


## ORIGINAL ARTICLE

# The tracking of lipopolysaccharide through the feto-maternal compartment and the involvement of maternal TLR4 in inflammation-induced fetal brain injury

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**Abstract**

**Problem:** Exposure to intrauterine inflammation (IUI) has been shown to induce fetal brain injury and increase the risk of acquiring a neurobehavioral disorder. The trafficking of the inflammatory mediator, lipopolysaccharide (LPS), in the pregnant female reproductive tract in the setting of IUI and the precise mechanisms by which inflammation induces fetal brain injury are not fully understood.

**Method of study:** FITC-labeled LPS was utilized to induce IUI on E15, tissues were collected, and fluorescence was visualized via the Spectrum IVIS. Embryo transfer was utilized to create divergent maternal and fetal genotypes. Wild-type (WT) embryos were transferred into TLR4<sup>-/-</sup> pseudopregnant dams (TLR4<sup>-/-</sup><sub>mat</sub>/WT<sub>fet</sub>). On E15, TLR4<sup>-/-</sup><sub>mat</sub>/WT<sub>fet</sub> dams or their WT controls (WT<sub>mat</sub>/WT<sub>fet</sub>) received an intrauterine injection of LPS or phosphate-buffered saline (PBS). Endotoxin and IL-6 levels were assessed in amniotic fluid, and cytokine expression was measured via QPCR.

**Results:** Lipopolysaccharide trafficked to the uterus, fetal membranes, placenta, and the fetus and was undetectable in other tissues. Endotoxin was present in the amniotic fluid of all animals exposed to LPS. However, the immune response was blunted in TLR4<sup>-/-</sup><sub>mat</sub>/WT<sub>fet</sub> compared with WT controls.

**Conclusion:** Intrauterine administered LPS is capable of accessing the entire fetoplacental unit with or without a functional maternal TLR4. Thus, bacteria or bacterial byproducts in the uterus may negatively impact fetal development regardless of the maternal genotype or endotoxin response. Despite the blunted immune response in the TLR4-deficient dams, an inflammatory response is still ignited in the amniotic cavity and may negatively impact the fetus.

**KEYWORDS**

fetal injury, inflammation, pregnancy, TLR4

Brown and Maubert should be considered joint first authors of this manuscript.

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## 1 | INTRODUCTION

Preterm birth is the leading cause of morbidity and mortality of infants in the United States. Intrauterine infection, such as chorioamnionitis, is strongly associated with preterm birth.<sup>1-3</sup> In addition, intrauterine infection and subsequent fetal exposure to inflammation is one of the greatest determinants of adverse neurobehavioral outcomes in exposed offspring.<sup>4,5</sup> In utero infection, whether bacterial or viral, has been linked to increased incidence of autism spectrum disorder,<sup>6-8</sup> schizophrenia,<sup>9-11</sup> and cognitive delay, suggesting that exposure to prenatal inflammation can adversely affect the developing brain and its impact may be lifelong.<sup>12</sup>

In an effort to better understand the etiology of inflammation-induced fetal brain injury, we and others have employed animal models to help elucidate the pathogenesis of this adverse outcome.<sup>11-21</sup> We have shown that an intrauterine infusion of lipopolysaccharide (LPS), a component of the cell wall of gram-negative bacteria, mimics localized infection and results in an increase in fetal brain cytokine expression, white matter injury, a significant decrease in the arborization of fetal cortical neurons as well as behavioral deficits in the offspring.<sup>14,22-25</sup> Prenatal exposure to inflammation also results in altered gene expression within specific brain regions of inflammation-exposed neonates, providing evidence that the effects of a prenatal inflammatory insult can persist into post-natal life.<sup>14,22,23</sup> Other laboratories have used similar animal models and have discovered analogous changes in fetal brain morphologies associated with exposure to inflammation, in addition to motor and cognitive deficits.<sup>18,19,24-27</sup> While the collective knowledge gained from these animal studies demonstrates the negative impact of inflammation/infection on the developing fetal brain, the precise mechanisms by which bacteria or its byproducts remains elusive. With the goal of preventing adverse outcomes from intrauterine inflammation, further understanding of how inflammation leads to fetal brain injury is necessary.

Bacteria or their byproducts are known to trigger inflammatory responses through activation of Toll-like receptors. Toll-like receptors, TLRs, are a family of transmembrane receptors that recognize specific molecular patterns associated with a variety of microbial pathogens.<sup>28</sup> Specifically, TLR4 functions as the primary signaling receptor for gram-negative bacteria and, therefore, is critical for LPS signaling.<sup>29</sup> TLR4 is present in both maternal (uterine, cervix, and decidua) and fetal tissues (fetus proper, trophoblasts, and immune cells in the placenta).<sup>30</sup> Hence, LPS could be signaling through maternal, fetal or both, maternal and fetal TLRs and triggering an inflammatory cascade. In order to design effective therapies to prevent fetal brain injury, it must be determined whether TLR signaling in the mother, fetus or both is essential for fetal brain injury in the setting of intrauterine inflammation.

