TLR-4-Dependent and -Independent Mechanisms of Fetal Brain Injury in the Setting of Preterm Birth

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Abstract

In this study, we sought to assess how essential activation of toll-like receptor 4 (TLR-4) is to fetal brain injury from intrauterine inflammation. Both wild-type and TLR-4 mutant fetal central nervous system cells were exposed to inflammation using lipopolysaccharide in vivo or in vitro. Inflammation could not induce neuronal injury in the absence of glial cells, in either wild-type or TLR-4 mutant neurons. However, injured neurons could induce injury in other neurons regardless of TLR-4 competency. Our results indicate that initiation of neuronal injury is a TLR-4-dependent event, while propagation is a TLR-4-independent event.

Keywords

prenatal inflammation, LPS, neuronal injury, TLR-4

Introduction

Preterm birth, occurring in approximately 12% of all pregnancies in the United States, is a major contributor to perinatal and neonatal morbidity and mortality.¹ There is a strong association between intrauterine inflammation and preterm parturition.^{2,3} Specifically, the presence of histological chorioamnionitis, as a marker of intrauterine inflammation, is associated with over 85% of early spontaneous preterm births (<28 weeks).⁴ The presence of intrauterine inflammation in a preterm birth is of high clinical relevance as prenatal inflammation has been demonstrated to be associated with an increase in adverse neurobehavioral outcomes for exposed offspring.⁵⁻⁹ Epidemiological data have demonstrated an association between prenatal inflammation and the subsequent development of cerebral palsy.¹⁰⁻¹⁵

Despite the emphasis of cerebral palsy as the main adverse outcome of interest in ex-preterm children, an emerging body of evidence demonstrates that prenatal inflammation is also associated with a spectrum of neurobehavioral disorders.^{10,12,16-22} In fact, recent studies demonstrate that ex-preterm infants have high screen positive rates for autism spectrum disorders (ASDs) and that chorioamnionitis and preterm birth are specific risk factors for ASD.²³⁻²⁸ Ex-preterm children have been demonstrated to have an astonishing prevalence of higher order neurodevelopmental impairment by the time they reach school-age.^{5,29-31} In some studies, up to 50% of ex-preterm infants experience difficulties in executive functioning as well as in the areas of attention and socioemotional behaviors, often requiring special academic support.³²⁻³⁵ Despite this growing body of evidence correlating

exposure to prenatal inflammation (as occurs in many preterm births), the mechanisms by which inflammatory pathways lead to fetal, and ultimately, neonatal brain injury remain elusive.

An essential step in the activation of inflammatory pathways is triggering of the innate immune system. The innate immune response is the first line of defense against microbial pathogens or their by-products. Lipopolysaccharide (LPS), a component of the cell wall of gram-negative bacteria, binds and activates toll-like receptor 4 (TLR-4), triggering a signaling cascade promoting cytokine release, chemokine activation, and nuclear factor κ B stimulation.³⁶⁻³⁹ While innate immunity serves an important teleological role to protect the host, activation of the innate immune response in the central nervous system (CNS) can have detrimental effects. The role of the TLR signaling in brain injury is complex and just beginning to be revealed with the use of knock-out mice.⁴⁰⁻⁴⁴ Recent work demonstrates that the activation of TLRs is likely to play a critical role in brain injury from both systemic and local inflammatory

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challenges.^{45,46} Recently, the presence of TLR-4 in the CNS was determined to be necessary for both an immune response in the brain to systemic inflammation as well as LPS-induced oligodendrocyte injury.^{47,48} While the specific cell in the CNS responsible for responding to LPS remains unclear, recent reports demonstrate that LPS can induce white matter injury, a common finding in ex-preterm children. While TLR-4 has been shown to be present on microglia and activation of TLR-4 on these glial cells is proposed to be an essential event for inflammation-induced brain injury,^{48,49} whether neurons (specifically fetal neurons) express a functional TLR-4 remains unclear.⁵⁰⁻⁵² Furthermore, it remains unknown whether, in the setting of intrauterine inflammation, stimulation of TLR-4 in the fetal brain is essential and/or the primary mechanism leading to brain injury and subsequent adverse neurobehavioral outcomes in exposed offspring.

