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Exposure to systemic and intrauterine inflammation leads to decreased pup survival via different placental mechanisms

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ABSTRACT

Problem: Exposure to systemic maternal inflammation (i.e., maternal sepsis, influenza, human immunodeficiency virus, or pyelonephritis) and intrauterine (IU) inflammation (i.e., chorioamnionitis or preterm labor) have been associated with adverse perinatal sequelae. Whether systemic and localized inflammation leading to adverse outcomes have similar placental mechanisms remain unclear.

Method of Study: We conducted a study by murine modeling systemic and localized IU inflammation with injections of either intraperitoneal (IP) or IU interleukin-1β (IL-1β) and compared fetoplacental hemodynamic changes, cytokine/chemokine expression, and fetal loss.

Results: IU IL-1β exposure reduced offspring survival by 31.1% and IP IL-1β exposure by 34.5% when compared with control pups. Despite this similar outcome in offspring survival, Doppler analysis revealed a stark difference: IU group displayed worsened fetoplacental hemodynamic changes while no differences were found between IP and control groups. While both IU and IP groups had increases in pro-inflammatory cytokines and chemokines, specific gene expression trends differed between the two groups, once again highlighting their mechanistic differences.

Conclusion: While both IP and IU IL-1β exposure similarly affected pup survival, only IU inflammation resulted in fetoplacental hemodynamic changes. We speculate that exposure to maternal systemic and IU inflammation plays a key role in fetal injury by utilizing different placental inflammatory pathways and mechanisms.

1. Introduction

Both maternal systemic and intrauterine (IU) inflammation have been associated with perinatal sequelae. Pro-inflammatory cytokines that are released during maternal inflammation have been shown to rapidly spread to the fetal circulation and injure developing organs that are vulnerable to the effects of inflammatory stressors. Past studies have shown that the fetal intracellular signaling cascade gets triggered by these circulating maternal cytokines and ultimately leads to neonatal morbidity and perinatal sequelae, especially within the premature newborn (Hanzl, 2009; McAdams and Juul, 2012). Amniotic fluid and umbilical cord blood have been used to measure the level of pro-inflammatory cytokines affecting the fetus. Increased cytokine levels in these fluids signified the impending onset of fetal inflammatory response syndrome (FIRS) and other neonatal morbidities (Gotsch et al., 2007; Witt et al., 2005; Yoon et al., 1999). Furthermore, recent murine research has shown that systemic and intra-amniotic inflammation produces different cytokine profiles in both dams and pups, suggesting the possibility of different inflammatory mechanisms (Garcia-Flores et al., 2018). In the same experiment, Garcia-Flores et al. found that treatment with exendin-4 led to significantly improved neonatal outcomes for systemic, but not local, inflammation, further supporting the idea that systemic and local inflammation have different pathophysiological mechanisms.

Interleukin-1β (IL-1β) is one of the pro-inflammatory cytokines invoked during the maternal inflammatory response that has been demonstrated to perpetuate the initial insult. Studies have shown that IL-1β is essential for inflammatory processes and exhibits detrimental effects via its ubiquitously expressed receptor, interleukein-1 receptor (IL-1R) (Basu et al., 2015; Bry et al., 2007; Cayabyab et al., 2003; Edelson et al., 1999; FAN et al., 2010; Kakkera et al., 2005; Nikiforou et al., 2016). The further release and secretion of IL-1β release is dependent

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on the P2 × 7 receptor, and this propagation of IL-1β in the pregnant mother is implicated in fetal brain injury and perinatal survival (Tsimis et al., 2017). High levels of IL-1β have also been indicated in women with chorioamnionitis (Roberto Romero et al., 2016), which is a condition that often leads to perinatal complications as shown by both human and animal studies (Burd et al., 2012; R. Romero et al., 1989; Yoon et al., 1997). Furthermore, in pregnant rodents and non-human primates, injections of IL-1β have been shown to induce preterm births (PTB) (Nadeau-Vallée et al., 2016, 2015; Romero et al., 1991; Sadowsky et al., 2006). We have also confirmed that systemic inflammation induced by IL-1β in mouse models leads to adverse perinatal outcomes in our previous study (Novak et al., 2019).

Moreover, IL-1β has been shown to induce vasoconstriction and atherogenesis, leading to endothelial dysfunction and the formation of thrombi (Vila and Salaices, 2005). This connection between vascular pathology and inflammatory states has led researchers to study the link between maternal inflammation and fetoplacental hemodynamic changes in order to better understand the mechanism that drives perinatal sequelae during maternal inflammation. However, current studies are inconclusive about the exact mechanistic link between maternal inflammation and fetoplacental blood flow. Fricke et al. have shown that systemic maternal inflammation induced by intraperitoneal injection of lipopolysaccharide (LPS) leads to placental injury and fetal intestinal damage without any changes in the placental blood flow (Fricke et al., 2018). However, in our recent study, we have found that IU inflammation induced by IU injection of LPS led to placental malperfusion, fetal cardiac dysfunction, and fetal brain damage (Eloundou et al., 2019). The exact placental differences caused by systemic and localized (IU) inflammation remain unknown.

Therefore, we sought to analyze fetoplacental hemodynamic changes, mediators involved in the inflammatory response, and fetal survival rate following the mother's exposure to systemic and local (IU) IL-1β in a mouse model. We hypothesized that exposure to IL-1β-induced maternal systemic and IU inflammation may lead to oxidative stress, pathologic vascular changes in the placenta, and fetal complications albeit with different placental cytokine responses and different degrees of fetoplacental hemodynamic changes.

2. Materials and methods

2.1. Animal preparation

All animal care and treatment procedures were approved by the Animal Care and Use Committee of Johns Hopkins University (Hopkins-IACUC Protocol No. MO14M326). CD1 pregnant dams (Charles River Laboratories, Wilmington, MA, USA) were used for this study. The dams were randomized to receive either intrauterine (IU) or intraperitoneal (IP) injections of IL-1β or phosphate-buffered saline (PBS) at embryonic day 17 (E17) as follows: (1) PBS-IP; (2) PBS-IU; (3) IL-1β-IP; (4) IL-1β-IU. To simulate a model of IU inflammation (IUI), we modified a well-established model of IUI by injecting IL-1β, instead of LPS, using methods as previously described (Burd et al., 2010, 2009). To simulate a model of maternal systemic inflammation, pregnant dams were subjected to a well-established model of systemic inflammation via IP injection as previously described (Novak et al., 2019).

Mice were anesthetized with isoflurane (Baxter # NDC 10019-360-60, Deerfield, IL, USA) before undergoing a mini-laparotomy. After dressing the abdominal area, a 1.5-cm medial incision was performed in the lower abdominal wall. For animals designated to receive IU injections, either 0.5 μg of mouse recombinant IL-1β (Sigma-Aldrich, St. Louis, MO, USA) in 100 μl phosphate-buffered saline (PBS) or 100 μl PBS alone (vehicle) was injected between the first and second amniotic membranes of the lower right uterine horn with care to not enter the amniotic cavity. For animals designated to receive IP injections, either 1 μg of IL-1β in 100 μl PBS was injected onto the opened abdominal cavity. Previous animal studies using a mouse model showed that 0.5 or 1 ug/mouse of IL-1β induces PTB, when administrated through the r intrauterine or systemic route. The rate of preterm birth was about 70–75% (Yoshimura and Hirsch, 2005; Nadeau-Vallée et al., 2014; Romero et al., 1991).