Our objectives for this study were (a) to determine whether intrauterine administered LPS can reach the fetoplacental unit using labeled LPS and (b) to determine whether a functional TLR4 in the mother is necessary for fetal brain injury in the setting of intrauterine inflammation.

## 2 | MATERIALS AND METHODS

### 2.1 | Animals

For all animal experiments, wild-type (WT) CD-1 outbred, timed-pregnant mice were purchased from Charles Rivers Laboratory and naturally occurring TLR4 mutant strain, C3H/HeJ, (TLR4<sup>-/-</sup><sub>mat</sub>) mice were purchased from Jackson Laboratory. C3H/HeJ mice have a point mutation in the TLR4 gene resulting in a dominant negative effect on LPS signal transduction. These mice are developmentally and immunologically normal despite their inability to ward off gram-negative bacterial invasion.<sup>31</sup> Timed-pregnant, CD-1 animals were shipped on day 8-12 after mating and acclimated in our facility 3-7 days before use. Non-pregnant CD-1 and TLR4<sup>-/-</sup><sub>mat</sub> females were utilized in embryo transfer experiments. All of the experiments were performed in accordance with the National Institutes of Health Guidelines on laboratory animals and with approval from the University of Pennsylvania's Committee on Animal Care and Use (Protocol # 804658).

### 2.2 | Embryo transfer technique

Divergent maternal and fetal TLR4 genotypes were created by performing embryo transfer experiments. Briefly, embryos at the 2-cell stage were harvested from superovulated, CD-1 females (WT, 5.0 IU of PMSG/HCG, Sigma-Aldrich) mated with CD-1 males (WT). Using pseudopregnant TLR4<sup>-/-</sup><sub>mat</sub> females as surrogates, 22-25 embryos were surgically transferred per female unilaterally. Aseptic technique was used throughout the surgery. These pregnant animals will be referred to as TLR4<sup>-/-</sup><sub>mat</sub>/WT<sub>fet</sub>. As a control for the embryo transfer procedure, CD-1, 2-cell embryos were surgically transferred to pseudopregnant CD-1 females. All embryo transfers were performed by the transgenic core facility at the University of Pennsylvania following IACUC guidelines.

### 2.3 | Mouse model of localized intrauterine inflammation

A mouse model of intrauterine inflammation was used for these studies as previously described.<sup>22,23,32-34</sup> The procedure was performed on day 15 of gestation (E15), with the normal gestation within this strain lasting approximately 19-20 days. Isoflurane anesthesia was used with an induction chamber (Vet Equip). Continuous isoflurane/oxygen anesthesia was administered via mask, and upon achievement of deep anesthesia, a minilaparotomy was performed in the lower abdomen. The right uterine horn was exposed through the incision to allow visualization of the first two gestational sacs (those most proximal to the cervix). Lipopolysaccharide (LPS, from *Escherichia coli*, 055:B5, Sigma-Aldrich) was infused at a concentration of 250 µg in 100 µL sterile phosphate-buffered saline (PBS) into the uterus between the lower two gestational sacs. Care was taken to avoid puncture of the amniotic cavity. Control animals

received anesthesia and intrauterine PBS. PBS was applied to the exposed uterus prior to returning it to the abdomen. The fascia and skin were closed with staples (Autoclips, Clay-Adams, from Fisher Scientific). The entire procedure varied between 2 and 5 minutes per mouse.

## 2.4 | Experimental groups

### 2.4.1 | TLR4<sup>-/-</sup><sub>mat</sub>/WT<sub>fet</sub>

On E15 of gestation, TLR4<sup>-/-</sup> female mice, pregnant with WT fetuses (TLR4<sup>-/-</sup><sub>mat</sub>/WT<sub>fet</sub>), received an intrauterine infusion of LPS (250 µg/100 µL, n = 5) or PBS (n = 3), as previously described. Six hours post-injection, dams were euthanized with CO<sub>2</sub> and amniotic fluid, placentas, uteri and fetal brain were collected.

### 2.4.2 | WT<sub>mat</sub>/WT<sub>fet</sub>

Naturally conceived, CD-1, wild-type animals (WT<sub>mat</sub>/WT<sub>fet</sub>) served as controls and received intrauterine injection of LPS (250 µg/100 µL, n = 6) or PBS (n = 6) on E15. Six hours post-injection, dams were euthanized with CO<sub>2</sub> and amniotic fluid, placentas, uteri and fetal brain were collected.

