cells that were subsequently immortalized (Swan71, ACH-3P, HTR8/SVneo) (**Figure 2B**). Since they are widely available, immortal, simple to propagate, and relatively stable in culture, these cell types are often chosen for initial experiments, which can be easily replicated and expanded in scale. Further, these cells can grow in simple media cocktails and on plastic culture dishes, making them a cost-effective choice. They have been used to assess treatments to prevent or limit infections. For example, treatment with palmitoleate reduced virus-induced apoptosis in ZIKV-infected HTR-8/SVneo, JEG-3 and JAR cells (105) and treatment with a non-nucleoside RNA polymerase inhibitor reduced virus replication in ZIKV-infected HTR8-SVneo cells (106). HTR8-SVneo cells were used to characterize pathogenicity of a ZIKV glycan-deficient mutant, which was shown to be less neuroinvasive in mice, yet the mutant virus displayed similar growth characteristics in this trophoblast cell line as did the wild-type (107).

These cell lines, however, have several limitations that can affect experimental design and interpretation. First, aside from BeWo cells, which are typically used to model SCT fusion, other immortalized trophoblast cell lines have a limited to non-existent capacity to differentiate into other trophoblast subtypes in culture. This characteristic restricts the utility of immortalized trophoblast cell lines in assessing viral effects on trophoblast differentiation. Further, there are crucial differences in the extent to which diverse trophoblast cell lines are able to form tight cellular junctions (108) and express and respond to Toll-like receptor activation (109, 110) when compared to primary trophoblast cells. Thus, experiments aiming at elucidating viral infection patterns across the placental barrier and immune responses to infections at this site become difficult to interpret when using immortalized or cancer-derived trophoblast-like cell lines. HTR8/SVneo cells (an extravillous-like trophoblast cell line) have the capacity to mount an antiviral response. Following infections with the flaviviruses ZIKV, DENV4, and YFV, infection patterns and immune responses were assessed in this cell line (111). All three viruses exhibited similar tropisms, yet ZIKV induced a stronger inflammatory response (involving IL-6, IL-8, CCL2, CLL3, and CCL5) but a lower IFN response when compared to DENV and YFV. HTR8/SVneo cells also responded to the presence of viral ssRNA in a TLR8-dependent manner (112). JEG-3 cells, which are typically cultured in two-dimensions, demonstrated an increased capacity to form syncytium, resist ZIKV infection, and basally expressed higher levels of IFN-stimulated genes when cultured in a three-dimensional system (48). Knocking down TLR7 and/or TLR8 by using siRNA also increased ZIKV replication in JEG-3 cells (113). Researchers often utilize one of these trophoblast cell lines alongside primary cultures to evaluate consistencies. For example, ZIKV-infected Swan71 and first-trimester primary cells (VCT) showed a similar induction of IFNβ and interferon stimulated genes (114).

Trophoblast Stem Cells (TSC) and Trophoblast Organoids

Recently, new models of first-trimester trophoblast have been developed that will greatly enhance our knowledge of early pregnancy susceptibility to a variety of insults, including infection. Human trophoblast stem cells (TSC) can be cultured long-term without differentiation in two dimensions (2D) or stimulated to differentiate into SCT or EVT (34) (**Figure 2C**). These TSC can be established from blastocyst outgrowths or isolated from first trimester placental digests (34) and are permissive to genetic manipulations (115–117). Similarly, human trophoblast organoids have been derived from first trimester placentas and provide a three-dimensional (3D) representation of the villous structures of the placenta that contains both SCT and VCT (35, 118, 119) (**Figure 2D**). Upon stimulation, these organoids will differentiate to form outgrowths of EVT that are highly invasive and move through a Matrigel substratum (35, 119). One caveat, however, is that these organoids routinely grow "inside-out", with the SCT structures forming cavities in the center of the organoid, when compared to the *in vivo* structure of the placental villi. This poses logistical hurdles when planning infection-based studies. To truly model vertical transmission during pregnancy, the SCT should be the first trophoblast subtype to contact virus, which would typically be introduced *via* maternal blood *in vivo*. It is therefore imperative that virus microinjection studies be optimized to allow direct exposure of "internal" SCT to virus using existing "inside-out" trophoblast organoids or that suspension and scaffold-based techniques be developed to invert the polarity of these organoids (120, 121).

Interestingly, even though both TSC and trophoblast organoids are derived from first-trimester placenta, each has a bias in terms of the specific *in vivo* trophoblast lineage counterpart it most closely resembles (122). Using distinct proliferation media, multiple groups have demonstrated that TSC's are nearly all positive for ITGA2 (> 90%) (122, 123), a

marker of the proliferative cells at the base of the cytotrophoblast cell columns (124), yet trophoblast organoids contain large areas of syncytium, numerous VCT (TP63+) and a small portion of ITGA2+ cells (< 23%) (35, 122). Further, there is also conflicting evidence about how well these TSC form multicellular organoid structures. When directly comparing TSC-derived organoids (TSC-organoids) and trophoblast organoids derived directly from first trimester tissue, the TSC-organoids formed significantly fewer SCT and secreted lower levels of HCG into the spent culture media (122). Other groups have demonstrated the ability of TSC-organoids to form SCT (115, 125–127) but it should be noted that none of these groups directly compared them to trophoblast organoids. It is still unclear why these results differ; however, we suspect that distinct methodologies might play a role. Sheridan et al. (122) passaged the TSC's in organoid form for more than five passages, whereas the other groups analyzed only directly converted 3D cultures. Perhaps, once these TSC-organoids are passaged, they might lose the ability to spontaneously form SCT to the same degree as *bonafide* trophoblast organoids.

Due to ethical concerns and legal restrictions, not all investigators have access to early placental tissues. However, several research groups have recently described the ability to generate first trimester-like trophoblast stem cells from embryonic or induced pluripotent stem cells (123, 126, 128–130). These technologies increase world-wide access to approaches that model first trimester human placental development but do not require primary tissues. Most recently, it has been reported that trophoblast stem cells can be isolated from term placentas (131, 132; Preprint: 133), an accomplishment that was previously thought to be impossible. That said, the exact identity and function of these proliferative trophoblast cells needs to be further defined. Do these proliferative trophoblast cells represent the same proliferative cells found in first trimester human placental tissues or are they an entirely different stem cell population only found in later pregnancy? Such questions need further clarification, but the models may provide an exciting platform for disease-based studies given that some diseases of human pregnancy only manifest clinically in late pregnancy and the pathology of the placenta at the time of isolation of these term trophoblast stem cells is known. Lack of information on pregnancy outcomes remains a seemingly insurmountable limitation of stem cells isolated from many first trimester specimens.

BMP4 Induced Trophoblast

Exposing pluripotent stem cells to bone morphogenetic protein 4 (BMP4) has also been employed as a useful model to study trophoblast differentiation (**Figure 2E**). The complete differentiation cocktail includes BMP4, an ACTIVIN/transforming growth factor (TGF)β inhibitor (A83-01) and a fibroblast growth factor (FGF)2 inhibitor (PD173074) and has been named BAP treatment and hence BAP-derived trophoblast (134). This model has been utilized by our group to investigate infections of the human placenta with ZIKV (102, 135) and SARS-CoV-2 (136), and by other groups with protocol modifications (137, 138). We found that these BAP-derived trophoblast were highly susceptible to ZIKV infection and that the African-lineage strains induced more severe cell lysis when compared to the contemporary Asian-lineage strains commonly associated with fetal microcephaly (102, 135). These findings led us to speculate that a maternal infection in early pregnancy with an African-lineage strain would actually result in a preclinical, early pregnancy loss which would abrogate the possibility of vertical transmission and potential fetal abnormalities. Interestingly, the BAP-derived trophoblast cultures also express reduced levels of C19MC miRNAs when compared to other trophoblast models, including trophoblast cell lines and term placenta (99, 102, 139) which could be a potential reason for their increased susceptibility to ZIKV. Another study suggests that stem-cell-derived trophoblast are equally lysed by Asian and African lineage-strains of ZIKV; however, in this study BMP4 was used in the absence of the other BAP cocktail ingredients and for only a single day to induce differentiation prior to infection (137), whereas our studies induced differentiation for four days with the complete BAP cocktail (102, 135). In fact, the ability of the African-lineage strains of ZIKV (in comparison to strains of the Asian-lineage) to induce massive cell death has been reported in other models, including stem cell-derived neural progenitor cells (140) and mouse embryos (141).