Routine laparotomy closure was performed, and the dams recovered in their respective cages. Pregnant mice were sacrificed 24 h after the injection (E18) to collect tissue samples of placenta and fetal brain for biochemical and histological analyses.

2.2. Doppler examination

Doppler examination was performed for the uterine artery (UtA), placental and fetal sides of the umbilical artery (UA), and fetal Myocardial Performance Index (MPI or Tei index) six hours after IL-1β or PBS-exposure in the mice. Briefly, general anesthesia was induced by inhalation of 5% isoflurane and oxygen at 3 l/minute and maintained with 1.5% isoflurane and oxygen at 2 L/minute. Doppler waveforms of the UtA were obtained at the level where the main UtA branches from the internal iliac artery. The systolic/diastolic (S/D) ratio, resistance index (RI), pulsatility index (PI), and the presence of early diastolic notch were evaluated in UtA. Doppler waveforms of the placental and fetal sides of UA were obtained at the placental cord insertion site and fetal abdominal cord insertion site, respectively. RI, PI, and the presence of absent end-diastolic flow (AEDF) were evaluated in the UA. S/D ratio was calculated as (peak systolic velocity/end-diastolic velocity). RI was calculated as [(peak systolic velocity – end-diastolic velocity)/peak systolic velocity]. PI was calculated as [(peak systolic velocity – end-diastolic velocity)/mean velocity]. To measure the Tei index, Tissue Doppler Imaging (TDI) of the mitral annulus was obtained from the apical four-chamber view. The Tei index was calculated as [(isovolumic contraction time + isovolumic relaxation time)/ejection time]. Doppler waveforms of UA and Tei index were obtained from fetuses on both sides of the uterus (1 to 2 fetuses on the right, 1 to 2 fetuses on the left). Doppler waveforms of UtA were measured from both the right and left side. All Doppler waveforms were taken at an angle as close as possible to 0°. Vevo 770 (VisualSonics, Toronto, Ontario, Canada) was utilized for the Doppler evaluation.

2.3. RNA extraction and quantitative polymerase chain reaction (RT-qPCR)

Quantitative polymerase chain reactions (qPCR) were completed based on protocols described by Manangeeswaran et al. (Manangeeswaran et al., 2016). Specimen was isolated from the first through fourth gestational sacs of the right uterine horn and fresh frozen on dry ice, followed by long-term storage at −80 °C. Total RNA for each placental and fetal brain specimen was obtained using RNeasy Plus Mini Kit (No. 74136, Qiagen, Valencia, CA, USA). For each specimen, 2 μg of RNA was used for complementary (c)DNA synthesis in a 40 μl reaction, using Bio-Rad iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA, Cat. No. 170-8891). qPCR reaction was completed by using CFX384 Touch Real-Time PCR Detection System (Bio Rad, Hercules, CA, USA) using manufacturer's protocol. The primers for NFκB1 (Mm.PT.58.30400172), IL-1β (Mm.Pt.58.44004828), IL-8 (Mm.PT.58.9981538), tumor necrosis factor alpha (TNF-α) (Mm.PT.58.12575861), heme oxygenase-1 (HO-1) (Mm.PT.58.8600055), nitric oxide synthase -1(NOS1) (Mm.PT.58.12129024), hypoxia-inducible factor 1α (HIF1α) (Mm.PT.58.11211292), C-X-C motif ligand (CXCL) 2 (Mm.PT.58.42151692), CXCL3 (Mm.PT.58.29283216), CXCL5 (Mm.PT.58.43548565), CXCL9 (Mm.PT.58.5726745), CXCL10 (Mm.PT.58.43575827), CXCL11 (Mm.PT.58.42838989), β-actin (Mm.PT.39a.22214843), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Mm.PT.39a.1) were obtained from Integrated DNA Technologies (Coralville, IA, USA). The primer for 18S (Cat. No. 4310893E) was obtained from Applied Biosystems (ThermoFisher Scientific).

2.4. Western blot analysis

Placentas were isolated from the first through fourth gestational sacs of the right uterine horn and fresh frozen on dry ice, followed by long-term storage at −80 °C. Proteins from homogenized placenta lysed in radioimmunoprecipitation assay (RIPA) buffer (containing protease inhibitors) were quantified using Bradford's method (Bio-Rad Laboratories). The segregated proteins (10 μg/lane) were isolated by 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes (Bio-Rad) and blocked with 5% skim milk (Bio-Rad) in Tris-buffered saline (TBS; Bio-Rad) with 0.1% Tween 20 (Sigma-Aldrich). Membranes were incubated with primary antibodies against C—X—C motif chemokine receptor 2 (CXCR2) (Abcam, Cambride, MA, USA), a receptor for IL-8, CXCL2, CXCL3, and CXCL5, or α-Tubulin (Abcam), a control marker for protein expression, diluted in TBS-Tween 20 with 5% skim milk at 4 °C overnight. Membranes were then washed with PBS containing 0.1% Tween 20 (Sigma-Aldrich) and incubated for 1 h with their respective secondary antibodies conjugated to HRP (Sigma-Aldrich). ECL (GE Healthcare) was used for detection using the ImageQuant LAS 500 (GE Healthcare, Little Chalfont, U.K.), and densitometric analysis was performed using ImageJ (National Institutes of Health; http://rsb.info.nih.gov/ij/). Resulting values were quantified relatively to the expression of α-Tubulin in each group.

2.5. Immunohistochemistry

Twenty-four hours after IL-1β or PBS injection, fresh placentas were dissected and fixed in 4% paraformaldehyde (PFA) (Affymetrix Inc., Cleveland, OH, USA) for histology. Tissue was then immersed in 30% sucrose (Sigma-Aldrich, St. Louis, MO, USA). Using Leica CM1950 cryostat (Leica Biosystems Inc., Buffalo Grove, IL, USA), the specimens were cut at 20 μm thickness and mounted on positively charged slides (Fischer Scientific). Placentas were cut transversally. For Immunohistochemistry (IHC), slides were incubated in PBS solution containing 0.05% Triton X-100 (Sigma-Aldrich) and 5% normal goat serum (Invitrogen, Carlsbad, CA, USA) for 30 min. Placental tissues were incubated with rabbit anti-vimentin antibody (1:200, Abcam, Cambride, MA, USA) and anti-CXCR2 antibody (1:200, Abcam). Donkey anti-rabbit Alexa Fluor 568 and donkey anti-rat Alexa Fluor 488 fluorescent secondary antibodies were utilized (1:500, Life Technologies, Grand Island, NY, USA) along with DAPI counterstaining. All images analyzed were obtained from Zeiss AxionPlan 2 Microscope System (Jena, Germany). Placental data were obtained at the middle level. Analyses were performed with ImageJ by evaluators blinded to group identification.

2.6. Fibrinogen and fibrin measurement

Histochemical staining (rapid phosphotungstic acid hematoxylin, PTAH, Polyscience Inc, Warrington, PA, US) was applied to the placenta for fibrin staining, according to the manufacturer's protocol. Analyses were performed with ImageJ by evaluators blinded to group identification.

2.7. Statistical analyses

The US-based indices were compared using Chi-squared test for discrete data and using a One-way ANOVA with Tukey post HSD test or Kruskal-Wallis Test with Bonferroni-Dunn Correction for continuous variables. Vimentin, CXCR2, IBA1 and fibrin positive expression were determined through quantitative analyses; specifically, the percentage of positive expression area was found within the image using Image J (v1.48, http://imagej.nih.gov/ij/, National Institute of Health, Bethesda, MD, USA), and means of the measures were compared using using a One-way ANOVA with Tukey post HSD test or Kruskal-Wallis Test with Bonferroni-Dunn Correction. Statistical significance was set at $P < 0.05$. Data analyses were performed with GraphPad Prism version 5 (GraphPad Software, La Jolla, California, USA).