### 2.4.3 | CD-1/CD-1ETs

As a procedural control for the embryo transfer, CD-1 embryos were transferred into pseudopregnant CD-1 females (CD-1/ CD-1 ETs). Controls received intrauterine injection of LPS (250 µg/100 µL, n = 3) or PBS (n = 3) on E15. Six hours post-injection, dams were euthanized with CO<sub>2</sub> and amniotic fluid, placentas, uteri and fetal brain were collected.

## 2.5 | Tissue collection

For the TLR4<sup>-/-</sup><sub>mat</sub>/WT<sub>fet</sub>, WT<sub>mat</sub>/WT<sub>fet</sub>, and CD-1/CD-1 studies, amniotic fluid, placentas, uteri, and fetal brains were collected. Amniotic fluid was collected and pooled from each gestational sac and centrifuged at 2000 g for 5 minutes at room temperature to remove cellular debris. The supernatant was then stored at -80°C. Placentas, uteri, and fetal brains were collected, rinsed in PBS, immediately flash frozen in liquid nitrogen and stored at -80°C.

## 2.6 | Analysis of amniotic fluid—endotoxin and IL-6

Amniotic fluid was collected from timed-pregnant WT<sub>mat</sub>/WT<sub>fet</sub> animals and TLR4<sup>-/-</sup><sub>mat</sub>/WT<sub>fet</sub> animals at 6 hours post-injection and analyzed for the presence of endotoxin and IL-6. Endotoxin levels were measured via the Limulus Amebocyte Lysate (LAL) Assay (Lonza Walkersville Inc), according to the manufacturer's instructions. IL-6 levels were determined using an IL-6 ELISA (R & D Systems)

according to manufacturer's instructions. The murine IL-6 ELISA had a minimal detectable dose of 1.3-1.8 pg/mL.

## 2.7 | RNA Isolation, cDNA Synthesis, and QPCR

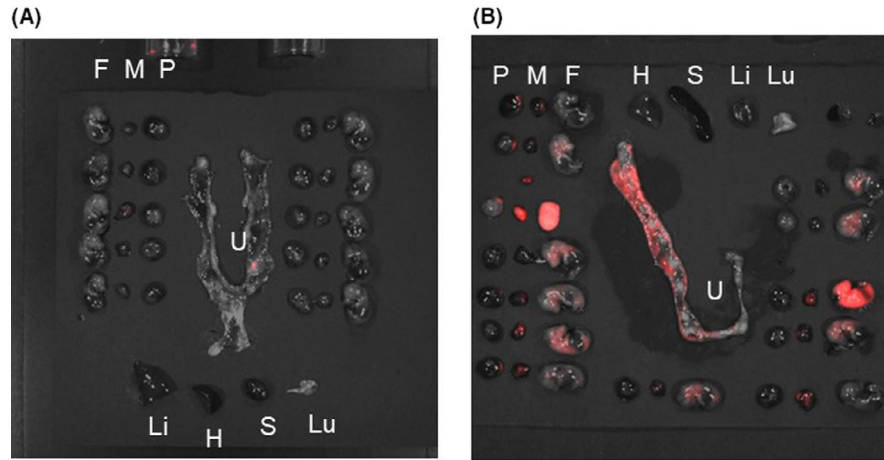
Messenger RNA (mRNA) expression levels of several cytokines were determined in placental, uterine, and fetal brain samples from animals exposed to in utero LPS or PBS treatment using quantitative polymerase chain reaction (QPCR) as previously published.<sup>15,16</sup> Briefly, placental, uterine, and fetal brain RNA was extracted with TRIzol reagent (Invitrogen), and cDNA was synthesized using random hexamers and the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Specific primers for murine IL1-beta (IL-1β), TNF-alpha (TNF-α), and IL-6 were purchased from ThermoFisher. To account for potential differences in starting material, 18S rRNA was also quantified (ThermoFisher). The target and 18S quantitative polymerase chain reactions were performed on the sequence detector PCR machine (Applied Model 7900, PA Applied Biosystems), as previously described.<sup>23</sup> Relative quantitation of 18S rRNA and target mRNAs were calculated and fitted to a five-point standard curve. Standard curves were obtained for each target gene using serial dilutions of the "standard" cDNA solution which was prepared by mixing small aliquots of all cDNA samples in equal proportion. Resulting cDNA quantities were normalized to the amount of 18S cDNA in each sample. Each reaction occurred in separate tubes and was performed in triplicate.

