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The Absence of TLR4 Prevents Fetal Brain Injury in the Setting of Intrauterine Inflammation

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Abstract

Background: Exposure to intrauterine inflammation during pregnancy is linked to brain injury and neurobehavioral disorders in affected children. Innate immunity, specifically Toll-like receptor (TLR) signaling pathways are present throughout the reproductive tract as well as in the placenta, fetal membranes, and fetus. The TLR pathways are mechanistically involved in host responses to foreign pathogens and may lead to brain injury associated with prenatal inflammation. **Objective:** We aimed to determine whether the activation of the TLR4 signaling pathway, in the mother and fetus, is critical to fetal brain injury in the setting of intrauterine inflammation. **Methods:** A mini-laparotomy was performed on time pregnant C57B6 mice and 2 knockout mouse strains lacking the function of the *Tlr4* and *Myd88* genes on embryonic day 15. Intrauterine injections of *Escherichia coli* lipopolysaccharide or saline were administered as described previously. Dams were killed 6 hours postsurgery, and placental, amniotic fluid, and fetal brain tissue were collected. To assess brain injury, quantitative polymerase chain reaction (qPCR) analysis was performed on multiple components of the NOTCH signaling pathway, including *Hes* genes. Interleukin (IL) IL6, IL1β, and CCL5 expression was assessed using qPCR and enzyme-linked immunosorbent assay. **Results:** Using an established mouse model of intrauterine inflammation, we demonstrate that the abrogation of TLR4 signaling eliminates the cytokine response in mother and fetus and prevents brain injury associated with increased expression of transcriptional effectors of the NOTCH signaling pathway, *Hes1* and *Hes5*. **Conclusions:** These data show that the activation of the TLR4 signaling pathway is necessary for the development of fetal brain injury in response to intrauterine inflammation.

Keywords

intrauterine inflammation, pregnancy, brain injury, TLR4 signaling, NOTCH signaling

Introduction

A substantial body of epidemiological and clinical data supports the causative link between maternal infections during pregnancy and adverse neurobehavioral outcomes in exposed children, including cerebral palsy, autism, attention-deficit and hyperactivity disorder, and others.[1-4] Microscopic examination and magnetic resonance imaging of neonatal brains in preterm children reveal complex neuropathological changes, such as decreased brain volume, gray and white matter injury, and microglial and astroglial activation,[3,5-7] which likely lead to neurological dysfunction later in life. The emergence of animal model systems of inflammation-induced brain injury[5,8] has unequivocally demonstrated that gestational exposure to microbial pathogens can cause cellular and behavioral deficits similar to those observed in humans.[5,9-16] A mouse model of intrauterine inflammation has contributed to these studies by recapitulating the most common clinical route of prenatal infection and showing that an intrauterine inflammatory environment can lead to neuronal and glial injuries.[17] However, despite continuing research efforts directed toward a better understanding of the cellular and molecular underpinnings mediating the effect of prenatal inflammation on fetal brain development and postnatal function, the mechanisms underlying brain injury are still poorly understood.

Intrauterine inflammation is detected in about 20% of all pregnancies. The most common pathogenic bacterial species associated with intrauterine inflammation include *Escherichia coli*, Group B *Streptococcus*, *Mycoplasma*s, *Ureaplasma*

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parvum, and *Ureaplasma urealiticum*.[8,18-21] The presence of these bacterial species in the feto–maternal compartment is recognized by Toll-like receptors (TLRs) type 2 and 4 which bind to the stereotypic pathogen-associated molecular patterns produced by gram-positive and gram-negative bacteria, respectively.[22,23] Activated ligand–TLR4 receptor complexes generate the molecular signal that is passed onto 2 alternative pairs of adaptor proteins: (1) Toll/interleukin (IL) 1 receptor domain-containing adapter protein and Myeloid Differentiation primary response gene 88 (MYD88) and (2) TIR domain-containing adapter-inducing interferon β (TRIF) and TRIF-related adaptor molecule, thereby initiating MYD88- and TRIF-dependent downstream signal transduction, respectively.[22] In contrast, TLR2 receptors signal solely through the MYD88-dependent intracellular signaling cascade.[24] The MYD88- and TRIF-dependent signaling pathways engage different molecular partners and induce changes in gene expression which lead to the production of distinct, yet partially overlapping, sets of cytokines, chemokines, and other immune mediators.[22,23] For example, the production of IL 6 (IL6) and 1β (IL1β), among others, is known to be mediated by the MYD88-dependent signaling cascade, while TRIF-dependent signaling activates the expression of a chemokine C-C motif chemokine ligand 5 (CCL5) as well as other cytokines.[25,26] While the involvement of the MYD88 and/or the TRIF-dependent signaling cascades in inflammation-induced brain injury remains largely unexplored, it has been well established that fetal exposure to increased inflammation within the womb can have detrimental consequences on the developing brain.[6,27]