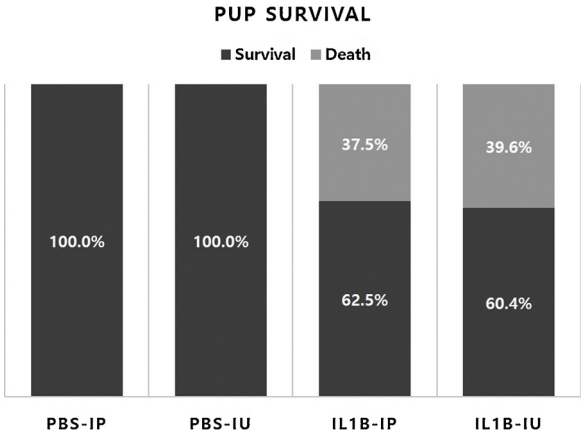

Fig. 1. Both intrauterine (IU) and intraperitoneal (IP) IL-1β exposure are associated with similarly decreased pup survival rates IL-1β (0.5 μg in 100 μl PBS) or PBS (100 μl) was injected in IU or IP cavity on embryonic day (E)17. Pup survival was assessed 24 h after IL-1β or PBS exposure on E18. IL-1β-exposure in the IU and IP cavities resulted in similarly lower pup survival rates when compared to the phosphate-buffered saline (PBS)-exposure groups. 6 dams for each group, *$p < 0.05$, compared with PBS-exposure group.

3. Results

3.1. Both IP and IU injection of IL-1β led to similarly decreased pup survival

Pup survival was assessed 24 h after IL-1β or PBS-exposure. IP and IU injections of IL-1β resulted in similarly decreased pup survival rates of 62.5% and 60.4%, respectively. There was a 100.0% survival rate for the pups of the two control groups, PBS-IP and PBS-IU (Fig. 1).

3.2. IU injection of IL-1β resulted in uterine arterial insufficiency in dams, umbilical arterial insufficiency, and abnormal function of the fetal heart in pups

Doppler examination was performed for the uterine artery (UtA) (Fig. 2a), placental and fetal sides of the umbilical artery (UA) (Fig. 2b), and fetal Myocardial Performance Index (MPI or Tei index) (Fig. 2c) six hours after IL-1β or PBS-exposure in the mice. The systolic-to-diastolic (S/D) ratio, resistance index (RI), and pulsatility index (PI) in the uterine artery (UtA) were significantly increased in the dams exposed to IL-1β via IU injection when compared to the two control groups, PBS-IP and PBS-IU, and compared to the group with IP injection of IL-1β. (Fig. 2d) Also, the rate of early diastolic notching in the UtA was significantly higher in the IL-1β-IU group than in the two control groups and the IL-1β-IP group. Similarly, the S/D ratio, RI, and PI in both the placental and the fetal sides of the umbilical artery (UA) were significantly higher for the IL-1β-IU group compared to the two control groups and the IL-1β-IP group (Fig. 2e). And the IL-1β-IU group demonstrated increased instances of either absent or reversal of end-diastolic flow in both the placental and the fetal sides of the UA when compared to all the other groups. These changes in the UtA and the UA were not noted in the IL-1β-IP group.

The overall Tei indices of the pups in the IL-1β-IU group were significantly increased compared to the pups in the two control groups and the IL-1β-IP group (Fig. 2f). And the rate of abnormal Tei indices (> 0.44) was significantly higher in the pups in the IL-1β-IU group (70.0%) compared to the pups of PBS-IP (0.0%, $p < 0.01$), PBS-IU (0.0%, $p < 0.01$) and IL-1β-IP groups (8.3%, $p < 0.01$). No significant changes were noted in the pups of the IL-1β-IP group compared to the