Important limitations of the BAP-derived trophoblast model are the short-lived nature of the cultures when grown in two dimensions and the heterogenous nature of the trophoblast generated during differentiation. Over the years, groups have questioned the validity of this model based upon the idea that stem cells from the epiblast might not have the ability to give rise to trophectoderm (126, 130, 139, 142). However, numerous studies and stringent comparisons with *in vivo* trophoblast indicate that these cells certainly represent trophoblast (143–146). A lingering question for BAP-derived trophoblast surrounds the precise trophoblast lineages and the developmental stage of placentation recapitulated by the model. It is clear that the transcriptome of SCT generated from BAP treatment is distinct from *in vitro*-derived SCT collected from term placentas (144). Recently, BAP-derived trophoblast were subjected to single nuclei RNA sequencing and demonstrated the presence of multiple transcriptomic cell clusters representing those seen for SCT in first-trimester placenta (24, 146). This same analysis revealed that only a few of the identified transcriptomic cell clusters exhibited cellular markers that were weakly associated with the EVT-like population isolated from primary first trimester placentas, suggesting the possibility that BAP treatment perhaps may not generate *bona fide* EVT (146). This finding would support the postulated identity of BAP-derived trophoblast as primitive syncytium, i.e., the invasive placenta prior to villous formation, since SCT would be highly represented at this early point in peri-implantation placentation whereas migratory EVT-like cells would be rare (102, 147, 148).

Blastoids

While it is impossible to validate the expression profile of the primary SCT (a.k.a., primitive trophoblast) formed in early pregnancy *in vivo*, advancements in extended embryo culture

(149, 150) and in the culture of blastoids generated from pluripotent stem cells (151–154) could provide important insights into this stage of human placental development (**Figure 2F**). Blastoid structures can also be directly generated by reprogramming fibroblast cells (155) or through the use of extended-potential stem cells (156). Very little is known about blastocyst implantation and very early placentation in humans and these models offer a unique opportunity to study the impact of maternal infections during the peri-implantation phase of pregnancy. They also allow improved comparisons to previous works on peri-implantation rodent and non-human primate placentation. The establishment of reproducible and efficient blastoid protocols are highly important as work with human embryos is challenging due to the rarity of samples and their restricted access for many research groups.

THE EFFECT OF GESTATIONAL AGE

The placenta's many functions include nutrient exchange between mother and fetus, physical support for the fetus, immune protection, and maintenance of a maternal physiology that advances fetal development *via* production of placental hormones. The placenta must simultaneously respond to continuous changes in fetal growth and development, maternal physiology, as well as to pathogenic and environmental stressors across gestation (157–159). The available routes for viral breaching of the placental barrier and the ability of virus to infect and be transported across this barrier also change throughout gestation as does the degree of insult to the developing fetus infected by a pathogen *via* vertical transmission.

RuV, the causal agent of Rubella (German measles), was the first reported example of a teratogenic virus (160). Although no longer prevalent in the United States due to the development and widespread use of an effective vaccine (MMR, effective against measles, mumps, and rubella), there are still many Rubella cases worldwide in places where vaccination programs are not as common (161). The fetal effects of maternal RuV infection during pregnancy are closely linked to the timing of infection and thereby the risk for vertical transmission. The risk of congenital infection is significantly higher when the mother is infected with the virus within the first 12 weeks of pregnancy and this risk decreases dramatically by the third trimester (162). Very little is known about how RuV crosses the placenta. In one case study, viral antigen was detected in the basal plate and in the endothelial cells within the chorionic villi of the placenta at week 35 of gestation following primary maternal infection in the 13th week of pregnancy (163). This is indicative of a persistent mode of infection, which would allow for prolonged exposure of the placenta/fetus to the virus when infection occurs early in pregnancy.

Prospective cohort studies of primary HCMV infections indicate that the risk for intrauterine vertical transmission is the highest when maternal infection occurs in the third trimester of pregnancy (164, 165). This risk of transmission correlates with the proposed mechanism that HCMV traverses the SCT *via* FcRn-facilitated transcytosis, a potential transmission pathway that becomes increasingly robust throughout later pregnancy (56). Even so, the risk for congenital defects is highest when the mother is infected in her first trimester (166).

The risk for vertical transmission of ZIKV and associated fetal abnormalities is also strongly and positively associated with first trimester infections (167–171). Although several initial case studies reported fetal abnormalities associated with third-trimester infections (69, 172), larger cohort studies indicated that the risk for microcephaly and severe brain defects in the fetus is limited to pregnancies in which maternal infection occurred in the first trimester (171). *In vitro* models also suggest that ZIKV infection is enhanced in placental trophoblast from the first trimester when compared to that from term pregnancy. Our group demonstrated that the stem-cell derived trophoblast (BAP) that most closely resembles the primitive placenta of post-implantation human pregnancy are highly susceptible to ZIKV infection (102, 135). One study suggests that cross-talk between maternal decidual and fetal trophoblast cells may also help to determine susceptibility to ZIKV infection throughout gestation (28). To this point, decidual cells from first trimester pregnancies were infected by ZIKV at much higher rates than equivalent cells collected at term. Further, conditioned media from ZIKV-infected first trimester decidual cells enhanced ZIKV infection of primary term SCT, which had previously been shown to be resistant to ZIKV infection (28, 47). VCT isolated from term placentas also showed the presence of actively replicating ZIKV, suggesting that vertical transmission of the virus could occur in the third trimester if ZIKV has been able to cross the SCT barrier by then (16).

As discussed above, there are examples of stark, gestational-age-dependent differences in the risk for placental infection and vertical transmission after maternal exposure to viruses. Therefore, studies indicating that trophoblast generated/collected from term placentas are relatively resistant or susceptible to a particular virus may not directly translate to similar levels of susceptibility in the first trimester.

CONCLUSION

The placenta is critical for protecting and nourishing the developing fetus. While there are many mechanisms in place to prevent harmful substances and pathogens from entering and traversing the placenta, there are several examples of viruses that can do just that and thereby disrupt placental and fetal development. Advancements in *in vitro* trophoblast modeling and a burgeoning understanding of placental development and function will promote an improved capacity to study placental infections from a more critical viewpoint. A long history of experimental inconsistencies and a diverse set of *in vitro* placental models that may not recapitulate *in vivo* events have led to contradictions concerning how (and to what degree) the human placenta is infected with different viruses. Future studies using more robust and standardized model systems will

hopefully generate more unified results. More consistent results, in turn, should allow for the development of new diagnostic tests and strategies to prevent and/or treat viral infections in pregnancy.

AUTHOR CONTRIBUTIONS

MS wrote the manuscript. JZ, AF, and DS provided critical feedback and review and editing of the manuscript. All authors contributed to the article and approved the submitted version.

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Viral Infections and Temporal Programming of Autism Spectrum Disorders in the Mother's Womb

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Recent reports have suggested a tight relationship between viral infections and neurodevelopmental disorders. In this regard, fetal brain damage can be caused by direct viral infection or induced immune responses and cytokine storm. Although recent years have seen phenomenal progress in diagnosing autism spectrum disorders (ASD) and identifying genetic and epigenetic causative factors contributing to this group of neurodevelopmental disorders, almost 60% cases in children remain of unknown etiology. Little is known about the collective pathophysiology of ASD. In this regard, epidemiological data suggest that viral infections during pregnancy are associated with high risk of having an autistic child. Although SARS-CoV-2 infections have been documented in pregnant women, we do not yet know whether COVID-19 pandemic will contribute to the onset of autism-like features in the offspring or impact autistic individuals. We hypothesize that ASD are programmed in the mother's womb and that uterine, not peripheral, immune activation is the initial trigger to induce fetal brain developmental anomalies. We further hypothesize that exposure to infections only during a temporal window of pregnancy impact the onset of ASD-like pathology, particularly in the male fetus/offspring. We will discuss the role of uterine regulatory T cells and their inflammatory trans-differentiation in the pathophysiology of ASD and comment on possible therapeutic intervention options.

Keywords: ASD autismspectrumdisorders, UIA uterine immune activation, Tregs regulatory T cells, Th17 T helper 17 cells, IL17a interleukin 17a isoform, poly I, C polyinosinic, polycytidylic acid

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INTRODUCTION

Obstetric infections are particularly dangerous to both the mother and the developing fetus as they may orchestrate events that interfere with normal fetal developmental programs (1–4). The placenta is now considered a specialized immune organ, and in this regard, intrauterine infections may adversely affect the immune balance regulated by the placenta in concert with the maternal immune system (5, 6). Viruses have evolved contemporary ways to evade the immune system and to cause diseases (7). Local cytokine storm and functionally or proportionally altered immune cell profiles have been the main consequences of viral infections (8, 9). Other co-infections by bacteria or parasites may further compound these immune responses. In the case of fetal growth and survival, these infections will have enormous deleterious effects. The well-known

congenitally acquired infections/pathogens that cause morbidity and mortality in newborns are called TORCH (*Toxoplasma gondii*, Others like *Treponema pallidum*, Rubella, Cytomegalovirus, Herpes Simplex Virus) infections. The TORCH group has been expanded to include Parvovirus B19, human immunodeficiency virus (HIV), *Varicella zoster* virus, Hepatitis C virus (HCV), Zika virus (ZIKV), and *Plasmodium falciparum* among others (5, 10–14). These maternal infections are passed either transplacentally or during the birth process. These pathogens may act independently or in concert to cause neonatal morbidities. More importantly, an array of literature now suggests an association between viral infections and poor fetal brain development and childhood diseases such as autism, schizophrenia, bipolar disorders, microcephaly and other serious brain disorders (14–16). As a matter of fact, several of these adverse neurobehavioral outcomes share the perinatal inflammation pathogenesis.