Specifically, previous studies have shown that intrauterine exposure to bacterial pathogens or their by-products leads to increased cytokine production in both maternal and fetal tissues.[11,12,14,28] An increase in cytokine expression in the fetal brain has been thought to directly contribute to perinatal brain injury which may occur via multiple potential mechanisms.[6,29] One of those mechanisms may involve an interaction with other well-characterized signaling pathways, such as NOTCH, in regulating various cellular processes during brain development.[30-33] For example, the members of the gp130 family of cytokines, including IL6, cooperate with the NOTCH signaling pathway in maintaining the pool of embryonic neural progenitor cells.[31]

Expression of multiple components of the NOTCH signaling pathway is known to be altered in the setting of traumatic,[34,35] ischemic,[36] hyperoxia,[37] and inflammation[38]-induced injury. In addition, an injection of *E coli* lipopolysaccharide (LPS) into the postnatal rat brain leads to an induction of NOTCH1 receptor expression and increased levels of NOTCH1 ligands in microglial cells.[39] While the specific roles of increased NOTCH signaling vary depending on the cellular mechanisms involved in different types of injuries, an alteration in NOTCH pathway expression in the brain indicates ongoing brain damage and thus can serve as a biomarker of brain injury.[38] The members of the NOTCH signaling pathway include ligands Delta-like (DLL) 1, 3, 4; Jagged (JAG) 1 and 2; receptors NOTCH 1 to 4; a transcriptional factor Recombining binding factor suppressor of hairless; and the downstream transcriptional effectors Hairy and Enhancer of Split (HES) and Hairy and Enhancer of Split related with YRPW motif (HEY).[39] Upon ligand binding, activated NOTCH receptors undergo several proteolytic cleavage events that culminate in the release of the intracellular portion of the protein, the NOTCH Intracellular Domain (NICD). The NICD translocates into the nucleus activating the transcription of the downstream *Hes* and *Hey* target genes.[40] Understanding the role of the TLR signaling pathway in mediating immune responses and recognizing that immune responses may beget fetal brain injury, the objective of this study was to determine whether the activation of the TLR4 signaling pathway in the mother and fetus was necessary for fetal brain injury in the setting of intrauterine inflammation. As a metric of fetal brain injury, we assessed the cytokine response and NOTCH pathway expression in the developing brain.

Materials and Methods

Mouse Model of Inflammation-Induced Fetal Brain Injury

C57B6 timed pregnant mice were purchased from Jackson Laboratories (Bar Harbor, Maine); *Tlr2*$^{-/-}$, *Tlr4*$^{-/-}$ double knock out (*Tlr2/4* DKO)[41,42] and *Myd88*$^{-/-}$ knockout (*Myd88* KO) strains[43] were kindly provided by Dr Sunny Shin. Embryonic day 0 (E0) was considered a mating day, and E1 was determined based on presence of the copulatory plug. *Myd88* KO mice and their wild-type counterparts were given water containing sulfamethoxazole (0.4 g/L) and trimethoprim (0.008 g/L) prepared using 1:100 dilution of Sulfatrim pediatric suspension (STI Pharma LLC, NDC 54879-007-16) to avoid lethality from bacterial infection as reported previously.[44] A mouse model of intrauterine inflammation was created as described previously.[17] Briefly, a mini-laparotomy was performed on wild-type and KO mouse strains under isoflurane anesthesia at E15, with normal gestation lasting 19 days.[13,17,45] The right lower uterus was exposed allowing visualization of the lowest 2 gestational sacs. Mice then received intrauterine injections of a common TLR4 ligand LPS from *E coli* (055: B5; Sigma, St Louis, Missouri, L2880), 250 μg per animal. This commercial LPS preparation contains approximately 3% of the endotoxin protein that was shown to induce TLR2 signaling.[46] To avoid a potential activation of the TLR2 signaling pathway by this contaminant in the experiments where we eliminated the TLR4 response, we used a double knockout line in both *Tlr2* and *Tlr4* genes (*Tlr2/4* DKO). The following experimental groups were utilized for these studies: *Myd88 KO experiment*: Saline-treated wild-type animals n = 6, saline-treated *Myd88* KO animals n = 4; LPS-treated wild-type animals n = 6, LPS-treated *Myd88* KO animals n = 4; *Tlr2/4 DKO experiment*: Saline-treated wild-type animals n = 6, saline-treated *Tlr2/4* DKO n = 6, LPS-treated wild-type animals n = 6, and LPS-treated *Tlr2/4* DKO n = 6.