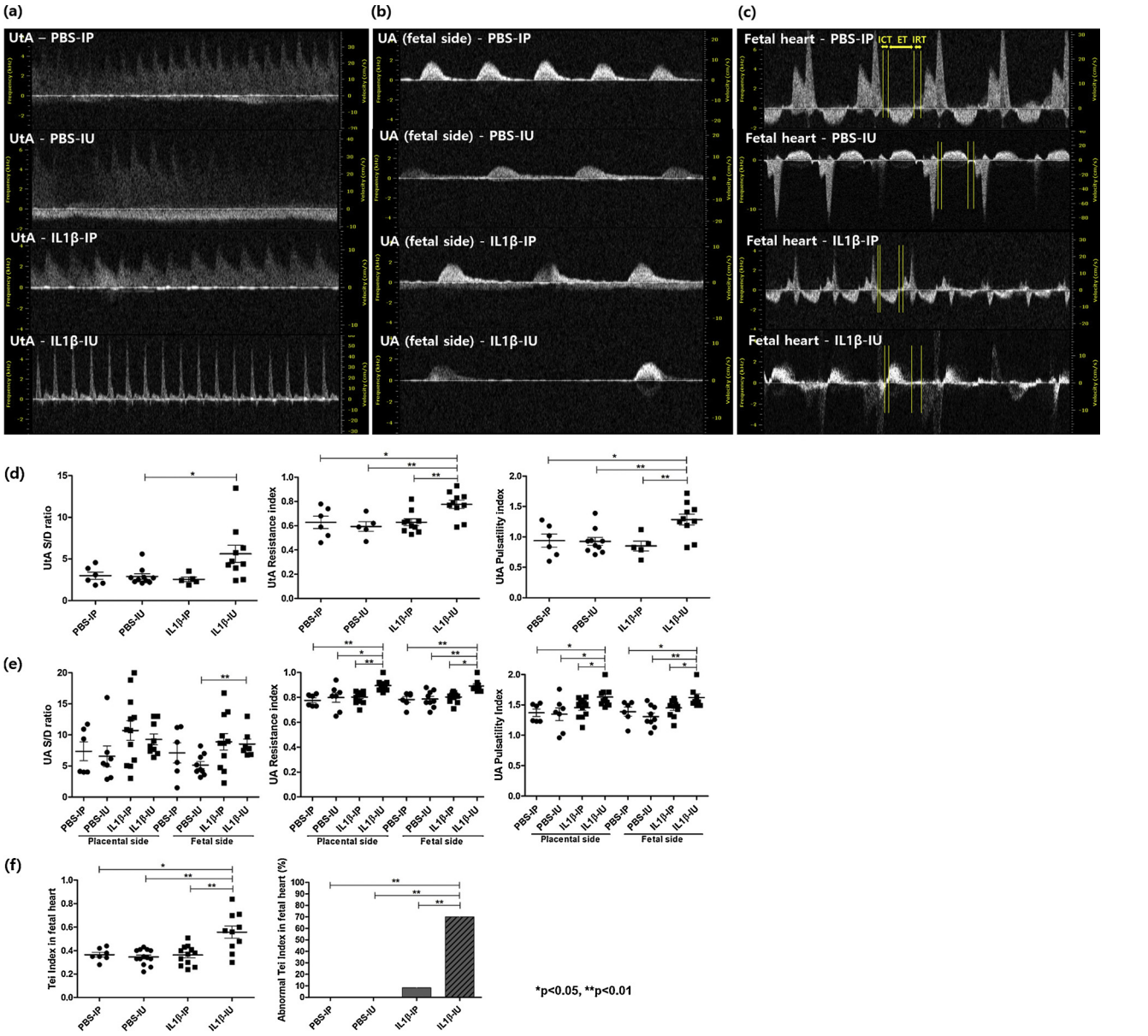

Fig. 2. Uterine and umbilical arterial insufficiency and abnormal Tei index associated with only IL-1β-IU group Doppler waveforms were interrogated six hours after IL-1β or PBS-exposure in the PBS-IP, PBS-IU, IL-1β-IP, and IL-1β-IU groups. Doppler was used to assess the (a) uterine artery (UtA), (b) fetal side of the umbilical artery (UA); and (c) left ventricular (LV) function of the fetal heart. (d) The systolic/diastolic (S/D) ratio, resistance indices (RI), and pulsatility indices (PI) in UtA (n = 6 dams for PBS-IP, n = 5 dams for PBS-IU, n = 10 dams for IL-1β-IP, n = 10 dams for IL-1β-IU), and (e) the same parameters measured in UA were significantly increased in IU IL-1β-exposed pups compared to PBS-exposed pups (n = 6 pups for PBS-IP, n = 7 pups for PBS-IU, n = 12 pups for IL-1β-IP, n = 9 pups for IL-1β-IU, one pup from one dam). (f) Rate of abnormal Tei index (> 0.44) and Tei index in the fetal LV were significantly higher in IU IL-1β-exposed pups compared to PBS-exposed pups. In contrast, there were no significant hemodynamic differences between the PBS- and IP IL-1β-exposed pups (n = 7 pups for PBS-IP, n = 13 pups for PBS-IU, n = 12 pups for IL-1β-IP, n = 10 pups for IL-1β-IU, one pup from one dam), *$p < 0.05$, **$p < 0.01$.

pups of all the other groups.

3.3. IU injection of IL-1β associated with upregulation of pro-inflammatory cytokines and hypoxia-induced proteins in the placenta of the dams

RT-qPCR of the placenta revealed that IU injection of IL-1β led to significantly increased expressions in pro-inflammatory cytokines and chemokines (IL-8, TNFα, CXCL2, CXCL3, and CXCL5) and hypoxia-induced proteins (i.e., HO-1, HIF1α, and NOS) when compared to PBS-IP, PBS-IU, and IL-1β-IP groups (Fig. 3). IP injection of IL-1β resulted in a significantly higher expression of CXCL9, a pro-inflammatory chemokine, when compared to the PBS-IP group; however, no other significant changes in the gene expressions of our target proteins (Fig. 3) could be found.

3.4. IU injection of IL-1β induced increased fibrin deposits in the placenta

PTAH staining of the placenta revealed a significantly increased area of fibrin staining in the IL-1β-IU group when compared to PBS-IP, PBS-IU, and IL-1β-IP groups (Fig. 4). The area of positive PTAH staining was 3.83 ± 0.64% in IL-1β-IU group, while PBS-IP, PBS-IU, and IL-1β-IP groups had areas of 1.00 ± 0.34% ($p < 0.01$), 0.80 ± 0.21% ($p < 0.01$), and 1.72 ± 0.18% ($p < 0.05$), respectively. However, this change was not noted in the IL-1β-IP group when compared to its respective control group, PBS-IP.

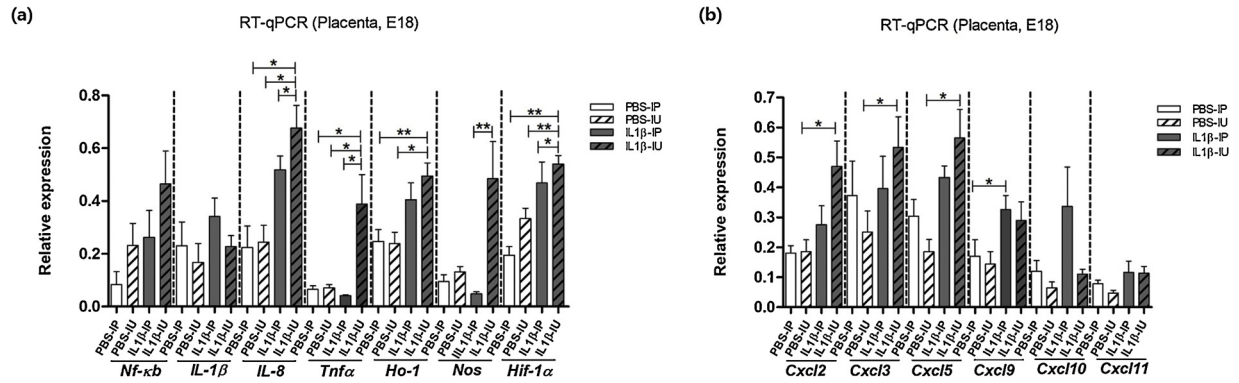

Fig. 3. IU and IP exposure to IL-1β associated with different patterns of placental gene regulation Placental gene expression (RT-qPCR) was assessed in all the mice 24 h after exposure to IL-1β or PBS. In IL-1β-IU group, qPCR revealed a significant upregulation of (a) IL-8, TNFα, HO-1, HIF1α, and (b) CXCL2, 3, 5 when compared to the PBS-IU group. With IL-1β-IP group, there was only a significant increase in the level of CXCL9 when compared to the PBS-IP group. Comparing the IL-1β-IU with the IL-1β-IP group, placental gene expressions of IL-8, TNFα, NOS, and HIF1α were significantly higher in the IU group. 6 pups for each group (one pup from one dam). For the quantification of gene expression, ß-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used. *p < 0.05.

3.5. IU injection of IL-1β led to decreased vimentin in the placenta

IHC staining for vimentin, an endothelial cell marker, demonstrated that IL-1β-IU group had significantly decreased vimentin expression in the placenta when compared to PBS-IP, PBS-IU, and IL-1β-IP groups (Fig. 5). The vimentin marker indicated that the area of positive vimentin expression was 25.14 ± 0.60% in IL-1β-IU group, while PBS-IP, PBS-IU, and IL-1β-IP groups had areas of 30.90 ± 1.36% ($p < 0.01$), 29.05 ± 1.43% ($p < 0.05$), and 30.23 ± 1.85% ($p < 0.05$), respectively.