## 2.8 | LPS Localization

CD-1, outbred, time-mated mice at embryonic day 15 were anesthetized with isoflurane and underwent a minilaparotomy to expose the uterus, as previously described. Briefly, fluorescently-labeled LPS (250 µg/100 µL PBS, FITC tagged [495/519] ThermoFisher) or PBS was injected between the first and second gestational sacs in the right uterine horn. Surgical staples were used to close the incision, and mice were returned to their home cages. Six hours post-surgery, the dams were euthanized with carbon dioxide and organs were collected. The reproductive tract (ovaries, uterus, cervix, and vagina), fetuses, fetal membranes, placentas, maternal uterus, liver, spleen, heart, and lung were harvested and visualized via the IVIS Spectrum (Perkin Elmer) fluorescence imaging system. All imaging studies were completed at the Small Animal Imaging Facility (SAIF) at the University of Pennsylvania.

## 2.9 | Statistical analysis

Data were analyzed using GraphPad Prism Software (Version 4). One way analysis of variance (ANOVA) with a Tukey's post hoc test was used to analyze IL-6 levels because the data were normally distributed and the variances were similar. *t* Tests analyses were utilized to analyze all other experimental results, using an unpaired *t* test if the data were normally distributed with similar variances. If data were normally distributed and the variances were not similar then an unpaired *t* test with Welch's



**FIGURE 1** Detection of Alexa Fluor<sup>®</sup> labeled LPS within the female reproductive tract of a pregnant mouse. Pregnant mice received an intrauterine injection of FITC-labeled LPS or PBS at E15. Six hours post-injection, tissues were harvested and imaged via the Spectrum IVIS. A, There was no detection of fluorescence, in any tissue, in the PBS-injected animals. B, In the FITC-labeled LPS-injected animals, the uterus (U), fetal membranes (M), the placenta (P), and the fetus (F) displayed a dramatic increase in fluorescence intensity compared to the PBS controls. However, there was no fluorescence detected in the maternal liver (Li), maternal heart (H), maternal spleen (S), and maternal lung (Lu) in the LPS-exposed animal

correction was employed. If data were not normally distributed, a Mann-Whitney was performed. Statistical significance was defined  $P < .05$ .

### 3 | RESULTS

#### 3.1 | Intrauterine LPS was detected in the uterus, fetal membranes, and placenta

In order to localize LPS within the reproductive tissues after an intrauterine infusion, CD-1 timed-pregnant animals received an intrauterine injection of FITC-tagged LPS. As expected, there was no fluorescence detected in the PBS-infused animals (Figure 1A). However, in the FITC-labeled LPS-injected animals, fluorescence was detected in the maternal uterus, the fetal membranes, the placenta and, the fetus. Fluorescent signal was not detected in the maternal liver, maternal heart, maternal spleen, or maternal lung (Figure 1B).

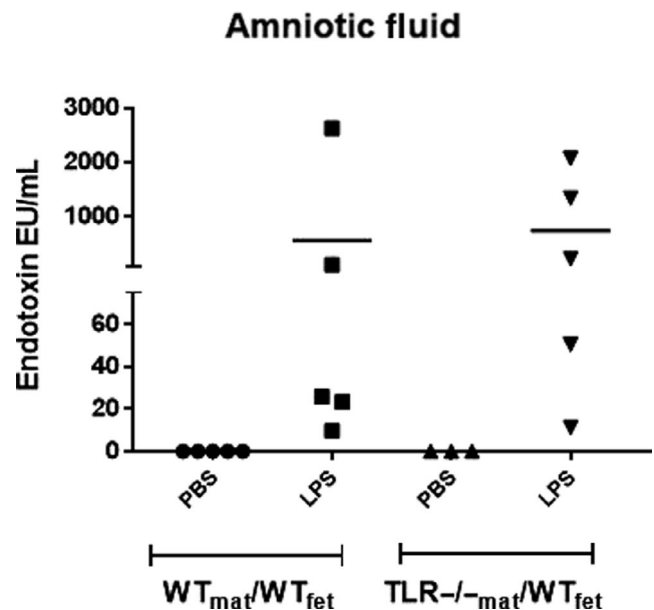
#### 3.2 | Endotoxin levels are elevated in the amniotic fluid of both $WT_{mat}/WT_{fet}$ and $TLR4^{-/-}_{mat}/WT_{fet}$ animals

Intrauterine infusion of LPS resulted in the increased detection of endotoxin within the amniotic fluid of both  $WT_{mat}/WT_{fet}$  and  $TLR4^{-/-}_{mat}/WT_{fet}$  animals (Figure 2). There was no significant difference in the endotoxin levels among the groups treated with LPS, regardless of genotype. No endotoxin was present in the amniotic fluid of PBS-infused animals.