Autism spectrum disorders (ASDs) are characterized by symptoms such as early social impairment, repetitive behavior, communication challenges, and learning and speech impairments, among other social traits (17, 18). Children and adults with autism have impaired social cognitive ability and perception, common executive dysfunction, and delayed information processing. Genetic causes have a crucial role in the development of ASD-like disorders, but early exposure to environmental factors during brain development has been shown to significantly increase the severity of the disorder (19, 20). According to the World Health Organization (WHO), ASDs affect around 1 in every 160 children worldwide. However, in the United States, the prevalence is astonishingly high, at one in every 55 children. The male offspring show a prevalence of ASD that is four to five times higher than that of their female counterparts (21).

Since inflammation, cytokine imbalance, and viral neurotropism could have an impact on fetal brain development, the potential role of immunological dysregulation in autism has garnered particular attention (22, 23). Viruses may cause ASD by directly infecting the brain, by inducing local and/or systemic cytokine storm, or by altering maternal or offspring localized immune responses. Several prior studies have linked ASD to various viral infections (24, 25).

In this review, we will focus on the evidence that uterine infection/specialized inflammation during a narrow gestational window (second trimester) has deleterious effects on fetal neurodevelopment. How viruses can access cellular components of the placenta and the pathogenic mechanisms that facilitate the process of specialized inflammation at the maternal–fetal interface is a subject of intense discussion. We also discuss resistance mechanisms used by the fetus and maternal–fetal interface that protect viral infections and inhibit the onset of ASD-like diseases. We summarize the emerging body of evidence in both humans and animals that links viral infection with increased incidence of autism. We propose a mechanism of inflammatory transformation of a uterine immune cell type that could be the basis of the early onset of brain developmental defects associated with ASD.

Abbreviations: ASD, autism spectrum disorders; UIA, uterine immune activation; Tregs, regulatory T cells; Th17, T-helper 17 cells; IL17a, interleukin 17a isoform; LPS, lipopolysaccharide; Poly I:C, polyinosinic:polycytidylic acid; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; COVID-19, coronavirus disease 2019; ZKV, Zika virus; CMV, Cytomegalovirus; TNF-α, tumor necrosis factor alpha; STB, syncytiotrophoblast.

IMMUNE DEFENSE AT THE MATERNAL–FETAL INTERFACE DURING VIRAL INFECTION

The decidual lining of the uterus, which is high in leukocytes, is one of the first lines of defense for the mother and fetus at the maternal–fetal interface. The decidua is replete with effector T lymphocytes, regulatory T cells (Tregs), NK cells, innate lymphoid cells, and macrophages (26). As the trophoblast invades and establishes the placental vascular bed and interacts with decidual immune cells, a milieu of immune tolerance is established. In order to orchestrate immune-tolerance, decidual cells remain in close contact with invading extravillous trophoblasts (EVTs) during pregnancy, particularly first and second trimesters. Using cell-to-cell fusion and the production of interferons (IFNs), exosomes, and antimicrobial peptides, trophoblasts create a functional barrier that confers antiviral resistance (3, 27). Fetal macrophages, also known as Hofbauer cells, proliferate rapidly upon virus infection. Hofbauer cells are targets of many viruses, including CMV and ZIKV. It is not clear if Hofbauer cells act as a check on viral propagation or more as a reservoir for the virus itself (28, 29). However, despite the fact that decidual immune cells generally have a more anti-inflammatory profile than their blood-borne counterparts, there is evidence of inflammatory transformation of decidual natural killer cells (dNKs), macrophages, and T cells in response to viral and bacterial products (30, 31). When infected with the influenza RNA virus during pregnancy, pDC exhibited an increased response and type I IFN production (32, 33). This is in contrast to required physiological inflammation during embryo implantation. Post implantation, the maternal–fetal interface is dominated by an "anti-inflammatory" phenotype, which is associated with preponderance of regulatory NK cells, M2 macrophages, and regulatory T cells (Tregs) that are critical for fetal protection. Tregs appear to play a vital role in regulating inflammation in early pregnancy and developing a responsive uteroplacental environment through their potent anti-inflammatory regulation (34, 35). When encountered with pathogenic assault, Tregs may switch to Th17 phenotype and suffer from plasticity or acquire a dual phenotype of Treg-Th17 cells (36, 37). Depending on the pathogen and the gestational age, these cells may promote overlap (plasticity) with Th17 cells and reprogram their conventional role as suppressive T cells (37). Our unreported findings suggest that uterine immune activation during pregnancy may lead Tregs to trans-differentiate into Th17 cells, altering fetal brain development and causing an ASD-like behavioral phenotype in the male offspring (38) (**Figure 1**).

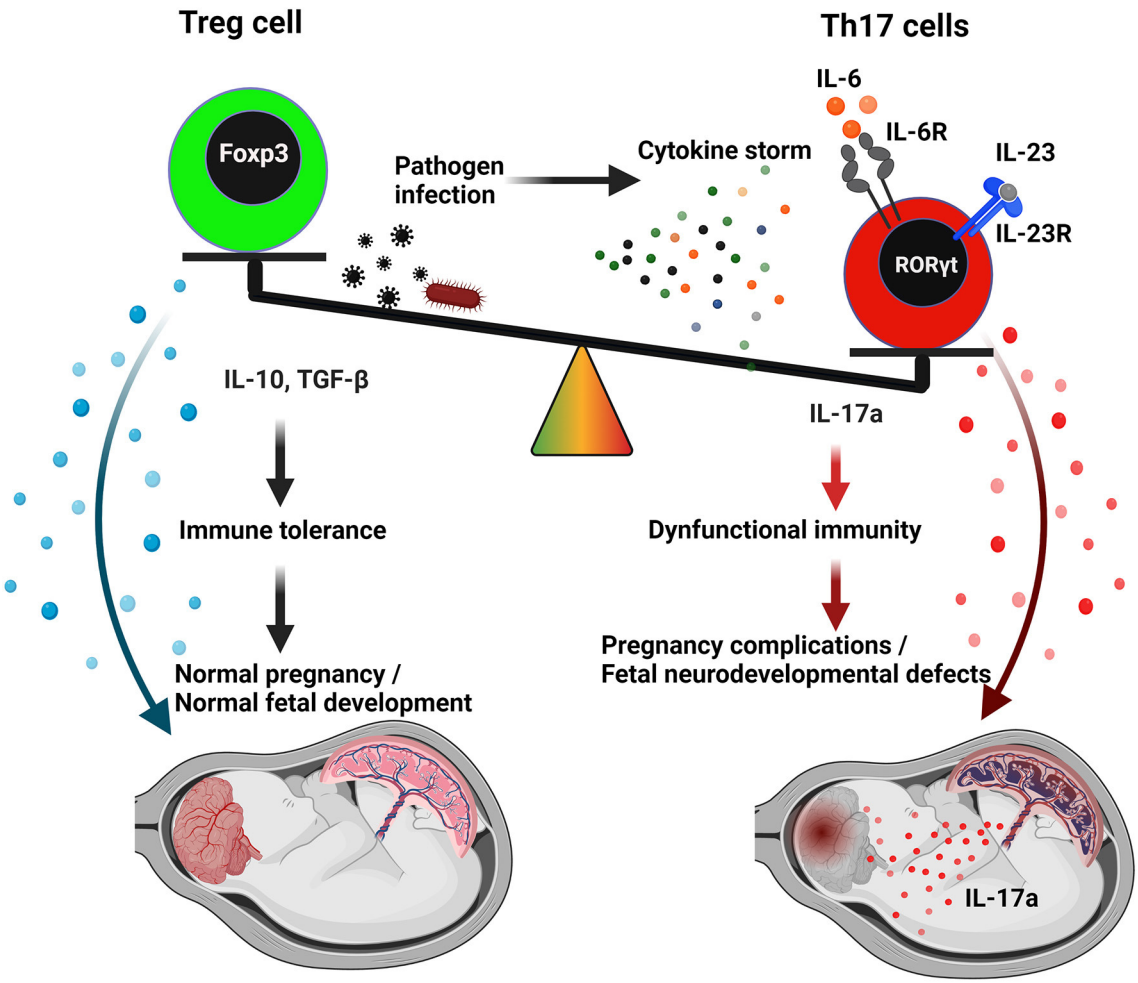

FIGURE 1 | A proposed model for Th17/Treg imbalance and IL-17a in viral infection mediating autism spectrum disorders (ASD). Uncontrolled systemic or uterine inflammation during pregnancy by pathogenic infection might generate cytokine storm effect. The principal component of cytokine storm, IL-6, could modify the uterine cytokine microenvironment and facilitate alteration of the Th17/Treg imbalance in favor of the Th17-population. Th17 cells, in an exacerbated proinflammatory response, can promote ASD through IL-17a. In contrast, Treg cells maintain self-tolerance and maintain normal fetal development through an anti-inflammatory response (IL-10, TGFβ). Th17 cells and Tregs, despite their seemingly diametrically opposed functions, share comparable features and differentiation mechanisms. IL-17 released by Th17 cells traverses the placenta and could potentially reach the fetal brain in the presence of an incomplete blood–brain barrier.