3.6. IU injection of IL-1β led to increased CXCR2 expression around placental vessels

IHC staining for vimentin, an endothelial cell marker, demonstrated that IL-1β-IU group had significantly decreased vimentin expression in the placenta when compared to PBS-IP, PBS-IU, and IL-1β-IP groups (Fig. 5). The area of positive CXCR2 expression was 32.62 ± 2.75% in IL-1β-IU group, while PBS-IP, PBS-IU, and IL-1β-IP groups had areas of 20.14 ± 1.26% ($p < 0.01$), 20.35 ± 0.69% ($p < 0.01$), and 21.09 ± 1.33% ($p < 0.01$), respectively. No significant changes in CXCR2 expression could be found in the IL-1β-IP group (Fig. 6). Western blot for CXCR2 in the placenta revealed that IL-1β-IU group had significantly higher expression of CXCR2 (0.76 ± 0.11) compared to

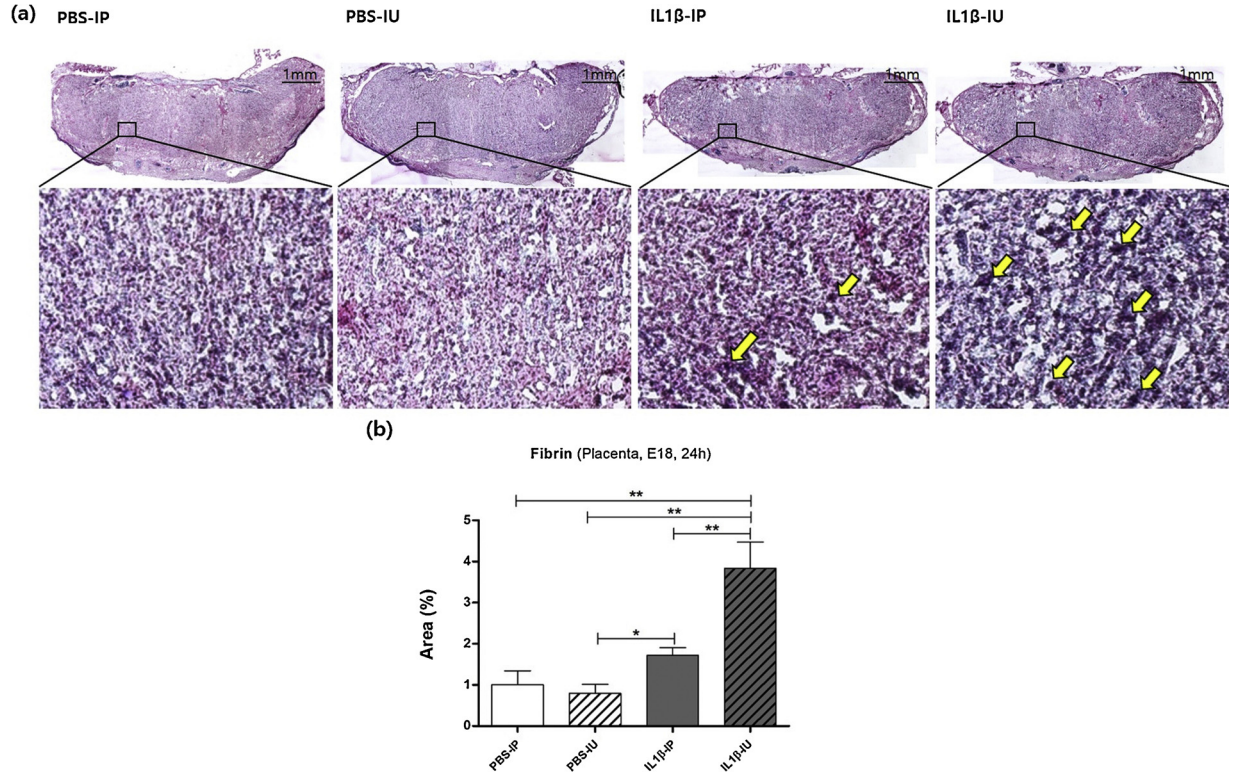

Fig. 4. Increased fibrin deposits associated with IU injection of IL-1β (a) Twenty-four hours after injection with either PBS or IL-1β, phosphotungstic acid-hematoxylin (PTAH) stain was applied to the placenta of all mice groups for fibrin staining. (b) The area for fibrin staining was significantly higher in the IL-1β-IU group compared to the IL-1β-IP group and the two control groups. However, this change was not noted in the IL-1β-IP group when compared to its respective control group, the IP-PBS group. 6 pups for each group (one pup from one dam), *p < 0.05.

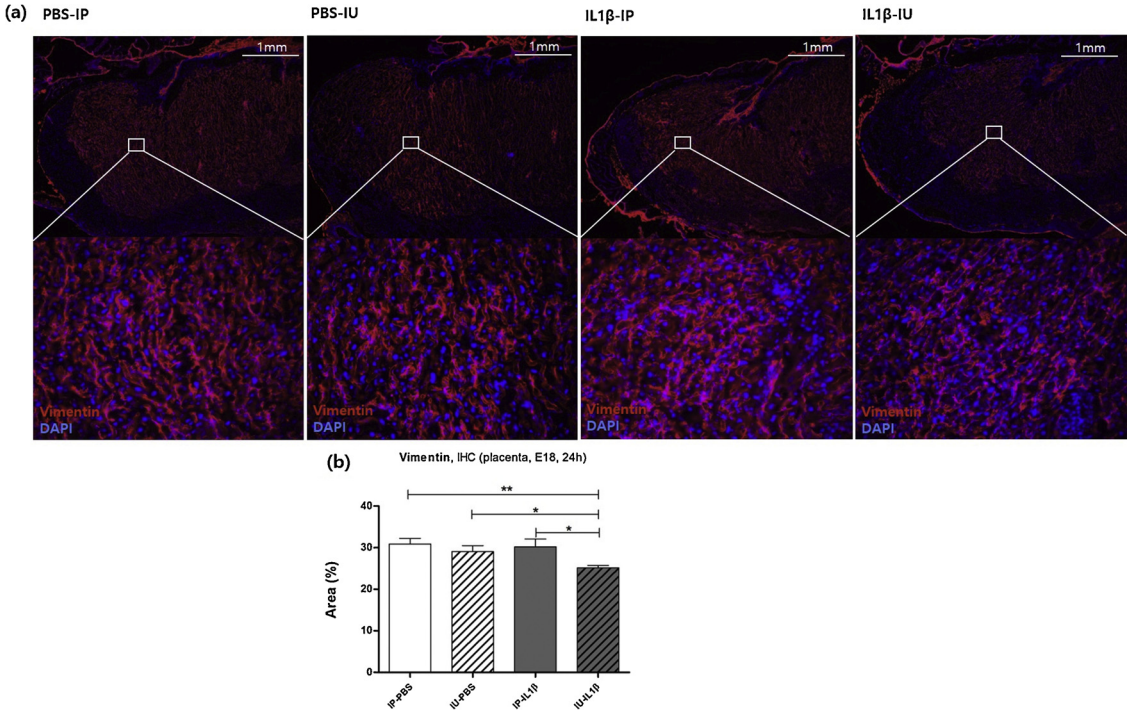

Fig. 5. IU injection of IL-1β led to decreased staining of vimentin in the placenta (a) IHC staining for vimentin (red) was applied to placentas of all mouse groups 24 h after injection with either PBS or IL-1β. (b) Quantitative measurements indicated that the area for positive vimentin expression was significantly lower for the IL-1β-IU group when compared to the IL-1β-IP group and the two control groups. However, there was no difference in vimentin staining between the IL-1β-IP group and the two control groups. 6 pups for each group (one pup from one dam),*$p < 0.05$, **$p < 0.01$ (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