In order to further investigate the mechanisms by which prenatal inflammation evokes fetal brain injury, we have used a mouse model of intrauterine inflammation, which closely mimics the most common human scenario of preterm birth.^{53,54} Using this mouse model, we have demonstrated that intrauterine inflammation induces a cytokine response in the fetal brain along with evidence of white matter injury.⁵⁵ Novel to our laboratory, we have also demonstrated that while intrauterine inflammation does not cause overt structural brain injury,⁵⁶ it does promote a specific neuronal insult.⁵⁷ Furthermore, we have demonstrated that this observed brain injury is present only in the setting of inflammation-induced preterm birth as preterm parturition from a noninflammatory stimulus does not cause brain injury.⁵⁸

Since we have demonstrated that inflammatory pathways are necessary for the observed brain injury in response to prenatal inflammation, it is necessary to determine the mechanisms by which inflammation induces such injury so that therapeutic strategies become a realistic goal. We hypothesize that the activation of TLR-4 in the fetal brain is essential for inducing neuronal injury in the setting of intrauterine inflammation. Using wild-type and TLR-4-mutant mice, this study sought to assess (1) whether there was a functional TLR-4 on fetal neurons, (2) whether activation of TLR-4 in neuronal and glial cells could initiate injury, and (3) whether neuronal to neuronal injury was a TLR-4-mediated event.

Materials and Methods

Mouse Model of Intrauterine Inflammation

All procedures were performed with Institutional Animal Care and Use Committee approval from the University of Pennsylvania School of Medicine. An established model of inflammation-induced preterm birth⁵³ was utilized for these studies as previously reported.⁵⁹⁻⁶¹ In this study, CD-1 outbred, timed pregnant mice were used (Charles River Laboratories, Wilmington, Massachusetts). This model serves to mimic the clinical scenario of the majority of spontaneous births with a local inflammatory response in the uterus but in the absence of overt infection/inflammation in the dam.⁶² Briefly, intrauterine injections of LPS (from *Escherichia coli*,

055: B5, Sigma Chemical Co, St Louis, Missouri) at a dose of 250 µg in 100 µL of phosphate-buffered saline (PBS) were administered on embryonic day 15 (E15) of a 19-day gestation as reported previously.^{53,54,60,63,64} Control dams received intrauterine infusion of saline. Another set of control dams received no intervention and was matched to the same gestational age. For survival surgery, pregnant mice were placed under a mask that kept a continuous flow of isoflurane/room air to obtain adequate deep anesthesia. A mini-laparotomy was then performed in the lower abdomen; LPS or saline was infused into the right uterine horn between the first and second gestational sacs (just cephalad from the cervix). Peritoneal closure was performed with sutures and the incision was closed with staples. At 6 hours postsurgery, dams were euthanized with carbon dioxide. Fetuses were harvested from the same uterine location (right and left lower uterine horns) for all dams. Whole fetal brains were collected for cortical neuronal cultures as well as assessment for differences in messenger RNA (mRNA) expression.

Cortical (Neuronal) Cultures

Cortical tissues were harvested from E15 fetal brains of CD1 untreated dams. To explore the role of TLR-4, cortical tissues were also harvested from E15 brains from a naturally occurring TLR-4-mutant strain, C3H/HEJ (Jackson Laboratory, Bar Harbor, Maine). No surgical intervention or treatment was administered to either mouse strain prior to collection. A total of 4 fetal brains per dam were used. The meninges, olfactory bulbs, brain stem, and cerebellum were dissected and separated in Petri dishes containing Ca⁺⁺/Mg⁺⁺-free Hanks balanced salt solution (Invitrogen, Carlsbad, California); pH 7.4, to isolate the cortex such that optimal cortical cultures were obtained. Each cortex was homogenized and placed in 4 mL of neurobasal medium (NBM; Invitrogen) containing 0.03% trypsin (Invitrogen) and incubated for 15 minutes at 37°C and 5% CO₂. The tissue was then removed and rinsed in 4.5 mL NBM with 10% fetal bovine serum (FBS) where it was titrated to inactivate the trypsin. For selective growth of neurons without the presence of astrocytes or microglia, which will be referred to as cortical culture, cells were maintained in specialized NBM supplemented with B-27 and 0.5 mmol/L L-glutamine (Invitrogen). Using 12-well culture plates coated with poly-L-lysine (1 mg/mL; Sigma-Aldrich), cells were consistently plated at equal density (10⁴ cells/mL). Cultures were maintained in a sterile incubator at 37°C.