PLACENTAL VIRAL INFECTION

The placenta is a temporary reproductive organ that allows the intermediation between the mother and the fetus during pregnancy. Because of its ability to modulate maternal immune responses, it is also called a potent immune organ. The syncytiotrophoblast (STB) forms and maintains the outer layer of villi by fusing the inner layer of proliferative progenitor cells known as villous cytotrophoblasts (CTBs) (39). CTBs also differentiate into invading trophoblasts primarily residing in the anchoring villi. These invading trophoblasts further differentiate into endovascular trophoblasts that are involved in spiral artery remodeling in the decidua. Invading extravillous trophoblast (EVTs) are involved in the cross-talk with decidual immune cells to regulate immune tolerance, angiogenesis, and fetal development (39, 40). Within the maternal–fetal contact, viruses can infect a variety of cell types and also traverse from one cell to another through caveolin-dependent endocytosis, macropinocytosis, or uptake processes, without the need for cellular receptors (41).

In the case of ZIKV, evidence of viral replication was found in proliferating villus and Hofbauer cells in the villous core. Through infected maternal blood macrophages, ZIKV or HIV can get into placental trophoblast cells and infect them (42, 43). Significant maternal viremia is required for placental infection to occur. By bathing terminal villi in maternal blood, the STB barrier serves as a common entry point for infectious agents to enter fetal blood and other organs later in the gestation. Viruses such as CMV, ZIKV, and SARS-CoV-2 may all infect the placenta directly by attaching to viral receptors on the maternal side of STBs, while other viruses can use antibody-dependent enhancement (ADE) to get through the STB barrier (44–46). Complex immune

evasion approaches are employed by members of the HSV and CMV families. These viral infections may be curtailed by robust IFN responses and anti-inflammatory activities of hormones and cytokines. In normal pregnancy, the placenta is equipped to protect the mother and baby from low viremia cases. However, overwhelming viremia and compromised local immunity may lead to infections in the placenta. CMV, ZIKV, and other infections have been shown to maintain vigorous viral replication in the placenta (47, 48). The evasion mechanisms employed by the viruses often target components in the type I interferon pathway. Congenital infections such as ZIKV, HIV, CMV, SARS-CoV-2, and others have been shown to suppress this pathway (49–52). Consequently, proinflammatory cytokines are normally induced, leading to the downstream events of tissue inflammation and damage (49, 50). The ZIKV protein NS5 antagonizes type I interferon-mediated induction of RIG-I pathway to neutralize the placental defense. Similar to ZIKV, CMV utilizes its IE1 protein to weaken the host's natural defense mechanisms (53, 54). When it comes to mimicking congenital pathogens, CMV and ZIKV are particularly effective since they both target the host interferon response while producing widespread infections during early pregnancy when innate defense in the placenta is challenged by immune evading mechanisms (53, 54).

In the context of the new COVID-19 pandemic and placenta infection, SARS-CoV-2 has been screened in placental sections, amniotic fluid, and cord blood. SARS-CoV-2 was found in the STB and villous fibroblasts of a COVID-19 patient with severe disease using transmission electron microscopy (TEM) (55). In the case of SARS-CoV-2, the spike protein must attach to angiotensin-converting enzyme 2 (ACE2) in order for the virus to enter the cells. Molecular analysis has revealed the presence of ACE2 in various components of the placenta, including syncytiotrophoblasts, endothelium, and vascular smooth muscle (56). Transmembrane protease serine 2 (TMPRSS2) and molecules, such as cathepsin B/L7 and furin, are required for active viral infection. Researchers have discovered the substantial expression of ACE2 in the placenta by single-cell RNAseq, but not TMPRSS2 in the placenta (57, 58). Recent studies using single cell RNAseq during early gestation indicated that ACE2 was expressed in the placenta, but TMPRSS2 expression was either absent or extremely low (58). It is then possible that other proteases that may contribute to viral replication in the placenta remain to be identified. There are a number of interesting aspects of placentation that ACE2 is involved in, including trophoblast migration and maternal vasodilation (59, 60). Uterine arterial malfunction in pregnant mice lacking the ACE2 gene was linked to reduced umbilical blood flow and placental hypoxia. Adverse pregnancy outcomes such as miscarriage, ectopic pregnancy, and hypertension have also been linked to ACE2 (60). There is therefore the possibility of placental anomalies and pregnancy consequences if SARS-CoV-2 affects the expression of ACE2 in the placenta. The presence of ACE2 in the placenta suggests that SARS-CoV-2 may be able to attach to it, resulting in the initiation of viral infection. Another way for the virus to breach the placental barrier is for it to be carried by blood cells. However, there is controversy regarding whether SARS-CoV-2 efficiently infects CTBs and/or EVTs (61, 62).

Although there are case reports of vertical transmission, evidence, so far, suggests that SARSCoV-2 does not transmit vertically (63). SARS-CoV-2 has not been discovered in cord blood, throat and nasopharyngeal swabs, urine, or feces from many neonates screened for the virus at birth. SARS-CoV-2-negative amniotic fluid samples have also been collected from COVID-positive pregnancies (64, 65). These observations suggest that SARS-CoV-2-mediated effects on fetal development must be regulated by local cytokine storm and/or uterine immune activation.

CYTOKINE STORM AND AUTISM SPECTRUM DISORDER

Cytokine storm disorders are caused by a complex interwoven network of cells, signaling pathways, and cytokines. It is considered that cytokines, such as interferon-γ, interleukin-1, interleukin-6, tumor necrosis factor (TNF)-α, IL17, and interleukin-18, play critical roles in immunopathology when their levels are increased during a cytokine storm (8, 9). The microbiome, genetic traits, and underlying conditions all influence the cytokine patterns. Innate immune cells recognize and respond to a wide variety of microbes by releasing cytokines that activate cells of the adaptive immune system *via* pattern recognition receptors that are not antigen specific (8, 66). Cytokine storms frequently result in an inflammatory response that is more of a Th1 type. To protect the body from intracellular infections, effector T cells release significant amounts of interferon-γ that induces delayed hypersensitivity reactions and activate macrophages (8, 67). There is evidence that Th17 cells can be the driving force behind a cytokine storm that is not dependent on interferon-γ (68).

The deleterious cytokine storm has been reported in response to several viral infections, including influenza H5N1 virus, influenza H1N1 virus, and SARS-CoV-2. MERS-CoV infection was also reported to induce increased concentrations of proinflammatory cytokines, IFN-γ, TNF-α, IL15, and IL17. A significant number of COVID-19-associated deaths have been linked to acute cytokine storm (67). Plasma cytokine levels of 41 COVID-19 confirmed cases in China revealed elevated levels of an array of cytokines (68). A recent study with a large cohort of COVID-19 patients showed that serum IL-6 levels could be used to predict the patient's prognosis. These studies reported that serum IL-6 level was significantly high in mortality cases compared with recovery cases. Additional studies also confirmed the significance of plasma IL-6 as a measure of COVID-19 severity. Even in pediatric COVID-19 patients, ranging from 2 months to 15 years, significant increase in the levels of IL-6, IL-10, and IFN-γ was reported (67, 69).

Systemic "cytokine storm" can potentially cause secondary immune activation at the placental niche (70, 71). In animal studies, cytokines have been found to have a critical role in mediating the effects of uterine immune activation on the developing embryo. Pregnant women who have been infected

with certain virus release proinflammatory cytokines at the maternal–fetal interface, amniotic fluid, and the fetal brain, which results in altered fetal behavioral outcomes. In animal models, IL-6, IL-17, TNF-α, and IL-1β have been identified as significant mediators of the fetal response to local inflammation, leading to ASD (72–74).

UIA-induced cytokine storm plays a central role in modulating the local immune system and possibly the brain immune responses. Cytokines act as messengers between the brain and the hypothalamus during infection, influencing the brain's response to fever and sickness (75). Increasing evidence points to the role of cytokines in higher-level brain processes, such as memory and cognition (76). Consequently, dysregulation of the immune system's cytokine signaling and/or regulation can result in a wide variety of neurological effects and complications. The importance of these interactions varies depending on when, how long, and how intense they are. For example, cytokines have varied effects on the developing and adult brains (76). For brain immune responses, neuronal stem cells (NSPCs) and immune cells communicate *via* cytokines, which can have both protective and harmful effects depending on the cytokine profile of each cell type (77, 78). The effects of cytokine and chemokine signaling on brain cell activity, proliferation, and survival are highly variable. Viral infections frequently change NSPCs, either directly through viral infection or indirectly through immune cell activity or cytokine/chemokine signaling (77). Multiple changes in the behavior of infected NSPCs can be induced by cytokines/chemokines that affect NSPC numbers, differentiation into other neural cells, migration to areas of injury, and eventually the development and repair of the human brain (79).