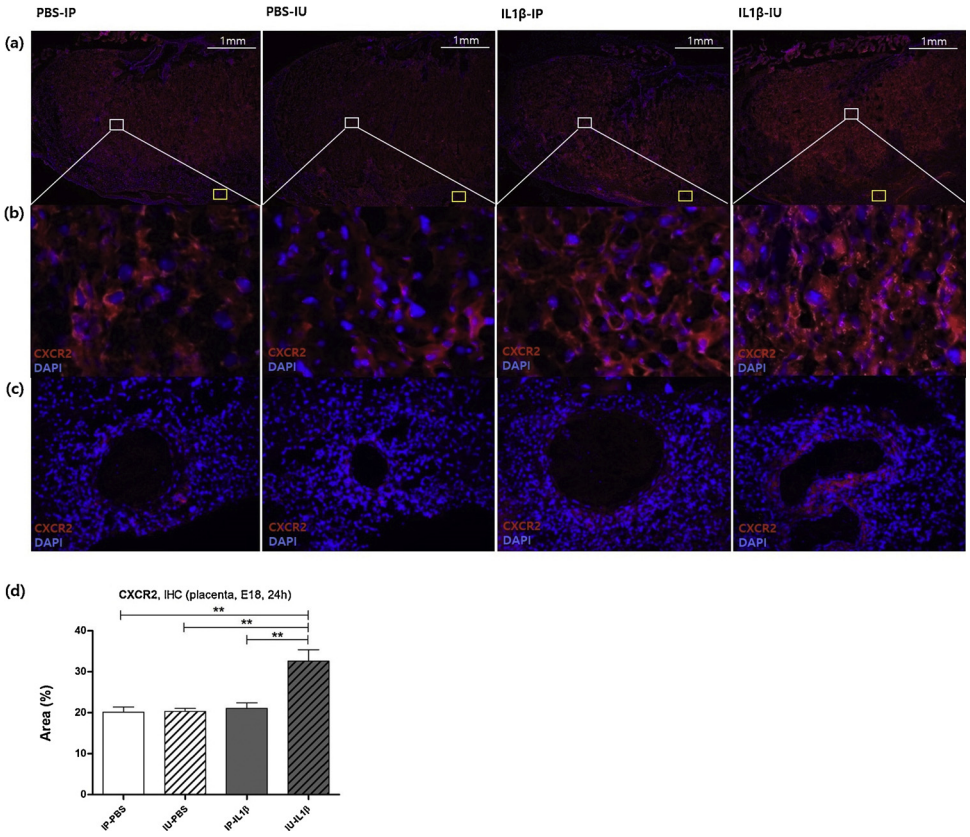

Fig. 6. IU injection of IL-1β led to increased staining of CXCR2 in the placenta IHC staining for CXCR2 (red) was applied to the placentas of all the mouse groups 24 h after injection with either PBS or IL-1β. The stained placenta was viewed under (a) 5x, (b) 40x, and (c) 20x magnification. (b) The labyrinth layer shows the increased CXCR2 staining in the IL-1β-IU group. (c) The bottom panel is the magnification of the inserts in top panel (yellow box). It shows that the increased CXCR2 staining in the IL-1β-IU group is mainly concentrated in the blood vessel wall of the decidua. (d) Quantitative measurement with ImageJ indicated that the area of CXCR2 staining was significantly increased for IL-1β-IU group when compared to the IL-1β-IP group and the two control groups. This change was not noted in the IL-1β-IP group when compared to the control groups. 6 pups for each group (one pup from one dam), **$p < 0.01$ (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

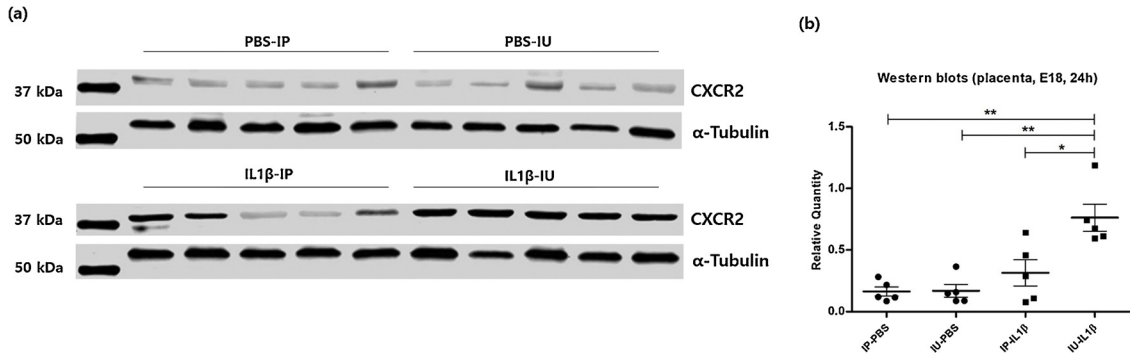

Fig. 7. Increased CXCR2 protein expression noted only in the IL-1β-IU group (a) Western blot analysis of CXCR2 expression in the placenta was performed for all mouse groups 24 h after injection with either PBS or IL-1β. (b) The relative quantity of CXCR2 protein was significantly increased in the IL-1β-IU group compared to its respective control group, the IU-PBS group. There was no change in the CXCR2 expression level of the IP-IL-1β group when compared with its control group. 5 pups for each group (one pup from one dam), *$p < 0.05$, **$p < 0.01$.

PBS-IU (0.17 ± 0.05, $p < 0.01$) and IL-1β-IP groups (0.32 ± 0.11, $p < 0.05$) (Fig. 7).

3.7. IU injection of IL-1β associated with upregulation of pro-inflammatory cytokines and chemokines in the fetal brain

RT-qPCR of the fetal brain revealed that IU injection of IL-1β led to significantly increased expression in pro-inflammatory cytokines and chemokines (IL-1β, TNFα, CXCL5, CXCL10, and CXCL11) (Fig. 8). IP injection of IL-1β resulted in a significantly higher expression of CXCL9, when compared to the PBS-IP group.

4. Discussion

Our study showed that while both local IU and systemic inflammation resulted in similar pup abortion rates, only IU IL-1β exposure resulted in fetoplacental hemodynamic changes, including fetal cardiac dysfunction, and significantly increased levels of pro-inflammatory cytokines and chemokines in the fetal brain. Past studies have shown that both systemic (i.e., maternal influenza (Zerbo et al., 2013), sepsis (Morgan and Roberts, 2013), human immunodeficiency virus (Altfeld and Bunders, 2016), or pyelonephritis (Szweda and Jóźwik, 2016)) and IU (i.e., chorioamnionitis (Cayabyab et al., 2003) or preterm labor (Burd et al., 2012)) inflammation in the mother result in perinatal sequelae, but no other studies have yet to research the mechanistic differences between systemic and IU maternal inflammation.

Pathology as a result of inflammation depends on the location, timing, intensity, and chronicity of the inflammatory process (Stone et al., 2019). IL-1β is an important cytokine in the inflammatory response that is produced by many cell types, including activated macrophages and microglia (Lopez-Castejon and Brough, 2011). A previous study had shown that IL-1β-induced inflammatory response *in utero* spreads from the uterine cavity to the placenta and fetal membranes (Nadeau-Vallée et al., 2017). This cascade of response induces a systemic inflammatory response in the fetus, which leads to an increase in fetal plasma levels of IL-1β, IL-6, and IL-8 along with increased leukocyte-induced transcription of IL-1β, IL-6, IL-8, and other proinflammatory genes (Nadeau-Vallée et al., 2017). The systemic fetal inflammatory response, especially during the early stage of development, has been shown to cause organ injuries, particularly in lungs, intestine, and brain (Nadeau-Vallée et al., 2017). Our study confirmed these past findings by demonstrating that both IP and IU maternal exposure to IL-1β resulted in increased expressions of pro-inflammatory cytokines and chemokines in the placenta, albeit with different patterns of expression, and similarly decreased pup survival rates.