#### 3.3 | LPS-infused, $WT_{mat}/WT_{fet}$ animals have significantly elevated IL-6 levels within the amniotic fluid compared to both the PBS controls and $TLR4^{-/-}_{mat}/WT_{fet}$ cohort

Amniotic fluid IL-6 levels were significantly increased in LPS-infused  $WT_{mat}/WT_{fet}$  animals compared with PBS controls (10.97-fold

increase,  $P = .0017$ , Figure 3). LPS infusion did increase amniotic fluid IL-6 levels 4.7-fold in the  $TLR4^{-/-}_{mat}/WT_{fet}$  cohort at this time point, but this enhancement was not statistically significant. The  $WT_{mat}/WT_{fet}$  animals exposed to LPS had significantly higher levels of IL-6 compared with their  $TLR4^{-/-}_{mat}/WT_{fet}$  counterparts (3.2-fold increase,  $P = .015$ ).



**FIGURE 2** Detection of endotoxin in amniotic fluid of  $WT_{mat}/WT_{fet}$  and  $TLR4^{-/-}_{mat}/WT_{fet}$  pregnant females. Animals were given an intrauterine infusion of LPS (250  $\mu$ g/animal) or PBS on E15 of gestation. Six hours after the injection, animals were killed, amniotic fluid was collected, and the presence of endotoxin was measured via the Limulus Amebocyte Lysate Assay. LPS-exposed animals had varying amounts of endotoxin present in the amniotic fluid while no endotoxin was detected in the PBS-injected animals

### 3.4 | WT<sub>mat</sub>/WT<sub>fet</sub> and TLR4<sup>-/-</sup><sub>mat</sub>/WT<sub>fet</sub> animals express different cytokine profiles in response to LPS injection

Intrauterine infusion of LPS resulted in a significant increase in placental cytokine expression in WT<sub>mat</sub>/WT<sub>fet</sub> animals compared with PBS-injected controls (Table 1). In contrast, there were no statistically significant alterations in placental cytokine expression in the TLR4<sup>-/-</sup><sub>mat</sub>/WT<sub>fet</sub> animals compared with controls.

Within the WT<sub>mat</sub>/WT<sub>fet</sub> uterus, LPS injection resulted in a dramatic enhancement of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  expression compared with PBS-injected controls (Table 1). However, alterations in IL-6 and TNF- $\alpha$  transcript abundance did not reach statistical significance. IL-1 $\beta$  was the only cytokine with significantly enhanced expression ( $P = .0021$ , Table 1). Conversely, LPS injection resulted in mild cytokine expression enhancement within the uterus of the TLR4<sup>-/-</sup><sub>mat</sub>/

WT<sub>fet</sub> animals. LPS injection did not alter IL-6 expression, but significantly, increased IL-1 $\beta$  and TNF- $\alpha$  (Table 1).

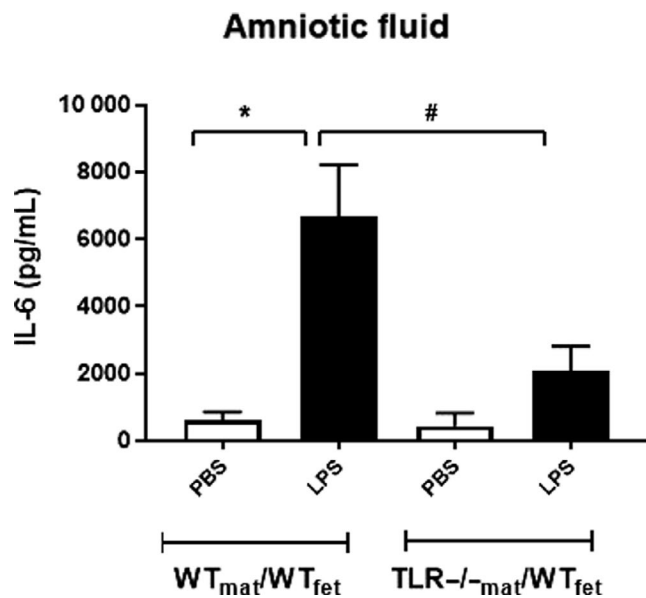
Lipopolysaccharide injection did not alter cytokine expression within fetal brains of the WT<sub>mat</sub>/WT<sub>fet</sub> dams and the TLR4<sup>-/-</sup><sub>mat</sub>/WT<sub>fet</sub> animals compared with PBS-injected controls (Table 1).

Embryo transfers of CD-1 embryos into pseudopregnant CD-1 dams served as a procedural control. The CD-1/CD-1 ETs had similar LPS-induced, tissue specific, cytokine expression profiles as their naturally occurring, wild-type counterparts (data not shown).

## 4 | DISCUSSION

This study provides evidence that (a) LPS injected into the intrauterine space gains access to the maternal-fetal compartment and may therefore freely interact with cells expressing functional TLR4 in the uterus, placenta, and/or fetus (b) a non-functional, maternal TLR4 abrogates the maternal immune response to a gram-negative bacterial challenge but does not prevent LPS from trafficking to the fetus and (c) a diminished maternal immune response may confer some protection to the fetus in the setting of intrauterine inflammation.