Surgical incisions were closed using staples, and dams were allowed to recover for 6 hours. All experiments were performed in accordance with the National Institute of Health Guidelines on Laboratory Animals with approval from the University of Pennsylvania's Animal Care and Use Committee.

Tissue Collection

Dams were killed for tissue collection 6 hours after the surgery. To reduce variation in gene expression due to possible differences in inflammatory response, fetal brains and placentas were collected from 2 fetuses on each side of the uterus surrounding the injection site (4 total) and pooled per dam to equal an N = 1. Amniotic fluid was collected from all amniotic sacs residing in the uterus of each dam. Leptomeninges were removed, and scalp was peeled off before brain collection. All tissue samples were immediately flash-frozen using liquid nitrogen and stored at −80%. Fetal brain and placental samples were later ground in liquid nitrogen using mortars and pestles while kept on dry ice and then returned to −80% till further processing.

RNA Extraction and Complementary DNA Synthesis

Tissue pellets were homogenized using 5-mm metal beads (QIAgen; Valencia, Santa Clarita, California, USA) in a Tissue Lyser II (QIAgen) at 30 Hz for 2 minutes after adding 1 mL of QIAzol Lysis Reagent (QIAgen Sciences) per 50 mg of tissue. The RNA was extracted according to the manufacturer's instructions. Briefly, after mixing with 1-bromo-3-chloropropane phase separation reagent (100 µL/sample; Molecular Research Center), the extracts were centrifuged at 12 000 *g* for 15 minutes at 4°C and RNA was precipitated after 10 minutes incubation with 500 µL of 100% ethanol per sample at room temperature. The RNA samples were pelleted at 12 000 *g* for 10 minutes at 4°C and rinsed with 75% ethanol. The pellets were resuspended in RNAse-free water, and RNA concentrations were determined by spectrophotometry at 260 nm (Nanodrop; Nano Drop Technologies, LLC; Wilmington, Delaware, USA). Two microgram of each RNA sample was used as a template in a 40 µL complementary DNA (cDNA) synthesis reaction containing random primers, 4 mmol/L deoxyribonucleotide triphosphate and a MultiScribe Reverse Transcriptase (50 u/µL; High Capacity cDNA Reverse Transcription Kit; Applied Biosystems; Waltham, Massachusetts, USA). The cDNA synthesis was performed according to the manufacturer's protocol.

Quantitative Polymerase Chain Reaction

Messenger RNA (mRNA) expression levels of several cytokines were determined in fetal brain samples from animals exposed to in utero LPS or saline treatment using quantitative polymerase chain reaction (PCR) as published previously.[12,14] The following TaqMan gene expression assays were used: Mm00446190_m1 (*Il6*), Mm00434228_m1 (*Il1*β), and Mm01302427_m1 (*Ccl5*), Mm00627185_m1 (*Notch1*), Mm00803077_m1 (*Notch2*), Mm01345646_m1 (*Notch3*), Mm00440525_m1 (*Notch4*), Mm00496902_m1 (*Jag1*), Mm01325629_m1 (*Jag2*), Mm01279269_m1 (*Dll1*), Mm00432854_m1 (*Dll3*), Mm00444619_m1 (*Dll4*), Mm01342805_m1 (*Hes1*), Mm00439311_g1 (*Hes5*), Mm00473576_m1 (*Hes7*), and Mm00468865_m1 (*Hey1*; Thermofisher Scientific, Waltham, Massachusetts). The cDNAs were diluted 1:5 with nuclease-free water and amplified using TaqMan Gene Expression Master Mix (Applied Biosystems) on Applied Biosystems 7900HT real-time PCR system. 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 (TaqMan VIC-TAMRA 4310893E-1408056 gene expression assay; (Applied Biosystems; Waltham, Massachusetts, USA)) in each sample. All reactions were performed in triplicate.