Through placental qPCR, we found that the IU IL-1β group showed significant increases in chemokines, CXCL2, 3, and 5, which are all high affinity ligands of CXCR2 (Stadtmann and Zarbock, 2012). We confirmed the significant increase in CXCR2 expression in the placenta, especially around placental vessels, of the IU IL-1β group via IHC. We believe that the activation of CXCR2 around placental vessels led to placental endothelial damage and fibrosis as evidenced by the abnormal Doppler findings and placental changes, such as increased fibrin deposits and decreased vimentin, in the IU IL-1β group.

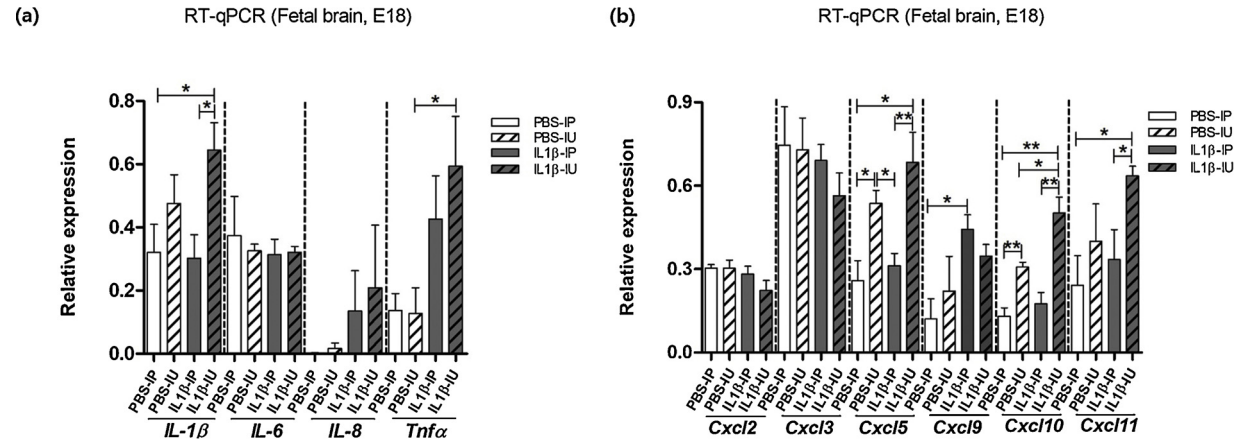

Fig. 8. IU and IP exposure to IL-1β associated with different patterns of Fetal brain gene regulation Fetal brain gene expression (RT-qPCR) was assessed in all the mice 24 h after exposure to IL-1β or PBS. In IL-1β-IU group, qPCR revealed a significant upregulation of (a) IL-1β, TNFα and (b) CXCL5, 10, and 11 when compared to the PBS-IU group. With IL-1β-IP group, there was only a significant increase in the level of CXCL9 when compared to the PBS-IP group. 6 pups for each group (one pup from one dam). For the quantification of gene expression, ß-actin and 18S were used. *$p < 0.05$, **$p < 0.01$.

Past studies have shown that CXCR2, by mediating the functions of its ligand chemokines, facilitates angiogenic changes and fibrosis in vascular endothelial cells (Li et al., 2017; Stadtmann and Zarbock, 2012). Activated CXCR2 attracts macrophages to the perivascular region. These macrophages then become activated to release cytokines responsible for inflammatory reaction and angiogenesis, such as TNF-α, IL-6 and VEGF (Li et al., 2017). Angiogenesis is a crucial process involved in the formation of fibrosis and collagen deposition in lungs (Keane et al., 2004). Additionally, chemokines and their receptors have been implicated in the pathogenesis of atherosclerosis via macrophage migration inhibition factor (MIF)-mediated chemotaxis. CXCR2, when complexed with CD74, not only acts as an MIF receptor but also as an integrin activator, which allows recruited monocytes to firmly adhere to the endothelium (van der Vorst et al., 2015). A past study analyzing the impact of CXCR2 deficiency on atherosclerosis has shown a reduction in lesion size and macrophage content in endothelial cells (Boisvert et al., 1998). Similarly, in a study of subarachnoid hemorrhage, depletion in CXCR2-positive neutrophils led to the inhibition of delayed cerebral vasospasm (Provencio et al., 2011). In a model of myocardial infarction, Tarzami et al. demonstrated that CXCR2-deficient mice had significantly smaller infarct size and decreased number of infiltrated immune cells in the infarct zone when compared to wild type mice (Tarzami et al., 2003). Overall, these past studies support our theory that the significant increases in CXCR2 around placental vessels of IU IL-1β group may play a critical role in the pathophysiology of vascular abnormalities in this group's dams and pups.

As briefly mentioned above, our study was the first to demonstrate that IU injection, but not IP injection, of IL-1β is associated with significantly increased fibrin deposits and decreased vimentin in the placenta. Although not significant, the IP IL-1β group's placenta also showed slight increases in fibrin deposits and decreases in vimentin when compared with the control group. Past research has shown that IL-1β induces CXCL1, a ligand for CXCR2, in a dose-dependent manner (Lee et al., 2015). Therefore, we believe that differences in placental fibrin deposits along with differences in the placental expression of CXCR2 between the IU and IP IL-1β groups may be a result of dose-dependent response to IL-1β. Systemic (IP) injection of IL-1β likely leads to an overall diluted dosage of IL-1β to reach the placenta when compared to local (IU) injection of IL-1β. This also explains why we still found some minor placental changes in the IP IL-1β group. Furthermore, in our experiment, IP exposure of IL-1β induced a significant increase in CXCL9, which is a ligand for CXCR3 (a marker of allograft rejection and anti-fetal rejection) (Maymon et al., 2018), This suggests to us that while systemic inflammation may lead to less placental thrombosis than IU inflammation, the pathophysiology of systemic inflammation still leads to an unfavorable environment for the fetus, as evidenced by the similar abortion rate in IP and IU dams.

Next, through Doppler exams, our study showed that IU injection, but not IP injection, of IL-1β resulted in uterine and umbilical arterial insufficiency in dams and pups, as shown by the increased arterial PI values. This result confirms and explains the findings of both Fricke et al., who found that mice exposed to IP injections of LPS had placental injuries without any alterations to the placental blood flow (Fricke et al., 2018), and our past study that demonstrated mice exposed to IU injections of LPS had ultrasonographically notable alterations to the placental blood flow (Eloundou et al., 2019).

Abnormal Doppler patterns for UtA and UA are associated with abnormal vascularization of spiral arterioles, superficial implantation, and maternal underperfusion (Brosens et al., 2013; Spinillo et al., 2012). And increased velocimetry in these arteries indicate an increased risk for fetal hypoxia, cardiovascular and central nervous system problems in the newborn, and adverse perinatal events, such as pre-eclampsia, preterm birth, and intrauterine growth restriction (IUGR) (Gaillard et al., 2013; Giles et al., 2003; Mone et al., 2014; Viscardi and Sun, 2001). Furthermore, past studies have shown that at 20 and 36 weeks gestational age (GA), increased uterine and umbilical arterial PI is associated with decreased placental area and weight, which results in IUGR (Mureşan et al., 2016; Salavati et al., 2019), and that an inverse correlation exists between placental thickness and UA S/D ratio and fetal biophysical profile (Akgündüz et al., 2015). Therefore, our study demonstrates a possibility for clinical utility in women presenting with IU inflammation (as in preterm labor with or without chorioamnionitis). These pregnancies may be monitored with ultrasound to stratify the risk for perinatal sequelae and to monitor for acute changes in the status of the fetus in preparation for labor.