Cortical Cocultures

Cocultures were also created for both CD1 wild-type and HEJ (TLR-4 deficient) strains. The E15 fetal cortex was removed as described above and transferred into 4.5 mL minimum essential media supplemented with 10% FBS, B-27, and 0.5 mmol/L L-glutamine establishing a coculture environment consisting of approximately 74% astrocytes, 23% neurons, and 2% microglia.⁵⁷ Cells were plated at the density of 2.5 × 10⁵ cells/mL

on poly-L-lysine-coated glass coverslips in 12-well culture plates. For each experiment, 3 to 4 fetal brains from 1 dam constituted 1 culture. Cultures were maintained in a sterile incubator at 37°C.

In Vitro Treatment Groups

In vitro treatment with LPS has been demonstrated in other studies to elicit an inflammatory response in CNS cells.^{49,65} To determine whether LPS activation of TLR-4 was necessary for neuronal injury in fetal CNS cells and whether TLR-4 was functional on both glial and neuronal cells, we exposed CD1 and HEJ cortical cultures (>95% neurons) and CD1 and HEJ cocultures (consisting of neurons, astrocytes, and microglia) to either sterile saline (10 µg/mL) in 1 mL NBM or LPS (10 µg/mL) in 1 mL NBM. All cultures were treated on day in vitro 1 (DIV1). To investigate whether neuronal to neuronal injury is a TLR-4-dependent event, both CD1 and HEJ cortical cultures were treated with either 1 mL of media harvested from cortical cultures produced from fetal brains exposed in vivo to intrauterine inflammation as described above, referred to as LPS exposed neuronal culture (LN media) or 1 mL of media harvested from cortical cultures produced from fetal brains exposed in vivo to intrauterine saline, referred to as control neuronal culture (CN media).

Immunocytochemistry

Morphology images were obtained from HEJ and CD1 cortical cultures treated on DIV10 with saline or LPS and CN or LN media. Plates were fixed and stained to assess morphological changes between treatment groups. Wells were rinsed in warm PBS and fixed with 4% paraformaldehyde in PBS for 20 minutes. Cells were permeabilized with 0.5% Triton X-100 (DiaSys Europe Ltd, Wokingham, UK) for 5 minutes at room temperature and then cells were washed twice with PBS. Double immunofluorescent antibody staining was used to visualize Neurofilament protein (NF-200; Sigma-Aldrich) or dendrites and cell bodies (microtubule-associated protein 2 [MAP-2]; Sigma-Aldrich). Both antibodies are diluted in PBS containing 1% FBS (MAP-2, 1:100; NF200, 1:400). Totally, 200 µL of both diluted antibodies were applied to each well, and plates were incubated at 37°C for 1/2 hours. Cells were then washed 3 times with PBS and incubated for 1 hour at 37°C with Alexa Fluor goat anti-mouse 488 (Invitrogen) and Alexa Fluor goat antirabbit 568 (Invitrogen) diluted 1:500 in PBS containing 1% FBS. A final rinse with PBS was performed, and coverslips were mounted using Prolong Gold (Invitrogen) onto glass coverslips. Confocal microscopy (Leica SP2 Confocal) was utilized to qualitatively evaluate the morphological changes through a ×40 objective lens.

Quantitative Analysis of Dendritic Processes in CD1/HEJ Cortical Cultures

To quantify dendritic processes emanating from the neuronal cell body at DIV3, between 30 and 60 neurons representing 4

different fetuses from 3 different dams for each treatment group were evaluated at a final image magnification of ×400 under the stringent condition that selected neurons cannot overlap or connect to other neurons. Fluorescent images are recorded and analyzed using a Dell Latitude D620 and processed with Image J1.37v.

Assessment of Inflammatory Cytokines in CD1/HEJ Cortical Cultures and CD1 Coculture

To quantify the concentrations of IL-6 and IL-1β cytokines after in vitro treatment with LPS, enzyme-linked immunosorbent assays ([ELISAs] Quantikine, R&D Systems, Minneapolis, Minnesota) were performed according to the manufacturer's protocol on media harvested on DIV3 from cell cultures. The absorbance at 450 nm was read on a microplate reader. Mean concentrations were determined in duplicate based on a standard curve.