Enzyme-Linked Immunosorbent Assay

Cytokine expression was analyzed in placental and amniotic fluid samples isolated from gestational sacs surrounding the injection sites using commercially available enzyme-linked immunosorbent assay kits for IL6 and CCL5 proteins (R&D Systems, Minneapolis, Minnesota). The assays were performed according to the manufacturer's instructions. Protein concentration was determined using BCA Assay (Pierce BCA Protein Assay Kit; Thermo Scientific) with a colorimetric detection at 450 nm based on standard curve (7.8-500 pg/mL). All tests were performed in duplicate. The resulting values for amniotic fluid and placental samples were normalized by volume and protein content, respectively.

Statistical Analysis

Statistical analysis was performed using Student *t* test (GraphPad PRISM4 software) for the comparisons between wild-type LPS and saline-treated groups and mutant (*Tlr2/4* DKO and *Myd88* KO) LPS and saline-treated groups. All graphic data represent mean values; error bars indicate standard errors of the mean. Welch correction was applied if the variances within compared groups were unequal.

Results

Absence of the TLR4 Signaling Pathway Eliminates a Cytokine Response in the Placenta, Amniotic Fluid, and Fetal Brain Following Intrauterine Exposure to LPS

To determine whether activation of the TLR4 signaling pathway is essential for a cytokine response in the fetus and placenta after in utero exposure to LPS, we compared cytokine expression in wild-type mice and a *Tlr2/4* DKO strain. The *Tlr2/4* DKO strain was chosen, as it allows to eliminate not only the LPS-induced TLR4 pathway activation but also a possible upregulation of the TLR2 signaling pathway caused by a contaminant in the LPS preparation (see Material and Methods).[46] MYD88- and TRIF-dependent cytokine production was examined by quantifying the levels of IL6 and IL1β and CCL5, respectively, in the placenta (Figure 1), amniotic