In the current study, FITC-conjugated LPS was localized to the uterus, the fetal membranes, the placenta, and the fetus, suggesting that LPS can interact directly with the TLR4-expressing cells in these tissues. Importantly, there was no fluorescence detected in maternal spleen, heart, lung, or liver, further confirming that our model of intrauterine inflammation is a local inflammatory insult and not systemic. However, it is important to note that it cannot be known whether or not the FITC label remains intact over the 6-hour period in the reproductive tract. It is possible that the label could be cleaved, and this could present a less accurate view as to the sites of LPS trafficking. However in a similar study, Kohmura and coworkers demonstrated that 1 hour after an intravenous injection of <sup>125</sup>I-LPS, radioactivity was detected in the placenta and the fetus.<sup>35</sup> While this study used a systemic model, and acknowledges that it is possible that both free <sup>125</sup>I and bound <sup>125</sup>I may exist, it provides further evidence of LPS's ability to navigate the maternal/fetal compartment.<sup>35</sup> Additionally, we demonstrate that LPS gains access to the amniotic fluid due to the presence of endotoxin within this compartment. Amniotic fluid cycles through the developing fetus and it was therefore not surprising that a fetal fluorescent signal was detected. In fact, fluorescence was detected in a number of fetuses demonstrating that the



**FIGURE 3** IL-6 protein expression in the amniotic fluid of WT<sub>mat</sub>/WT<sub>fet</sub> and TLR4<sup>-/-</sup><sub>mat</sub>/WT<sub>fet</sub> Pregnant females were treated with an intrauterine dose of LPS (250  $\mu$ g) or PBS on E15. LPS injection resulted in significantly increased IL-6 protein in WT animals (\*,  $P = .0017$ ) compared with controls. LPS did not significantly elevate IL-6 levels in the TLR4<sup>-/-</sup><sub>mat</sub>/WT<sub>fet</sub> animals. The WT<sub>mat</sub>/WT<sub>fet</sub> dams had a significantly higher immune response compared with their TLR4<sup>-/-</sup><sub>mat</sub>/WT<sub>fet</sub> counterparts (#,  $P = .015$ )

**TABLE 1** Fold changes in cytokine mRNA expression in placental, uterine, and fetal brain tissues

Cytokine	Placenta		Uterus		Fetal Brain	
	WT	TLR4 <sup>-/-</sup> <sub>mat</sub> /WT <sub>fet</sub>	WT	TLR4 <sup>-/-</sup> <sub>mat</sub> /WT <sub>fet</sub>	WT	TLR4 <sup>-/-</sup> <sub>mat</sub> /WT <sub>fet</sub>
IL-1 $\beta$	9.7 ( $P = .0030$ )	1.2 ( $P = .75$ )	16.5 ( $P = .0021$ )	3.3 ( $P = .032$ )	6.5 ( $P = .065$ )	-1.3 ( $P = .61$ )
IL-6	16.7 ( $P = .0034$ )	6.1 ( $P = .22$ )	102.5 ( $P = .05$ )	3.5 ( $P = .076$ )	3.1 ( $P = .068$ )	-0.67 ( $P = .58$ )
TNF- $\alpha$	14.1 ( $P = .001$ )	1.8 ( $P = .30$ )	25.1 ( $P = .055$ )	4 ( $P = .016$ )	10 ( $P = .10$ )	-0.56 ( $P = .43$ )

Note: Tissues were collected from WT<sub>mat</sub>/WT<sub>fet</sub> and TLR4<sup>-/-</sup><sub>mat</sub>/WT<sub>fet</sub> animals 6 h post-intrauterine LPS or PBS injection. Fold change was calculated by dividing the mean expression in LPS-exposed tissues by the mean expression in PBS-exposed tissues.



LPS injection migrates throughout the uterine horns. We and others have previously shown that intrauterine exposure to LPS results in an acute perinatal brain injury as evidenced by altered neuronal morphology, neurotoxicity, and an upregulation of pro-inflammatory cytokine expression.<sup>22,34,36</sup> Therefore, it is possible that LPS reaches the fetal brain and directly interacts with TLR4 on the endothelial cells on the blood-brain barrier, microglia, astrocytes, and/or immune cells, as we have previously shown.<sup>37</sup> Thus, we have shown in previous publications that any inflammatory response in the amniotic fluid or fetus activates macrophages, which in turn migrate to the blood-brain barrier and subsequently lead to an inflammatory response in the brain.<sup>37</sup> Labeled LPS has been previously utilized to track LPS exposure in a pregnant rat model. Specifically, Ashdown, et al<sup>38</sup> injected iodinated LPS into the intraperitoneal space in a pregnant rat and detected it within maternal tissues and the placenta but not in the fetal brain. Unlike our model, Ashdown et al used a different LPS serotype, a lower dose of LPS, and importantly, the LPS was administered intraperitoneal as opposed to our local intrauterine injection. These differences in model, dose, and administration may account for the lack of LPS detection within the fetus.