Quantitative Polymerase Chain Reaction for Expression of Neuronal Markers and TLR-4

Total RNA was extracted from cell culture wells with trizol (Invitrogen) according to product protocol. Complementary DNA (cDNA) was generated from 20 µg of RNA/sample using a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, California). To assess expression of neuronal markers, quantitative polymerase chain reaction (qPCR) was performed using equivalent dilutions of each sample on the Applied Biosystems Model 9700 sequence detector PCR machine, as reported previously.^{55,63,64} Primer sets to neuronal markers MAP-2 and nestin, conjugated to Taqman MGB probes, were used for qPCR (Applied Biosystems). The relative abundance of the target of interest was divided by the relative abundance of 18S in each sample to generate a normalized abundance for the target of interest. All samples were analyzed in duplicate. For detection of TLR-4, semi-qPCR was performed. Following amplification in the Thermacycler using 30, 32, or 35 cycles, PCR products were analyzed by electrophoresis on 1.5% agarose gels containing ethidium bromide (Invitrogen).

The MTT In Vitro Toxicology Assay to Assess Cell Viability

An 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT)-based in vitro toxicology assay kit (Sigma Aldrich) was used on CD1 or HEJ cortical cultures and CD1 or HEJ cocultures per the manufacturer's protocol. The assay is dependent on the cellular reduction in MTT by the mitochondrial dehydrogenase of viable cells. A 0.1 mg of MTT was applied to the well of each treatment group, followed by incubation at 37°C for 2 hours. Adding an equal volume of MTT solubilization solution dissolved the resulting blue formazan crystals. The samples were placed in cuvette and absorbance of dye product was spectrophotometrically measured at a wavelength of 570 nm (BioPhotometer, Eppendorf, Germany).

Statistical Analysis

Statistical analysis was performed using SigmaStat 3.5 (Aspire Software International, Ashburn, Virginia). Data were expressed as the standard deviation of the mean or as the median when the data are not normally distributed. Statistical significance was defined as a 2-sided $P < .05$. For statistical analysis of inflammatory markers in cell culture media, Student *t* test (for parametric data) or Mann-Whitney rank sum (for nonparametric data) was used. To analyze neuronal mRNA expression in the fetal brains, to investigate the number of dendritic processes, and to assess differences in cell viability, values were compared between LPS- and saline-exposed fetal brains using Student *t* test.

Results

LPS Is Effective in Eliciting Neuronal Damage in Cell Culture

To determine whether TLR-4 is functional on fetal central nervous cells in a culture environment, CD1 cocultures and HEJ cocultures consisting of neurons, astrocytes, and microglia were treated with LPS. Treatment of CD1 cocultures with LPS resulted in a significant release of pro-inflammatory cytokines with an increase in both IL-6 ($P = .002$, *t* test) and IL-1 β ($P = .002$; Figure 1) expression in LPS-exposed cocultures compared to saline control. Treatment of HEJ cocultures with LPS resulted in a slight increase in IL-6 ($P = .047$ *t* test) and IL-1 β ($P = .23$ *t* test) levels, however, this change is negligible compared to the magnitude of response elicited by LPS-induced CD1 cultures (Figure 1A).

In addition to activating a cytokine response, *in vitro* exposure to LPS was capable of evoking cell death in CD1 cocultures only. When cell viability was assessed in CD1 coculture, LPS-treated cells demonstrated a significant decrease in viable cells (saline: 2.56 ± 0.19 , LPS: 1.33 ± 0.19 , $P = .001$, *t* test) as measured by a lack of absorbance of enzymatic product. However, cell viability was unaffected by LPS treatment in HEJ cocultures (saline: 1.45 ± 0.32 , LPS: 1.67 ± 0.39 , $P = .4$, *t* test; Figure 1B). As a means to quantify the population of neuronal cells, neuronal markers, MAP-2, and nestin were evaluated by qPCR in cultures exposed and unexposed to LPS. In CD1 cocultures, mRNA expression of both markers was significantly decreased in LPS-treated cultures (MAP-2, $P = .02$; nestin, $P = .008$, *t* test), resulting in a 7- and 10-fold decrease in MAP-2 and nestin, respectively (Figure 2).