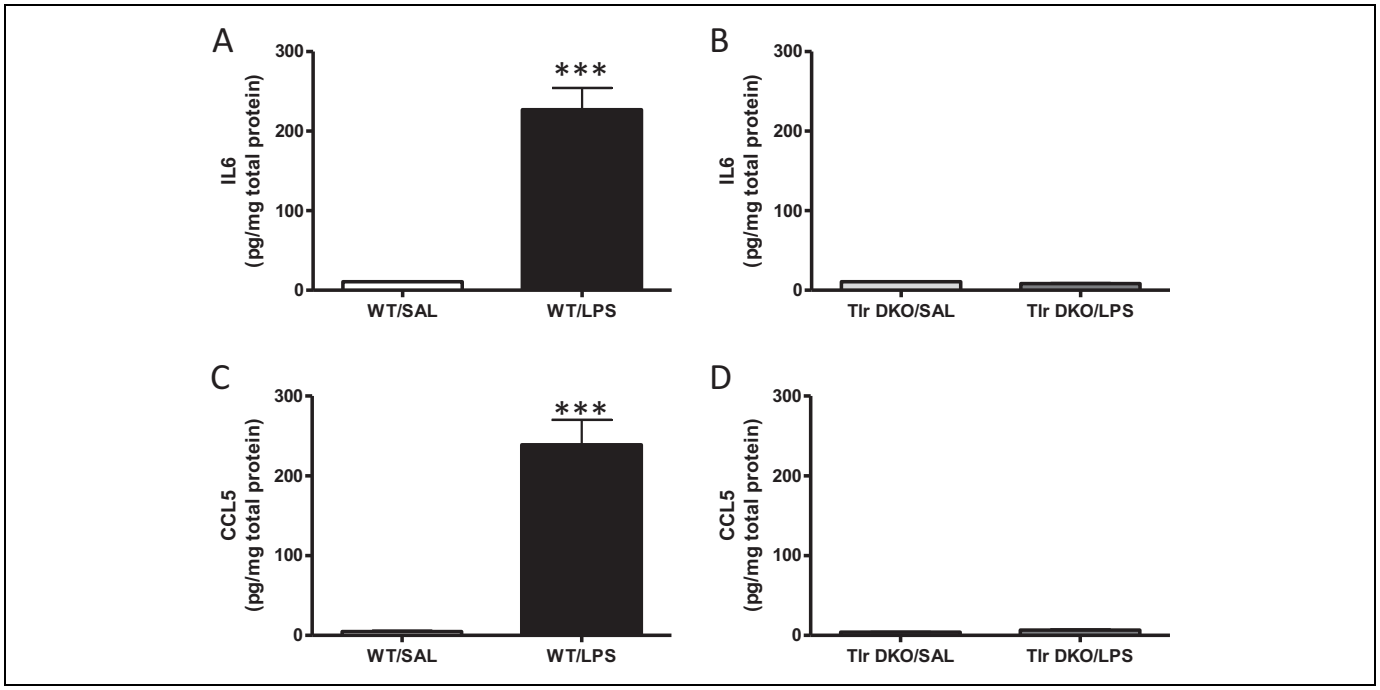

Figure 1. Interleukin (IL) 6 and CCL5 levels are unaltered in the placentas from Toll-like receptor (*Tlr*)2/*4* double knock out (DKO) animals after intrauterine exposure to lipopolysaccharide (LPS). The IL6 (A, B) and CCL5 (C and D) content was measured in the placentas from wild type (A and C) and *Tlr2/4* DKO (B and D) Animals after Exposure to LPS or Saline (SAL) using enzyme-linked immunosorbent assay (ELISA). The IL6 and CCL5 levels were increased in the placentas from LPS-treated wild-type, but not *TLR2/4* DKO, animals. ***<0.001 (Student *t* test).

fluid (Figure 2), and fetal brain (Figure 3) 6 hours after the LPS treatment administered on E15. As expected, we observed a significant increase in the levels of IL6 and CCL5 in the placental (Figures 1A and C, respectively) and amniotic fluid (Figures 2A and C, respectively) samples in wild-type dams exposed to intrauterine LPS. Similarly, in utero LPS exposure increased *Il6* and *Il1*β mRNA levels in the fetal brains of wild-type dams (Figures 3A and C, respectively). In contrast, the expression of *Il6*, *Il1*β, and *Ccl5* was not altered in the placenta (Figures 1B and D), amniotic fluid (Figures 2B and D), and fetal brain (Figures 3B and D) of the *Tlr2/4* DKO mice after in utero LPS administration. These findings indicate that the abrogation of the TLR4 receptor function prevents both MYD88- and TRIF-dependent cytokine production after in utero exposure to LPS in all tissues examined.

Absence of the TLR4 Signaling Pathway Prevents an Activation of the NOTCH Signaling Pathway in the Fetal Brain After Intrauterine Exposure to LPS

To determine whether intrauterine exposure to inflammation alters NOTCH pathway expression in the fetal brain, we measured mRNA levels of multiple pathway components, including ligands JAG1, JAG2, DLL1, DLL3 and DLL4, receptors NOTCH1, NOTCH2, NOTCH3, and NOTCH4 and transcriptional effectors HES1, HES5, HES7, and HEY1 6 hours after the LPS treatment. Our data demonstrate that several members of the NOTCH signaling pathway were altered in the setting of intrauterine inflammation: *Notch1*, *Hes1, Hes5*, and *Hes7* mRNAs were increased in the fetal brain of wild-type animals (Figures 4A, C, E, and G, respectively). The expression of other members of the NOTCH signaling pathway was not found to be significantly different after exposure to intrauterine inflammation (data not shown). The upregulation of *Hes1*, *Hes5*, and *Hes7* genes that are known to be induced by the NOTCH signaling pathway[47] strongly suggests an inflammation-induced activation of NOTCH signaling in the fetal brain.

To explore whether the absence of the TLR4 receptor function can prevent an LPS-induced increase in expression of the NOTCH pathway components in the fetal brain, we compared changes in *Notch1*, *Hes1, Hes5*, and *Hes7* expression in wild-type and *Tlr2/4* DKO strains exposed to intrauterine LPS or saline. Unlike what we observed in wild type dams, mRNA expression of these NOTCH signaling components was not significantly increased in the fetal brains of *Tlr2/4* DKO mice after exposure to LPS (Figures 4B, D, F, and H).