Individuals with autism are shown to have dysregulated interleukin-6 (IL-6). Children and adults with the disease have elevated amounts of IL-6 in their blood compared with healthy individuals. Postmortem brain tissues from children with ASD also show elevated levels of IL-6 (80, 81). According to immunohistochemistry examination of cerebellar sections, autistic postmortem brain specimens had considerably greater IL-6 staining (82). Autistic brain exhibits significantly higher IL-6 and its receptors in the brain (83, 84). In adults, circulating IL-6 from the peripheral tissues can pass the blood–brain barrier and affect a wide range of activities in the brain (85, 86). Multiple studies have shown that UIA triggers inflammatory response through proinflammatory cytokines in the embryonic brain. IL-6 mRNA and protein levels in the fetal brain have been found to increase in the wake of an UIA (87). This molecular interaction supports the feed-forward inflammatory cycle. The elevated expression of IL-6 has been seen in a number of central nervous system (CNS) illnesses (88), including those caused by HIV, CMV, and Zika viral infections (89–91). IL-6 induction appears to be responsible for many of the long-lasting behavioral changes seen in UIA-born offspring. The presence of IL-6 in the brain throughout neural development affects avoidance learning and causes autism-like behavior, whereas mice lacking IL-6 are more susceptible to infection and have impairments in fear conditioning (92, 93). Exposure to IL-6 in the womb alters the offspring's NSPC pools for the rest of their lives. In the fetus, maternal IL-6 treatment increases the number of cortical and forebrain neural precursors (94). Neuropathology, GABA dysregulation, and immune system alterations are all linked to IL-6 during the course of a child's life (95). Infection during pregnancy has the same effect. IL-6 has a wide range of effects on the developing brain. Neuronal self-renewal, migration, cell survival, and neurite outgrowth are all influenced by IL-6 and its family members (96–98). It is also possible that exposure to IL-6 during critical periods of pregnancy will change the synaptic networks of neurons in offspring. IL-6 overexpression causes a reduction in glutamate receptor expression *in vitro* and *in vivo*, as well as an increase in the ratio of excitatory to inhibitory synapses in the brain (99, 100). This is especially relevant in the context of autism, where a disproportionate excitatory-to-inhibitory neuronal ratio is thought to play a role in the development of the disorder. In this way, maternal IL-6 not only affects the pre-natal NSC pool but also has an effect on the post-natal NSPC pool. As a result of the changes in fetal NSPC activity, the SVZ pools of adult NSC display increased proliferation and neurogenesis (97, 98). Instead of increasing NSC proliferation and astrogliogenesis, when localized IL-6 was expressed from ZIKV infected microglia, there was a decrease in neurogenesis. This is in contrast to circulating maternal IL-6 (101). IL-6 has also been reported to have some protective effects on neuronal stem cells. There is a reduction in both the stem and immature neuron populations *in vitro* after infection with HSV1. When an active infection is present, however, IL-6 from microglia prevents these effects from occurring (102). It is possible that the setting of the inflammatory milieu, together with the presence of other cytokines, will have an impact on the effects of IL-6. However, subsequent human studies have cast doubt on the role of gestational IL-6 alone in the development of autism. Increased IL-6 levels in mid-pregnancy maternal serum and amniotic fluid were found to be linked with developmental problems but not autism in a longitudinal analysis.

Additionally, IL-6 plays a critical role in regulating the balance of proinflammatory Th17 cells and Tregs (103). Similar to IL-6, this upregulation of IL-17a in the pre-natal environment can also lead to abnormal fetal neurodevelopment and has been consistently associated with ASD (104). Furthermore, expression of IL-17RA in the brain dramatically increases during UIA. Cortical dysplasia is a prevalent characteristic in children with ASD (105, 106). The subtle pathogenic changes in the fetal brain that may occur in response to viral infections during pregnancy changes are depicted in **Figure 2**. This has been confirmed in mouse models. Interestingly, the architectural organization of neurons in the cortex was disrupted in embryonic mice injected IV with IL-17a at an early stage of development (E14.5). Indirect evidence supports the role of IL-17 in direct neuronal damage. Different neuronal populations express IL-17 receptor (104). IL-17 disrupts blood–brain barrier (BBB) tight junctions *in vitro* and *in vivo* and promotes CNS inflammation (107). IL-17 may contribute to CNS tissue damage by affecting the cells that express IL-17 receptor, such as microglia, endothelial cells, astrocytes, and neurons (108, 109). IL-17a was found to be sufficient to generate ASD-like traits in male offspring early and persistently. This is consistent

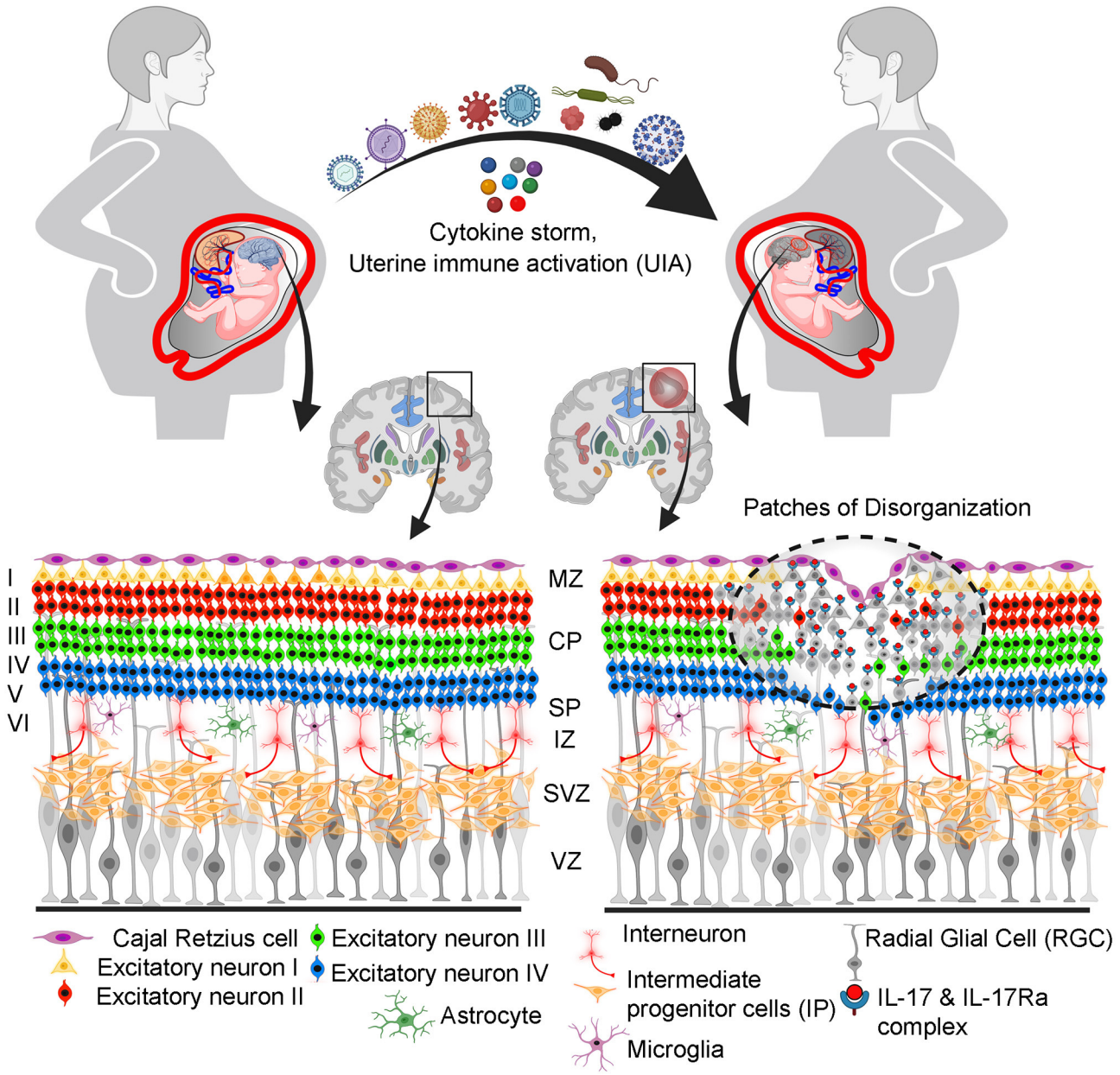

FIGURE 2 | Proposed pathophysiological pathway illustrating how prenatal maternal viral infection and associated cytokine storm disrupted fetal brain programming leading to increased risk of ASD. Among cytokines, IL-17a binds to receptors in the upper layer neuronal cells and alters their identity and plasticity. This can lead to changes in the cortical layer organization and "patchy disorganization" in some regions of the somatosensory cortex. This disorganization potentially affects fine orchestration, proliferation, neurogenesis, migration, and gliogenesis (astrogliogenesis and oligodendrogenesis, and synaptogenesis). Cortical layering during embryonic development: MZ, marginal zone; CP, cortical plate; SP, subplate; IZ, intermediate zone; SVZ, subventricular zone; VZ, ventricular zone.

with the male preponderance, and behavioral findings in ASD, which suggests that persistent maternal IL-17 contributes to the pathogenesis of ASD in offspring (104, 110). Persistent IL-17 during pregnancy may have lingering effects even during adulthood where it may cause defects in adult glia, inhibitory synapses, and behavior (111). It is also necessary to examine if maternal IL-17 are directly responsible for the cytokine responses reported in the fetal brain. Contrary to its embryonic effect on ASD development, IL-17 may have opposite effects during high fever scenario in adults (112, 113). This behavioral recovery was followed by a reduction in neuronal activity in the primary somatosensory cortex dysgranular zone (S1DZ). This finding tends to support the hypothesis that certain children with autism spectrum disorder (ASD) demonstrate behavioral gains when experiencing inflammation followed by fever (113).