There is conflicting data in literature regarding the presence of TLR-4 on fetal neurons.^{51,66} After treatment of E15 and E18 CD1 cortical cultures with both control (saline) and LPS, we detected low expression levels of TLR-4 on neurons using real-time PCR (RT-PCR). However, it was only after several amplification cycles that expression was revealed (Figure 3). Additionally, qPCR was performed on DIV3 to quantify TLR-4 expression in CD1 cortical and

Figure 1. A, Quantification of cytokine levels in CD1 cocultures and HEJ cocultures on day in vitro 3 (DIV3) by enzyme-linked immunosorbent assay (ELISA). Bar graph representing means and standard deviations for interleukin 6 (IL-6) expression in cocultures stimulated with lipopolysaccharide ([LPS] $N = 6$) or saline ($N = 6$). Treatment of CD1 cocultures with LPS resulted in significantly increased levels of IL-6 (2796.5 ± 131.5 pg/mL) compared to treatment with saline (IL-6 = 11.378 ± 3.39 pg/mL, $P = .002$) as well as increased IL-1 β (LPS: 57.517 pg/mL, Sal: 0.45 pg/mL, $P = .002$). Treatment of HEJ cocultures with LPS elicited a slight IL-6 response (20.509 pg/mL) and negligible IL-1 β (1.6 pg/mL). B, Cell viability as assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Bar graph represents mean and standard deviation (SD) of spectrophotometric absorbance prior to and with treatment of LPS for CD1 (saline: $2.6 \pm .47$, LPS: 1.33 ± 0.48) or HEJ (saline: 1.45 ± 0.32 , LPS: 1.67 ± 0.39 , $P = .4$, *t* test) cocultures. The asterisk denotes $P < .05$.

cocultures. There was no difference in TLR-4 mRNA expression between CD1 saline or LPS-treated cortical and cocultures ($P = .71$, analysis of variance on ranks; data not shown). Collectively, these results demonstrate that there is a functional TLR-4 on fetal CNS cells, which are capable of responding to this dose of LPS although LPS does not seem to alter TLR-4 mRNA expression at this developmental time point.

Figure 2. Messenger RNA (mRNA) expression of neuronal markers, microtubule-associated protein 2 (MAP-2) and nestin in cocultures on day in vitro 10 (DIV10) by polymerase chain reaction (PCR). Bar graph represents mean and standard deviation (SD) for $n = 6$ per treatment group (saline in hatched bars, LPS represented in solid bar). Lipopolysaccharide (LPS)-exposed cocultures demonstrated a statistically significant decrease in both MAP-2 (21.576 ± 9.65 , $P = .02$) and nestin mRNA levels (51.013 ± 13.05 , $P = .008$) compared to saline-treated cocultures. The asterisk denotes $P < .05$, Student t test.

Initiation of Neuronal Injury Via LPS is Dependent on TLR-4

As it has been previously shown that cytokines are increased in fetal brains after exposure to intrauterine inflammation,⁵⁵ we investigated whether cytokine expression was altered specifically in cortical cultures from neuronal cells. On DIV3, IL-6 and IL-1 β expression levels were extremely low in media harvested from CD1 and HEJ cortical cultures, even in those cultures exposed to LPS. The differences between treatment groups did not reach significance ($P > .05$, t test; data not shown).

Prior studies have demonstrated damaging effects of LPS treatment on neurons, which are visible by immunostaining with the dendritic marker, MAP-2. Upon treatment with LPS,

the dendrites become sparse and disconnected.⁶⁷ We have shown that in vivo exposure to LPS results in a loss of MAP-2 staining and causes "MAP-2 beading," a sign of neuronal fragility.^{57,58} The HEJ neurons (TLR-4 mutants) treated in vitro with LPS appear morphologically similar to saline-treated neurons (Figure 4), resembling healthy aggregation patterns and dendritic processes. However, CD1 neurons treated in vitro with LPS have the characteristic MAP-2 beading typical of injury (Figure 4). Furthermore, exposure of either CD1 or HEJ neurons to in vitro treatment of either saline or LPS did not have a significant effect on the number of dendrites in culture (Figure 5). In addition, there was no significant effect on cell viability in CD1 or HEJ cortical cultures between saline and LPS 3 days after treatment (CD1: $P = .432$, HEJ: $P = .982$). The lack of response in HEJ cortical cultures to LPS is expected, as it is known that this strain of mice has a nonfunctional TLR-4. However, as CD-1 (with a functional TLR-4) cocultures, consisting of glia and neurons, responded to stimulation with LPS (Figure 1) while cortical cultures did not, these findings suggest that TLR-4 is not functional on fetal neurons even in TLR-4 competent mice.