Absence of MYD88, a Downstream Adaptor Protein in the TLR Signaling Pathway, Prevents Cytokine Production in the Placenta, Amniotic Fluid, and Fetal Brain After Intrauterine Exposure to LPS

The MYD88 is an adaptor protein that mediates 1 of the 2 major intracellular signaling branches downstream from

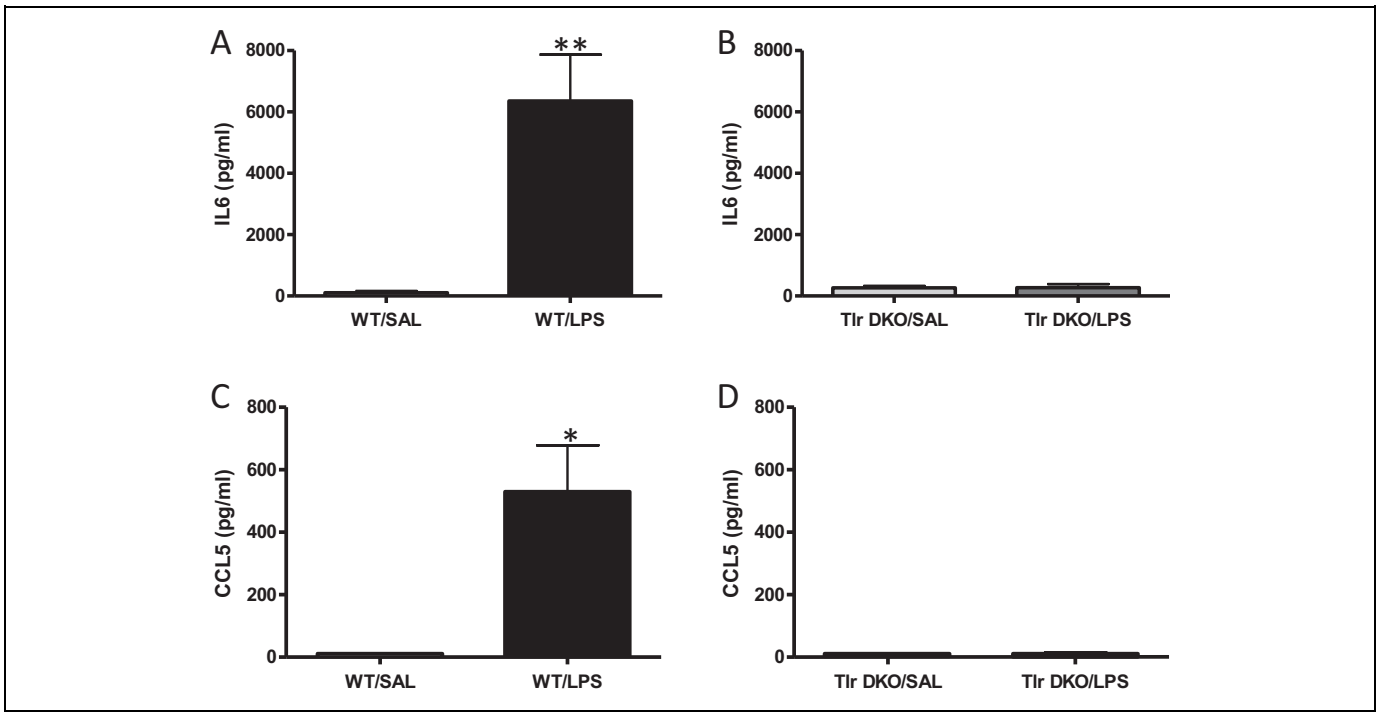

Figure 2. Interleukin (IL) 6 and CCL5 levels are unaltered in the amniotic fluid samples from Toll-like receptor (*Tlr*)2/*4* double knock out (DKO) animals after intrauterine exposure to lipopolysaccharide (LPS). The IL6 (A and B) and CCL5 (C and D) content was measured in the amniotic fluid samples from wild type (A and C) and *Tlr2/4* DKO (B and D) animals after exposure to LPS or saline (SAL) using enzyme-linked immunosorbent assay (ELISA). The IL6 and CCL5 levels were increased in amniotic fluids from LPS-treated wild-type, but not *Tlr2/4* DKO, Animals. *<0.05, **<0.01 (Student *t* test).