The experiments performed in the present study utilizing dams with an inactive form of TLR4 further emphasizes the importance of maternal immune system activation in the initiation and propagation of the inflammation that may harm the fetus. We have shown that in the face of a gram-negative bacterial challenge, a functional, maternal TLR4 is required for robust maternal immune activation. Both WT<sub>mat</sub>/WT<sub>fet</sub> and TLR4<sup>-/-</sup><sub>mat</sub>/WT<sub>fet</sub> animals demonstrated an increase in endotoxin within the amniotic fluid post-LPS injection revealing that both cohorts had similar levels of exposure to endotoxin, regardless of the maternal genotype. Therefore, the trafficking of LPS is independent of the presence of TLR4. The WT<sub>mat</sub>/WT<sub>fet</sub> animals demonstrated a statistically significant increase in IL-6 levels within the amniotic fluid compared with their TLR4<sup>-/-</sup><sub>mat</sub>/WT<sub>fet</sub> counterparts. The lack of a functional TLR4 in the TLR4<sup>-/-</sup> mothers prevented the LPS from initiating a strong maternal inflammatory response. However, though not statistically significant, there was a 4.7-fold increase in IL-6 levels in the LPS-exposed TLR4<sup>-/-</sup><sub>mat</sub>/WT<sub>fet</sub> animals (2100 pg/mL) compared with the PBS controls (440 pg/mL). While this increase was dramatically less than the increase seen in the amniotic fluid of the WT<sub>mat</sub>/WT<sub>fet</sub> animals (6700 pg/mL), it shows that the absence of a functional TLR4 in the mother does not completely abolish the inflammatory response in the fetoplacental unit. Furthermore, we provide evidence that LPS can cross the placental barrier and may interact with the functional TLR4 on fetal/placental tissues eliciting an increase in the cytokine levels within the amniotic fluid, albeit not as robust as the increase in cytokines experienced by the WT<sub>mat</sub>/WT<sub>fet</sub> animals. It is important to note that even minor enhancements in cytokine levels within the amniotic fluid are associated with white matter lesions in neonates. Specifically, Yoon and coworkers demonstrated that infants born with cerebral palsy had enhanced amniotic fluid levels of TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 and possessed ultrasonically detectable white matter lesions.<sup>20</sup> In the TLR4<sup>-/-</sup> mothers, exposure to LPS still resulted

in enhanced cytokine levels within the amniotic fluid, independent of a maternal immune response, suggesting that a clinical scenario exists where a mother could present with no symptoms of infection and an exposure to an inflammogen may still negatively impact the developing fetus.

The inflammatory threshold required to elicit fetal brain damage is unknown. Even a mild increase in cytokine levels within the fetomaternal compartment, similar to that seen in our TLR4<sup>-/-</sup><sub>mat</sub>/WT<sub>fet</sub> dams, may have a negative impact on fetal neuronal health. As previously mentioned, LPS injection resulted in increased IL-6 levels within the amniotic fluid of the TLR4<sup>-/-</sup><sub>mat</sub>/WT<sub>fet</sub> dams compared with the PBS controls. While this increase was not statistically significant, this elevation in IL-6 may be enough to exacerbate the immune response thus perpetuating the inflammation which ultimately may lead to fetal brain injury. In the current study, LPS administration did not result in a statistically significant increase in cytokine transcript abundance within the fetal brains of wild-type animals compared with their PBS controls. In the WT<sub>mat</sub>/WT<sub>fet</sub> fetuses, the expression levels of IL-1 $\beta$  (6.5-fold increase,  $P = .065$ ) and IL-6 (3.1-fold increase,  $P = .068$ ) were approaching significance but their  $P$  values exceeded our standard of significance of  $P = .05$ . This may be due, in part, to the utilization of fewer animals in the wild-type group ( $n = 6$ ) compared with our previous study ( $n = 9$ ).<sup>14</sup> In earlier reports, we have shown that intrauterine LPS administration resulted in enhanced fetal brain cytokine expression, suggesting that fetal brain inflammation resulting from intrauterine LPS exposure can occur despite the lack of significant enhancement of fetal brain cytokines in the current study.<sup>14,39</sup> Thus, we have shown that diminishing the maternal immune response results in decreased cytokine expression in the fetomaternal compartment potentially protecting fetal neurons from the consequences of excessive inflammation. It is important to note that it is difficult to ascertain at which point inflammation becomes excessive and leads to negative outcomes.