Ultrasound differences and fibrin deposit differences in IP and IU groups also provides some clinical implications for mothers experiencing systemic inflammation. Our results suggest that systemic (IP) inflammation in the mother leads to indolent coagulopathic inflammatory changes in the placenta, as evidenced by fibrin deposits. Furthermore, we showed that despite increased fibrin deposits in the IP group's placenta, the placental blood flow disturbance induced from these changes cannot be detected by an ultrasound Doppler exam. This is further supported by the results from Fricke et al. that showed that pregnant mice exposed to IP LPS had placental injuries without showing ultrasonographical Doppler wave changes to placental blood flow (Fricke et al., 2018). Therefore, we have shown that for mothers experiencing systemic inflammation, it is not clinically relevant to monitor them with serial ultrasound exams for any acute changes in the fetus. Rather, they need to be counseled on the possibility of long-term sequelae on the fetus from exposure to the indolent cycle of inflammation. For instance, recent study by al-Haddad et al. has shown that fetuses exposed to any form of maternal infection had an increased risk of autism and depression (al-Haddad et al., 2019). In the context of our research, while mothers experiencing systemic inflammation may not need serial ultrasound monitoring, they may still need to be appropriately counseled on the negative effect that systemic inflammation can have on fetal neurodevelopment.

Past studies have also shown the potential use for ultrasound in the context of a pregnancy complicated by inflammation and fetal inflammatory response syndrome (FIRS). The thymus, which is morphologically complete by 16–20 gestational weeks, is one of the major organs involved in producing T cells and maintaining the fetal immune system (Gordon and Manley, 2011). In fetuses exposed to a pro-inflammatory microenvironment due to maternal IU inflammation, the thymic cortico-medullary ratio was noted to be significantly decreased representing thymic involution in these fetuses (Sciaky-Tamir et al., 2015) After adjusting for gestational age-related size differences, fetal thymus size was associated with the severity of IU infection (Sciaky-Tamir et al., 2015). Furthermore, in women with preterm premature rupture of membranes (PPROM), normal fetal thymus size was correlated with no evidence of clinical or histological chorioamnionitis (Yinon et al., 2007). These studies, along with our research showing the importance of Doppler wave changes in umbilical and uterine arteries involved in IU inflammation, suggest a novel use of sonograms for assessing the severity of fetal involvement in mothers with IU inflammation, such as PPROM.

Our study also showed that IU injection of IL-1β resulted in abnormal function of the fetal heart in pups. Tei index, or myocardial performance index, is considered to be a useful predictor of cardiac function in the fetus, irrespective of fetal heart rate and blood pressure. Increased Tei index indicates a ventricular dysfunction in fetuses that experience adverse perinatal conditions, such as IUGR, preeclampsia, and diabetes (Bahtiyar and Copel, 2008; Friedman et al., 2003; Ghawi et al., 2013; Nair and Radhakrishnan, 2016). Beyond the immediate perinatal period, abnormal Tei index has also been used as a prognostic indicator for amyloidosis, dilated cardiomyopathy, ischemic heart disease, and congestive heart failure (Bashiri et al., 2006; Tei et al., 1995). Our results indicate that uterine inflammation may increase the overall fetal vascular resistance, as shown by aforementioned abnormal Doppler findings, leading to left ventricular overload and left heart failure. This strain on the left heart likely leads to the increased Tei index and

signifies a global cardiac dysfunction. Overall, this finding of cardiovascular dysfunction in fetuses exposed to IU inflammation supports past research that demonstrated that increased velocimetry in UtA and UA is associated with negative cardiovascular outcomes in the newborn (Szweda and Jóźwik, 2016).

Furthermore, our study showed that fetal brains exposed to local (IU) maternal inflammation had significantly increased expressions of pro-inflammatory cytokines when compared to those exposed to systemic (IP) maternal inflammation. Previous studies have similarly shown that maternal systemic inflammation caused by infectious organisms, such as influenza, is not significantly associated with increased risk for autism spectrum disorders (ASD) or developmental delays in the surviving offspring if the mother never harbors a fever during the infection. The association between ASD and maternal infection only becomes significant when the mother experiences a fever. However, even in that case, when the fever is controlled with anti-fever medication, the significant association once again disappears (Wilkerson et al., 2002; Zerbo et al., 2013). Other longitudinal cohort studies analyzing prenatal infections caused by influenza, rubella, toxoplasmosis, genital reproductive infections, and herpes simplex virus-type 2 infections have shown that prenatal infections are associated with schizophrenia in the offspring (Brown, 2006; Brown et al., 2005; Susser et al., 2000). However, these studies do not differentiate between systemic and intrauterine inflammation (IUI) and their results have not yet been replicated successfully in the current literature. These past studies along with our fetal brain qPCR and fibrin deposit results suggest that a fetus exposed to an indolent cycle of systemic inflammation may not have an increased risk for acute neuroinflammation but may still develop neurological problems in the long term.

On the other hand, intrauterine inflammation (IUI) has been repeatedly shown to cause fetal brain injury, such as cerebral palsy, via immune dysregulation in the developing brain (E. Tsimis, 2018). IUI is associated with long term neurodevelopmental problems in the offspring even in the absence of preterm parturition (Elovitz et al., 2011). Furthermore, IUI has been shown to cause fetal inflammatory response syndrome (FIRS), which is associated with elevated levels of inflammatory cytokines, development of periventricular leukomalacia, and motor and cognitive deficits in children (Burd et al., 2012; Gomez et al., 1998; Svigos, 2001). Adler et al. also demonstrated that mice that were exposed to prenatal IUI showed biomarkers of neurodegeneration, as evidenced by 24 h qEEG results at post-natal day (PND) 100 (Adler et al., 2014). These past studies, along with our fetal brain qPCR results, indicate that exposure to IUI may be associated with an increased risk for acute neuroinflammation that can ultimately lead to chronic detrimental effects in the surviving offspring.

Our study has several strengths. This is the first study to use Doppler examination to look at the blood flow and fetal heart function differences between IU and IP injection of IL-1β in dams and pups. Furthermore, our study provided the first ever evidence that exposure to IU, but not systemic (IP), IL-1β resulted in adverse fetoplacental hemodynamic changes and increased pro-inflammatory cytokines and chemokines in the fetal brain, even though both IU and systemic IL-1β exposure led to similar pup abortion rates.

However, there are several limitations to this current study. First of all, this study utilizes a mouse model for maternal IU and systemic inflammation to analyze the possible molecular pathways involved in their pathophysiology. Similar Doppler studies and molecular studies in human subjects should be done to confirm the IU and systemic inflammatory pathway differences found in mouse models. Secondly, we did not do an extensive analysis of angiogenic or angiostatic chemokines in our mice models. Thirdly, while we may have found the possible mechanism behind IU inflammation, our results do not shed light on the exact mechanism of systemic inflammation.

In conclusion, we propose that exposure to maternal systemic and IU inflammation plays a key role in fetal injury, albeit utilizing different placental inflammatory pathways and mechanisms. While further evaluation of angiogenic and angiostatic chemokines in these two groups should be investigated to understand the extent of the two pathways' differences, our study sets a necessary foundation for future studies and clinical trials on preventing perinatal sequelae from maternal inflammation.

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Author contributions

JL designed and performed the experiments, analyzed the data, and wrote the manuscript. NS performed the experiments, assembled the data, and wrote the manuscript. QN, JD, AC, SL, CN, JL and MM performed the experiments. IB designed and directed the experiments and analyzed the data. All authors read and approved all changes made by the final manuscript.

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