VIRAL INFECTIONS AND RISK OF AUTISM

Pregnant women are more vulnerable to infections. During pregnancy, viral infections may or may not develop clinical indications in the mother. Indirect or direct consequences on the fetal development are inevitable. Infections will trigger immune responses, particularly in the placental microenvironment. However, immune responses are not always pregnancy compatible. The observations from human and animal studies suggest that pregnancy when challenged with viral infections is more likely to have an offspring with ASD-like features and other neurodevelopmental anomalies (114, 115). Neurodevelopmental problems in the fetus can be caused by both DNA and RNA viruses that cross the maternal–fetal interface (116). Here we have reviewed a few of the viral infections during pregnancy that have been associated with the onset of ASD. We also suggest mechanisms encompassing neurological immunological pathways that could play a role in the programming of ASD during fetal neurogenesis.

Zika Virus

ZIKV is a single-stranded RNA virus of the Flaviviridae family. In general, *in utero* exposure to ZIKV is associated with birth defects such as microcephaly (117). Although ZIKV-associated neuro-immunological effects have been linked to the development of neurological diseases (117, 118), it is still not clear whether ZIKV infection during pregnancy significantly contributes to the incidence of ASD during the early years of infants (119). In a study of 216 infants during the Rio de Janeiro ZIKV epidemic of 2015–2016, the prematurity rate was very high (13%). Microcephaly was identified in 8 of 216 infants (120). With neurological Bayley-III and other assessments, ASD was diagnosed in the second year of life in previously healthy children. This suggests that ZIKV during pregnancy may primarily affect gestational age of birth and congenital defects such as microcephaly. It is noteworthy to state that improved neurodevelopmental outcomes were observed in female children, term babies, and maternal infection later in pregnancy (121). ASD developed in six of the 18 children with very low average performance. In another study, the findings in 156 infants from ZIKV infection during pregnancy and 79 infants without ZIKV infection suggested that there were minimal differences in neurodevelopmental outcomes at 24 months of age (122), suggesting that ZIKV infection if not timed for a temporal window of pregnancy may not lead to increased incidence of ASD. Although association between ZIKV infection during pregnancy and ASD is inclusive, more studies with larger cohorts are needed, particularly in the context of ZIKV infection during second trimester. Brazilian ZIKV strain may pass the placental barrier, infect progenitor cortical cells, and drive cell death by apoptosis and autophagy (123). Infection of cytotrophoblasts or the transmigration of infected primary human placental macrophages allowed this virus to infiltrate the embryonic neural cells (124). In newborns with congenital ZIKV infection, neuroimmune modulation may have a role in the development of autism. Proinflammatory cytokines, such as TNF-α- and IL-6, are generated at high levels in response to ZIKV infection and so predispose patients for this condition (125). Interestingly, ZIKV has been found to be highly specific for oRGs (radial glial cells) (126). Centrosomal abnormalities and early differentiation were observed in neural precursor cells (NPCs) infected with the virus. Early differentiation and maybe abnormal radial fiber migration of newborn neurons may have resulted from the breakdown of adherens junctions in the vRGs. The general view is that the ZIKV inhibits the proliferation of NPCs, triggers selective cell death, and shrinks the size of the brain in humans. Subsequently, detailed analysis of the structure of the brain of Zika-infected mice indicated a reduction in the number of VZ-like areas as well as the number of SOX2-positive RGs and TBR1-positive layer VI neurons (127, 128). Neurological problems have been documented in experimental studies *in vivo* and *in vitro* as a result of ZIKV neurotropism and the molecular signatures left by infection. Even after birth, infected nerve cells generate and release proinflammatory cytokines that are significantly linked to neuropsychological disorder. Moreover, ZIKV-infected human mesenchymal stem cells were also discovered to display ASD molecular markers in a distinct manner (129). Although there is no concrete evidence of ASD in newborns with maternal ZIKV infection, the altered neuro-immune axis may contribute to ASD.

Rubella

Rubella virus is a single-stranded, plus-sense RNA virus belonging to the *Togaviridae* family. Congenital rubella syndrome (CRS), which includes sensorineural hearing impairment, cataracts, heart problems, and/or brain and nervous system damage, can arise from rubella infection during early pregnancy, which is normally a self-limiting condition (130). In the 1960s, the prevalence of intellectual disability and autism in CRS patients in the United States was substantially greater than in the general population, with 42 and 7.4% incidence rate, respectively (131). The virus enters the bloodstream by infected cells and alveolar macrophages, which goes to the lymph nodes in the affected area and cause lymphadenopathy (132). Exactly how Rubella reaches the maternal-fetal interface has not been thoroughly investigated. According to one theory, monocytes in the basal plate diffuse into the intervillous space and/or lymphatic arteries as a result of persistent infection. CTBs, endothelial cells of villous capillaries, amniotic epithelium, and different cells of the basal plate were found to contain detectable virions in placentas with CRS (133). Of the various side effects of CRS is ASD. In the 1970s, 200 times higher incidence of ASD was reported in children with CRS (134). CRS and autistic children show similar traits of hyperactivity and spasticity. Another similarity is that in both autism and CRS, certain changes in the brain are thought to be the result of dysregulated immune system (134, 135). After 3 to 5 years following the exposure, 95% of the children with CRS were suspected of having developmental issues and sensory dysfunction, and 41% were suspected of having autism (134, 135). At molecular level, viral replication in the host cell impacts the expression of genes involved in the development of sensory organs in a direct and indirect manner. Consequently, the long-term impacts of Rubella on the developing embryo are compounded by the host–virus interactions. Another important similarity between CRS and

autism is that children with these disorders lack antibodies to rubella. Thus, introduction of rubella vaccines (RCVs) may result in significant reduction in CRS as well as neurodevelopmental and sensory issues in young children (136). Evidence for this supposition is available in small cohort studies.

Influenza Virus

Influenza is a contagious respiratory infection caused by influenza viruses A and B. Both influenza A and influenza B viruses are enveloped negative-sense RNA viruses belonging to the *Orthomyxoviridae* family. Pregnant women were among the most at risk during the 2009 H1N1 pandemic of influenza A (H1N1) (137). Third-trimester pregnant women with high temperature were more likely to develop severe disease during the 2009 H1N1 pandemic and in inter-pandemic periods, compared with those in earlier stages of pregnancy. Influenza virus infection can be more severe in pregnant women and their offspring, according to both animal research and clinical findings (137, 138). During pregnancy, influenza virus can have detrimental consequences on the fetus because of hormonal signaling imbalance, inflammation, or activation of the immune system against fetal tissue. As with other common viral pathogens, influenza during pregnancy has been linked to a range of neurodevelopmental issues, including ASD, bipolar disorder, and schizophrenia (139–141). In spite of the conflicting evidence, some epidemiological evidence suggests that pre-natal maternal influenza virus infection increases the likelihood of ASD in offspring. Autistic children were found among the offspring of ∼8% of pregnant women who had influenza or had been exposed to it during their pregnancy. Conversely, analysis from a large cohort (196–929) of infants delivered at Kaiser Permanente Northern California between January 1, 2000 and December 31, 2010 at a gestational age of at least 24 weeks) found no link between maternal influenza infection during pregnancy and an elevated risk of ASD (142). When it comes to ASD, a new study found that influenza infection during pregnancy was not related with an increased risk. There is a pertinent debate on the maternal influenza A immunization during pregnancy and risk for autism in the offspring (143). In a large study of 39,726 infants from pre-natally exposed H1N1 vaccine with 13,845 in the first trimester and 29,293 infants from unexposed group, the authors found no correlation between H1N1 immunization during pregnancy and autism (144).