The low-level inflammatory response observed in the TLR4<sup>-/-</sup> dams might be attributed to TLR2 stimulation from minor contaminants in the LPS preparation<sup>40</sup> as the LPS preparation used in this study has a low level of "endotoxin protein" (<3%), as do many of the commercially available LPS preparations. Alternatively, a study by Kayagaki and coworkers has identified an intracellular LPS sensor capable of initiating an LPS-induced inflammatory cascade.<sup>41</sup> This non-canonical pathway, is TLR4 independent, and relies upon the activation of the inflammasome, a large, multi-protein cytoplasmic complex that couples pathogen recognition with cytokine maturation and release. It therefore can be postulated that mice lacking a functional TLR4 can have increased cytokine release due to LPS internalization and subsequent stimulation of the inflammasome.

With these studies, in the absence of a competent maternal TLR4, a fetal inflammatory response from exposure to intrauterine inflammation is blunted in the placenta and the uterus. The dampening of the maternal immune response may protect the fetus and prevent fetal brain injury which is due to excessive inflammation. However, our study can only support this finding for short term brain injury, as

all experiments were conducted 6 hours after LPS exposure. Since LPS was detected in the amniotic fluid of TLR4<sup>-/-</sup> dams, it is possible that with prolonged exposure, placental/fetal TLR4 could respond to the available LPS and induce a fetal immune response and propagate fetal brain injury. Additionally, while not significantly different, IL-6 levels in the TLR4<sup>-/-</sup><sub>mat</sub>/WT<sub>fet</sub> were elevated. It is possible that trafficking of LPS to the fetoplacental unit will eventually engage TLR4 in fetal cells and be sufficient to induce fetal brain injury. TLR4 is known to be expressed on fetal skin, fetal lung, and intestinal epithelium.<sup>42-44</sup> As such, available LPS in the amniotic fluid would be in contact not only with fetal skin but also with the gut and pulmonary tract as amniotic fluid is swallowed by the fetus. If we were to extrapolate these findings to the clinical realm, some pregnant women might be exposed and/or have LPS (or other bacterial protein) present at the level of the uterus but do not have significant maternal immune response, for a variety of genetic, immunological differences, etc. And, if that LPS or bacterial byproduct could be trafficked, as we observed in this mouse study, to the fetal side of the placenta, then fetal injury could occur without a significant maternal (uterine) immune response and/or without any signs of intrauterine inflammation (no contractions, preterm labor). If that hypothesis is valid, then targeting the maternal immune response will only be an effective therapeutic strategy, if we can prevent trafficking of bacteria to the fetal side.

Compared with systemic inflammatory stimulation, the use of a localized model of prenatal inflammation better recapitulates what occurs clinically in humans<sup>26</sup>; however, this model does have limitations. Brain development in the mouse does not parallel human fetal brain development, specifically in regards to the timing of myelination.<sup>45</sup> Naturally, these studies are not feasible in humans and research involving non-human primates is excessively costly. In the current study, an intrauterine injection of LPS stimulated the inflammation in this mouse model of inflammation-induced fetal brain injury. Certainly, intrauterine inflammation can be created via a number of different means such as injecting live bacteria, heat-killed bacteria, and bacterial cell wall components into the uterine cavity. Using killed *E coli*, Filipovich and coworkers found similar increases in cytokine expression within the placenta and uterus 8 hours post-intrauterine injection suggesting that both heat-killed bacteria and LPS can act as inflammogens in the intrauterine space creating an adverse environment for the fetus.<sup>46</sup> In this study, we used a one-time infusion of LPS which may not represent what happens during human pregnancy. Understanding these potential limitations, the rodent model has demonstrated strengths in elucidating the pathogenesis of fetal brain injury from exposure to intrauterine inflammation that would not be possible with human studies.<sup>13-16,24-26,34,36,37,39</sup>

In conclusion, this study does support the concept that blocking the maternal TLR4 response and/or limiting the initial maternal immune response holds promise as a therapeutic approach to preventing fetal brain injury from exposure to prenatal inflammation. As such, Chin and coworkers demonstrated that (+)-naloxone, a TLR4 antagonist, is capable of preventing inflammation-induced preterm birth in a mouse model.<sup>47</sup> In another study, utilizing a rhesus monkey

model, a TLR4 antagonist prevented LPS-induced uterine contractility and lowered amniotic fluid cytokine levels.<sup>48</sup> These findings further support the concept that therapies preventing TLR4 activation and the downstream inflammatory cascade hold clinical promise of preventing inflammation-induced preterm parturition.

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