Neuronal to Neuronal Injury Is a TLR-4 Independent Event

Using methods described in Immunocytochemistry section, media from LPS-exposed cortical cultures (LN) and control cortical cultures (CN) were applied to cortical cultures from CD1 and HEJ (TLR-4 mutants) cortical cultures. Morphologic analyses demonstrate that primary HEJ neuronal cultures treated in vitro with CN media result in healthy neuronal morphology (Figure 6A), while treatment with LN media results in neuronal fragility in the form of MAP-2 beading and decreased aggregation of neurons on DIV10 (Figure 6B). Consistent with previous reports,⁵⁷ CD1 cortical neuronal cells suffered similar damage from LN media treatment (Figure 6C and D).

Confirming alternation in neuronal morphology, objective assessment of dendritic counts demonstrated that treatment of CD1 and HEJ cortical cultures with LN significantly reduce the number of dendrites compared with CN treatment (Figure 7). Cell viability of HEJ cortical cultures is not affected by LN treatment compared with CN (CN = 0.181 ± 0.06 , LN = 0.18 ± 0.09 ; $P = .993$). These experiments suggest that neuronal-neuronal injury is independent of TLR-4.

Discussion

These studies provide novel insight into the pathogenesis of neuronal injury in the setting of intrauterine inflammation. Specifically, we have demonstrated that (1) initiation of neuronal injury is TLR-4 dependent but that neurons are unlikely to be the cell on which TLR-4 is activated and (2) that propagation of neuronal injury is a TLR-4-independent event. These findings have important therapeutic implications.

Known mechanisms that lead to neurological and behavioral phenotypes in other disorders include abnormalities in synaptic

Figure 3. Toll-like receptor 4 (TLR-4) messenger RNA (mRNA) expression at day 2 and day 7 in culture (cortical) as determined by polymerase chain reaction (PCR). Fetal brains harvested at embryonic day 15 (E15) (A) or E18 (B) have low levels of TLR-4 expression at both 2 and 7 days in culture. Intensity increases at higher cycle numbers (indicated on right). Lane M is base marker. Lanes C1 to C3 represent TLR-4 expression in cortical cultures harvested from fetuses exposed to intrauterine saline (controls). Lanes L1 to L3 represent TLR-4 mRNA expression in cortical cultures from fetuses exposed to intrauterine inflammation (lipopolysaccharide [LPS]). Positive controls for TLR-4 mRNA expression were E18 whole fetal brains (E18), postnatal brains from P21 (PND) and from pregnant mouse uterus, which is known to highly express TLR-4. The last lane represents the negative control with water instead of complementary DNA (cDNA).

Figure 4. Representative confocal images of neurons on day in vitro 14 (DIV14). The HEJ cortical cultures were treated in vitro on DIV1 with saline (A) or lipopolysaccharide ([LPS] B). Treatment with LPS did not alter neuronal morphology while treatment of CDI cortical cultures with LPS (D) demonstrates neuronal fragility compared with saline-treated neurons (C).

communications and dendritic arborization.⁶⁸⁻⁷² Recent work from our laboratory suggests that neuronal injury is present in fetuses exposed to intrauterine inflammation and as such,

neuronal injury may be an important mechanism for adverse neurobehavioral outcomes in ex-preterm children.^{57,58} The data presented here reveal the presence of functional TLR-4

Figure 5. Bar graph representing mean and standard deviation (SD) of dendritic counts in cortical cultures exposed to lipopolysaccharide (LPS) or saline. In vitro stimulation with LPS did not alter the number of dendritic processes in CD1 (toll-like receptor 4 [TLR-4] competent) or HEJ (TLR-4 mutant) compared to saline. For CD1 cultures, the number of processes in saline-exposed cocultures was 4.3 ± 0.20 compared to 4.6 ± 0.30 for LPS-exposed cocultures ($P > .05$, Student *t* test). For HEJ cultures, the number of processes was not significantly different between saline- (4.6 ± 0.3) and LPS (4.5 ± 0.3 ; $P > .05$, Student *t* test)-exposed cultures.

on CNS cells, namely astrocytes and microglia, as an essential contributor to the initiation of neuronal damage in fetuses exposed to prenatal inflammation. Selective targeting of these specific cell types may be a future therapeutic strategy. Collectively, these data demonstrate that while fetal neurons express low levels of TLR-4 mRNA, that TLR-4 is not functional in fetal neurons. These findings are significant and novel in that they elucidate innate immune pathways in fetal, not adult, neurons.