Cytomegalovirus and HSV Infections and Risk of Autism Spectrum Disorders

CMV and HSVs (HSV1 and HSV2) are double-stranded DNA viruses, which belong to the herpesviruses class. Like other TORCH pathogens, CMV, HSV1, and HSV2 may cause pregnancy complications, including spontaneous abortion, intrauterine growth restriction, preterm birth, brain anomalies, or visual impairment (145). Although infections with herpesviruses may not be primary infections, these viruses are associated with persistent or latent infections and may impact pregnancy if reactivated. What is important is gestational age at the time of infection which may control the risk of vertical transmission to the fetus and disease level (145, 146). There is a 40% chance that a fetus will be infected if the mother is infected. CMV and HSV2 sero-positivity has been used to examine a relationship between infections by these viruses and the incidence of ASD (147). One study involving 442 mothers of children with ASD suggested that high levels of HSV-2 IgG antibodies in maternal mid pregnancy were associated with increased risk of ASD in male offspring. In this study, no association was found between ASD and the sero-positivity for *Toxoplasma gondii*, rubella virus, CMV, or HSV-1 (148). However, in another study, CMV sero-positivity was found to be a more potent trigger than HSV-2 to influence the onset of ASD (149). These observations suggest that this association with ASD needs to be further evaluated in prospective studies with larger cohorts of pregnant women, particularly keeping the gestational age of infection in mind. It is not clear what immune changes occur during primary or reactivated CMV or HSV infections. The ability of CMV to infect trophoblasts has been demonstrated (150, 151). Several investigations have documented the inflammatory pathology that results from the placental immune response against CMV. Paracrine apoptosis of uninfected cells occurs during CMV infection of CTB and SYN possibly as a result of excessive production of TNF-α. This inflammatory reaction at the maternal–fetal interface has adverse consequences on the fetal neurodevelopment. Children with congenital CMV infection who were previously asymptomatic at birth have been found to have ASD (152, 153). In children with neurological disabilities and cerebral cortical abnormalities, teratogenic consequences of CMV infection were found by neuroimaging. Forty-five fetuses from women with a positive pre-natal diagnosis of CMV infection were examined for neuronal damage. This virus had been detected in the brain's cortex as well as its white and gray tissues as well as the germinal matrix and the leptomeninges (154). Neurons, neuroblasts, glia, endothelium, ependymal, and meningeal cells were among the CMV-positive cells identified. In the third layer of the cerebral cortex, there was significant laminar necrosis, with numerous macrophages replacing the growing neurons (155, 156). Multifocal aggregates of $CD8^+$ T-lymphocytes and granzyme B^+ T-lymphocytes were seen in the necrotic and CMV-positive portions of the inflammatory infiltrate. Severely brain-damaged fetuses had a noticeable invasion of fetal activated $CD8^+$ T-cells (157–159).

Human Immunodeficiency Virus and Risk of Autism Spectrum Disorders

According to the World Health Organization, around 1.3 million women living with HIV became pregnant each year. Transplacental transmission of HIV can occur during delivery and/or post-natal breastfeeding. Vertical transmission *in utero* is estimated to occur at a rate of around 1%−2% and is associated to maternal CD4 levels and viral load (160). Lymphocytes infected with HIV endocytose or transcytose the virion particle if they come into contact with trophoblast cells. Aside from that, CTB, SYN, and Hofbauer cells have been found to contain HIV genetic material (161). These findings are in line with epidemiological studies, which show that although HIV can

be transmitted transplacentally, this is a rare occurrence (1%) (162, 163). On the other hand, the number of HIV-uninfected children who were exposed to HIV while in the womb or while breastfeeding is rising, and these children are referred to as HIV exposed uninfected (HEU). Many children who have been exposed to HIV but have not become infected have been shown to have ASD. Congenital infections, such as CMV and toxoplasmosis, are more common in HEU. As compared with uninfected individuals, those exposed to the HEU developed weaker cognitive functioning and poorer motor, mental, and language development. Researchers found that HEU diagnosed with an ASD-like pathology had higher leukocyte mitochondrial DNA content than controls, suggesting that mitochondrial malfunction may play a role in HEU's risk of developing ASD (164–166).

Coronavirus Disease 2019 and Possible Risk of Autism Spectrum Disorders

Although it is too early to examine an association between SARS-CoV-2 infection during pregnancy and the incidence of ASD or other neuropsychiatric disorders, infection-associated cytokine storm has been thought to be a possible risk factor for neurodevelopmental disorders in infants (167, 168). The common notion is that poor placental infection or vertical transmission, lack of trans-placental transfer of SARS-CoV-2 antibodies, and reduced co-expression of ACE-2 and TMPRSS2 may provide protection against placental infection and vertical transmission. However, cytokine storm induced by SARS-CoV-2 may reach the placenta and the fetus. In view of recent results indicating that pregnant women with SARS-CoV-2 infection have elevated levels of IL-6, IL-17, and other inflammatory cytokines (169, 170), fetal neurodevelopmental may be at risk. Although SARS-CoV-2 is rarely transferred to the fetus, it can breach the blood-cerebrospinal fluid barrier (BCSFB) by infecting epithelial cells expressing the ACE2 receptor (171). In light of maternal immune activation, it appears plausible to assume that infection with SARS-CoV-2 during the early stages of pregnancy could similarly result in serious fetal neurodevelopmental defects.

SEXUAL DIMORPHISM IN VIRAL PATHOGENESIS AT THE MATERNAL–FETAL INTERFACE

Although male fetuses are exposed to the same *in utero* environment, they have preponderance for generalized complications of pregnancy, showing higher vulnerability to placental inflammation, hypoxia, placental abruption, preeclampsia, eclampsia, and preterm birth (172, 173). These adverse pregnancy outcomes are invariably associated with increased risk of poor neurodevelopmental outcome. Emerging research points to a complicated relationship between infant's sex and maternal immune surveillance (174, 175). Differential crosstalk between male and female fetuses with the placenta might arise from the varying effects on innate immunity. Sex-specific differences in viral pathogenesis stem from gene dosage effect of X and Y chromosomes and specific sex hormone gradient (176, 177). There is evidence that sex-linked genes regulate innate, cellular, and humoral immune surveillance, significantly increasing the male susceptibility to adverse immune response (**Figure 3A**). The female placenta shares the same X-linked gene dosage as of the fetus as it is derived from the extra embryonic tissues. Gene dosage of X-linked genes, such as IL-13, IL-4, IL-10, XIST, TLR7, FOXP3, and Sox9 in female placenta could potentially contribute to female protection against viral infection compared with male through gene dosage and epigenetic modification. Taken together, sex-specific placental stress signaling and gene expression significantly impact the fetal adaptation in a sexually dimorphic manner to the *in utero* environment upon maternal immune response and stress (178–180). Epidemiological data reveal a higher mortality rate in male compared with female embryos due to viral infection. This unique immunological advantage to female is attributed to efficient humoral and cellular antiviral immune responses. Recent SARS pandemics (SARS-CoV-1 and SARS-CoV-2) also indicated sexual dimorphic IFN signaling, in blood and lungs, resulting in high morbidity in male patients (177, 181). Placental Type I and II IFN signaling at the maternal-fetal interface dampens viral pathogenicity. The propensity of placental Type I and Type II IFN signaling at the maternal–fetal interface during viral pathogenicity depends largely on sexual identity of the placenta. Sexual dimorphic antiviral response also arises from sex specific expression of TLR and IFN pathway genes (182, 183). Female immune cells show 10-fold higher expression of TLRs in comparison with its male counterpart (184). Furthermore, higher innate immune cell abundance in female confers higher immune protection against viruses. Interestingly, Treg abundance and proliferation rate is also higher in females. Interestingly, human female naive $CD4^+$ T cells preferentially produce IFN-γ upon activation, but human male naive T cells produce more IL-17 than their female counterparts. Additional sexual dimorphism in IL-17 expression from T cells also exists (185–188). This sexual dimorphism in immune responses originates from X- and Y- linked chromatin remodeling. In addition to that, sex-specific microRNA expression, such as miR-124 and miR-202-5p/3p, could be attributed to sex-specific immune responses. For example, gestational expression of miR-124 in XX cells leads to induction of $Foxp3^+$ Tregs by suppressing STAT3 signaling (189).