Using RT-PCR from neuronal cell culture and in vivo models of ischemic stroke, Tang et al reported that primary E15 mouse cortical neurons express TLR-4 mRNA. Furthermore, they demonstrated that TLR-4 expression was increased at both the mRNA and protein levels in response to interferon gamma (IFN- γ).⁵¹ However, these results do not convey functionality of TLR-4. Furthermore, this response is limited to ischemic conditions, in which injury was evoked via energy deprivation. This model differs from our intrauterine inflammation model, which may trigger different cellular pathways.

Prior in vitro work has demonstrated that glial cells respond to LPS and thus, have a functional TLR-4. In these studies, the glial and/or mixed glial cultures were exposed to varying doses of LPS. The LPS doses ranged from 10 ng to 100 μ g, with all doses eliciting an inflammatory response.^{65,73,74} The dose of LPS used in these studies is within the range of previously reported doses with a similar effect. Hence, we can extrapolate from these prior works and our findings that the dose of LPS

used for these studies was sufficient to induce an inflammatory response. Hence, the lack of a response to LPS in our cortical cultures is not from an insufficient LPS dose but from lack of a functional TLR-4 signaling system.

Using different mouse strains known to have functional and mutated TLR-4, we demonstrated that TLR-4 is not functional on fetal neurons but appears to be functionally active on fetal astrocytes and microglia. Prior reports demonstrate that glial cells created from the background strain for C3H/HEJ (that have functional TLR-4) respond to LPS as demonstrated by an immune response from microglia.⁷⁵ This report, along with our results, further demonstrate that lack of immune response in fetal neurons in wild-type mice is from a lack of a functional TLR-4. Our results also confirm other findings that demonstrate the robust presence of TLR-4 on microglia and astrocytes alone.⁷⁶ In previous studies, stimulation of quiescent microglia with LPS elicited a heightened immune response.⁷⁷ Other findings support the role of astrocytes and microglia as mediators of cytotoxic effects of LPS.⁶⁶ These reports along with our recent findings suggest that neurons do not, or cannot, respond to an inflammatory insult using the same mechanism as glia cells. We show that this is due to cell type-specific presence of functional TLR-4 in the fetal CNS. Therefore, targeting activation of TLR-4 as a therapeutic avenue may be beneficial for preventing the initiation of neuronal injury in the setting of intrauterine inflammation.

Previous work from our laboratory demonstrates that injured neurons can propagate neuronal injury.⁵⁷ Using TLR-4 mutant mice, we are now able to demonstrate that this neuronal-neuronal injury is a TLR-4-independent event as media from injured neurons (exposed to LPS in vivo) can induce similar injury on TLR-4 competent and incompetent neurons. While previous work has demonstrated that cytokines are neurotoxic and can mediate neuronal-neuronal injury,⁷⁸⁻⁸¹ our results suggest that neuronal-neuronal injury in the setting of intrauterine inflammation is not a cytokine-mediated event. Other endogenous mediators have been implicated in neuronal-neuronal injury; Qiu et al found that the nuclear protein high-mobility group box 1 is released after ischemic injury from neurons and may contribute to the initial stages of the inflammatory response.⁸² Additionally, recent work has demonstrated that microglia-derived superoxide is critical for the inflammation-induced selective loss of dopaminergic neurons.⁸³ Other possible mediators of neuronal-neuronal injury are heat shock protein 70 (Hsp-70) and prostaglandin release.^{84,85} All these mediators could be potential therapeutic targets for preventing ongoing neuronal injury in utero or postnatally.