A recent study highlighted a unique feature of the human placenta's ability to reactivate inactive X-chromosome at very early or late gestational period (190, 191). Unlike other tissues, these unique molecular features of the human female placenta highlight the possibility of inducing random X reactivation in an inimical intrauterine environment in the early stages of embryo development. This X-reactivation could potentially increase the gene dosage of certain X-linked genes as described above, leading to improved protection from viral infection. Sex-specific placental transcriptional analysis also reveals unique molecular adaptation under the same maternal *in utero* environment. The study highlighted the changes not only in the X- and Y-linked genes but also in immune regulatory pathways within autosomal gene cluster. The female placenta showed higher expression of

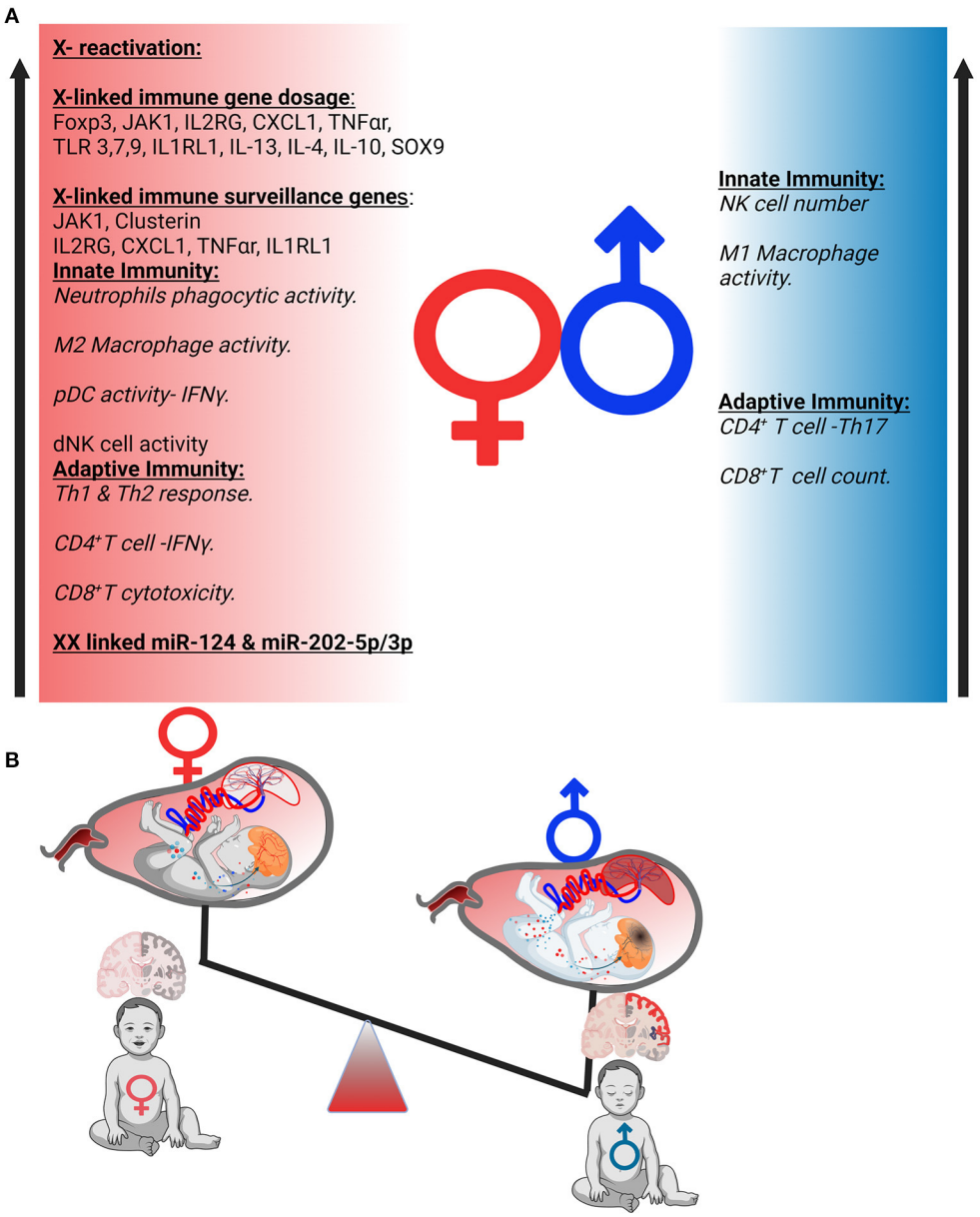

FIGURE 3 | Possible mechanism of sexual dimorphism in uterine immunity and autism spectrum disorders (ASD) development. **(A)** Sexual dimorphism of the immune responses. Immune components of both innate and adaptive immunity are differently regulated in females and males. **(B)** Sex-specific placental response to inflammation generated a differential gradient in both sexes that affects fetal brain differentially.

immune surveillance genes including JAK1, IL2RB, Clusterin, LTBP, CXCL1, IL1RL1, and TNF-α receptor (179, 192).

Sexual Dimorphism in Autism Spectrum Disorders Development

Sexual dimorphism has been observed in a range of neurodevelopmental disorders including ASD, schizophrenia, attention deficit hyperactivity disorder, and intellectual disabilities (193–195). The brain is highly vulnerable to environmental insults during early gestational period, as it undergoes rapid developmental processes, including neurogenesis, neuronal migration, and synaptogenesis. Systemic and uterine immune activation during pregnancy is associated with disruption in fetal neurogenesis and predisposes to neuropsychiatric disease in male offspring (196–198) (**Figure 3B**). These sex-specific responses serve as additional mechanisms in which to consider male ASD predominance (199). The male preponderance does not appear to be directly linked to genetic factors, as sex-skewed expression of neurodevelopmental risk genes has not been

discovered. Although male and female littermates are exposed to the same maternal *in utero* inflammation, it has been reported that behavioral shortfalls manifest mainly in the male offspring, mirroring the sex bias observed in placental immune response to viral infection (200). Because the placenta is the immunologic hub and the first site of fetal exposure to maternal inflammation, we propose that sex-specific reactions to uterine immune activation (UIA) that have deleterious impacts on fetal neurodevelopment may originate in the placenta.

CONCLUSION AND FUTURE PERSPECTIVE

Knowledge of placental molecular and immunologic pathways and their role in the transmission or protection from infection is critical to the care of pregnant women and the health of their newborn children. Infections during pregnancy can have serious implications. A variety of host–pathogen interactions specific to the maternal–fetal niche have been discovered as a result of the complexity and distinctive characteristics between the two hosts. Yet, the molecular mechanisms underlying infection-associated pathologies remain largely unknown, in part, due to the difficulties inherent in defining the interactions between the pathogen and the maternal and/or fetal hosts during pregnancy (201). There are significant differences in the placental architecture between humans and mice that prevent direct correlations of these findings to humans, despite the fact that mouse models have been useful for gaining valuable insights into numerous aspects of pregnancy. Even though pregnant women appear to be less susceptible to early infection than non-pregnant women, immunologic modifications with advanced pregnancy may hamper pathogen clearance, resulting in an increased severity of diseases caused by particular pathogens.

Neuronal development is a complex process that depends on the interaction between genetic and environmental factors. Various risk factors might affect embryonic development. Despite this, studies have shown that the inflammatory response to infections is a widespread and important feature. The identification of target cells for infection and study of possible neurodevelopmental effects require a better understanding of normal neurodevelopment and its comparison with the pathways that disrupt it.

Future research is critical to the development of tailored treatments that take into account the complex relationships between maternal and fetal tissues and how infections influence these interactions. Overall, research suggests that these pregnancy-related problems are linked to neurodevelopmental abnormalities, particularly ASD. The uterine immune system is affected by hormonal changes. Thus, therapeutic approaches to minimize the spread of infectious and other diseases by modifying the hormonal environment should be considered. In order to prevent and treat diseases, new preventive and therapeutic avenues that may interfere with the pathogen-placenta deleterious cross-talk are a possible prophylactic or therapeutic avenues. With the view of curtailing maternal and fetal inflammation during pregnancy, maternal immunizations may have long-term advantages for the offspring as well.

AUTHOR CONTRIBUTIONS

Both authors contributed equally to all aspects of the article.

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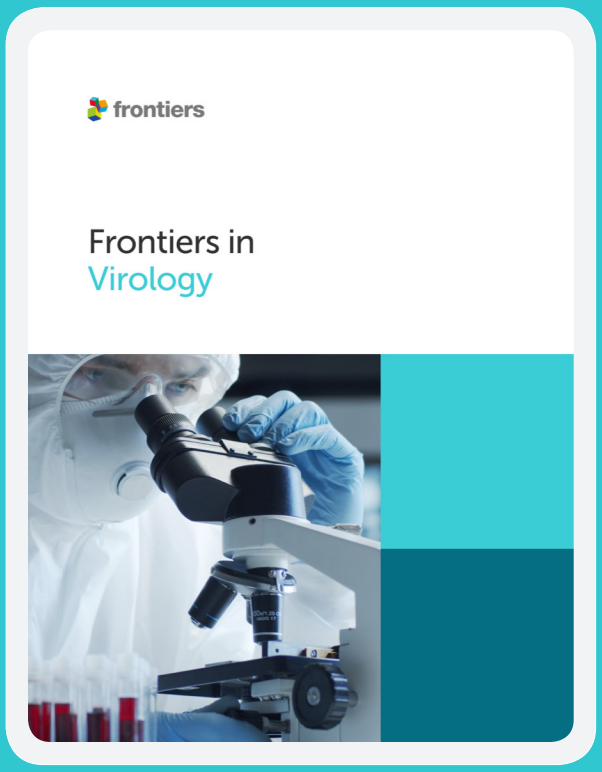

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