Due to the difficulty of studying parturition and inflammation-induced preterm birth in the human fetus, established mouse models offer an invaluable means to elucidate the effects of inflammation and parturition on the fetal brain. Yet, there are always limitations with the use of animal models, as extrapolating findings to the human situation require further validation. A notable strength of this model though is that it closely mimics the most common clinical scenario associated with preterm birth creating an intrauterine inflammatory

Figure 6. Representative confocal images of neurons on day in vitro 14 (DIV14). Both HEJ and CD1 cortical cultures were treated in vitro on DIV1 with control neuronal culture (CN) media (HEJ: A, CD1: C) or LPS exposed neuronal culture (LN) media (HEJ: B, CD1: D). LN-exposed neurons demonstrated neuronal injury, as evident by characteristic dendritic “beading.” This effect is clearly visible in a magnified image of dendritic processes in the insets.

environment.^{53,54} Other models that induce a systemic inflammatory response best serve to mimic fetal brain injury in the setting of systemic inflammation/infection in the human as might occur in the setting of sepsis.^{55,59,61,86-88} Yet, the exposure to systemic inflammation/infection is a less likely occurrence in human pregnancy than preterm birth or chorioamnionitis at term, as such our model of intrauterine inflammation serves as an appropriate model to study brain injury from prenatal exposure to inflammation. It is noted that the use of this mouse model is limited as myelination is delayed in the mouse fetus compared to the human fetus.⁸⁹ However, for these studies, the focus is on the neurons and glia, not oligodendrocytes so this is not a significant limitation to this work.

The use of primary neuronal cultures to dissect mechanisms of neuronal injury is well established.⁹⁰⁻⁹² However, this in vitro environment may lack other essential elements that compose the complete LPS signaling pathways in vivo. Certain distinct TLR signaling pathways are dependent upon recruitment of adapter proteins such as TIR domain-containing adaptor inducing IFN-beta (TRIF), which is critical for LPS signal transduction, or MyD88.^{93,94} The recruitment and activation of these cofactors in the TLR-4 complex and cascade may differ in cell culture experiments. Additionally, multiple forms of negative regulation such as intracellular factors downstream of TLR-4 pathway or negative feedback loops act to prevent an exaggerated inflammatory response,⁹⁵ which needs to be

Figure 7. Bar graph representing means and standard deviation (SD) of dendritic processes in cortical cultures from CD1 (toll-like receptor 4 [TLR-4] competent) and HEJ (TLR-4 mutant) exposed to media from LN (cortical cultures created after in vivo exposure to lipopolysaccharide [LPS]) or CN (control neurons). Treatment with LN significantly decreased the number of processes compared to CN in both CD1 (3.2 ± 0.10 vs 4.8 ± 0.03 , $P = .001$, Student *t* test) and HEJ cortical cultures (3.3 ± 0.3 vs 4.7 ± 0.3 , $P = .001$, Student *t* test). The asterisk denotes $P < .005$.

accounted for when assessing causative agents. However, since LPS was able to induce a response in coculture, TLR signaling pathways appear to be present and complete, at least in fetal glia cells.

Collectively, if our findings with these in vitro studies translate to in vivo events, then these results demonstrate 2 key targets for therapeutic interventions. In the setting of intrauterine inflammation, the initiation of neuronal injury appears to lie with glia cells and the activation of TLR-4. Clinically, if intrauterine inflammation could be detected prior to fetal brain injury, then targeting TLR-4 may serve to prevent fetal brain injury. However, targeting of TLR-4 would be insufficient if some neuronal injury has occurred prior to delivery. As demonstrated in our prior work, injured neurons can propagate neuronal injury.⁵⁸ What we demonstrate with this study is that this neuronal to neuronal injury is independent of TLR-4. This is a crucial finding. If upon delivery the fetus is “removed” from an inflammatory environment/stimulus, one might assume that there could be no further brain injury. Yet, our studies demonstrate that brain injury and specifically neuronal injury may be propagated through injured neurons and can persist in the absence of an ongoing inflammatory stimulus. Thus, altered neuronal-to-neuronal and neuronal-to-glia communication may continue into the postnatal period leading to adverse neurobehavioral outcomes.

In conclusion, gaining insight into the role of TLR-4 in the pathogenesis of fetal brain injury from intrauterine inflammation could provide new therapeutic targets to prevent long-term adverse neurobehavioral outcomes in ex-preterm children. These results offer evidence that therapeutic strategies to prevent long-term adverse neurobehavioral outcomes from exposure to prenatal inflammation must address both the initiation and propagation of fetal brain injury.

Declaration of Conflicting Interests

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