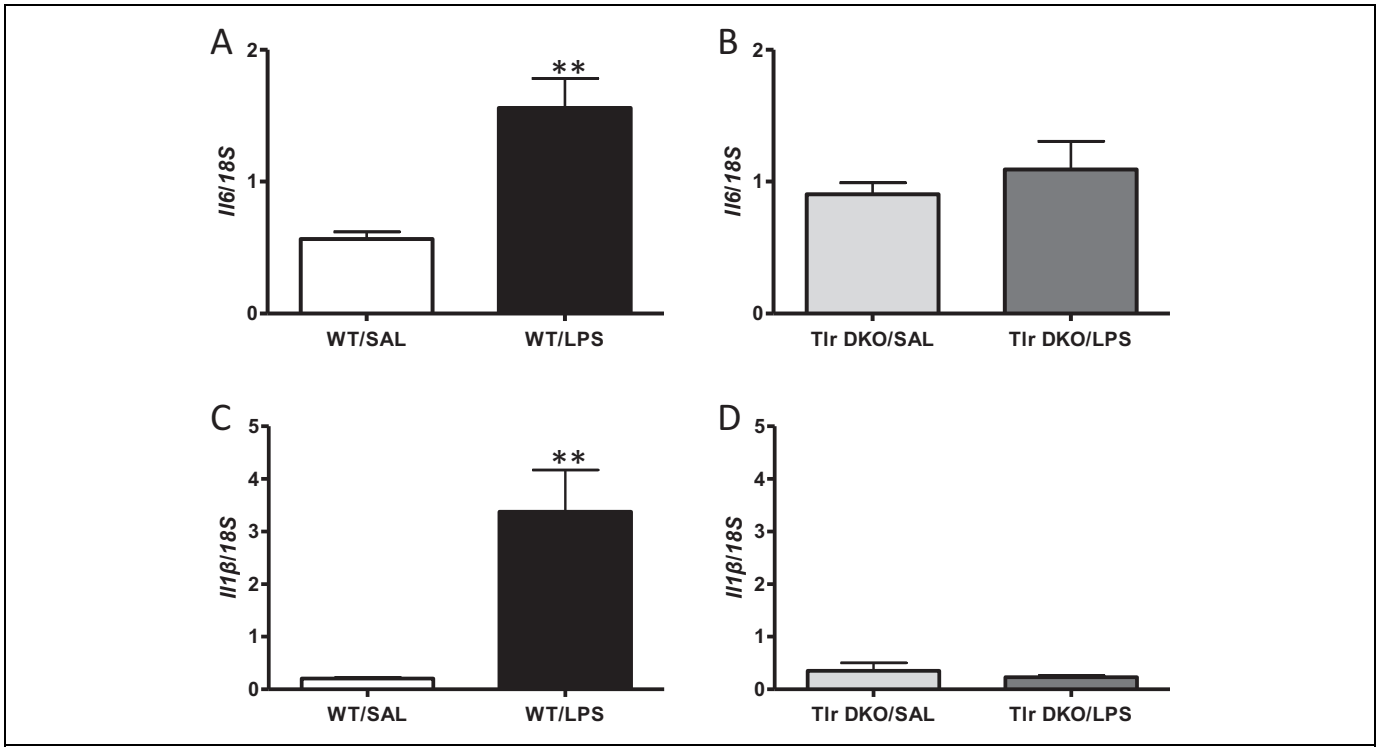

Figure 3. Interleukin (IL) *6* and *Il1*β levels are unaltered in fetal brains from Toll-like receptor (*Tlr*)2/*4* double knock out (DKO) *a*nimals after intrauterine exposure to lipopolysaccharide (LPS). The *Il6* (A and B) and *Il1*β (C and D) levels were measured in fetal brains from wild type (A, C) and *Tlr2/4* DKO (B and D) animals after exposure to LPS or saline (SAL) using quantitative polymerase chain reaction (qPCR). The *Il6* and *Il1*β content was increased in the brains from LPS-treated wild-type, but not *Tlr2/4* DKO, animals. **≤0.01 (Student *t* test).

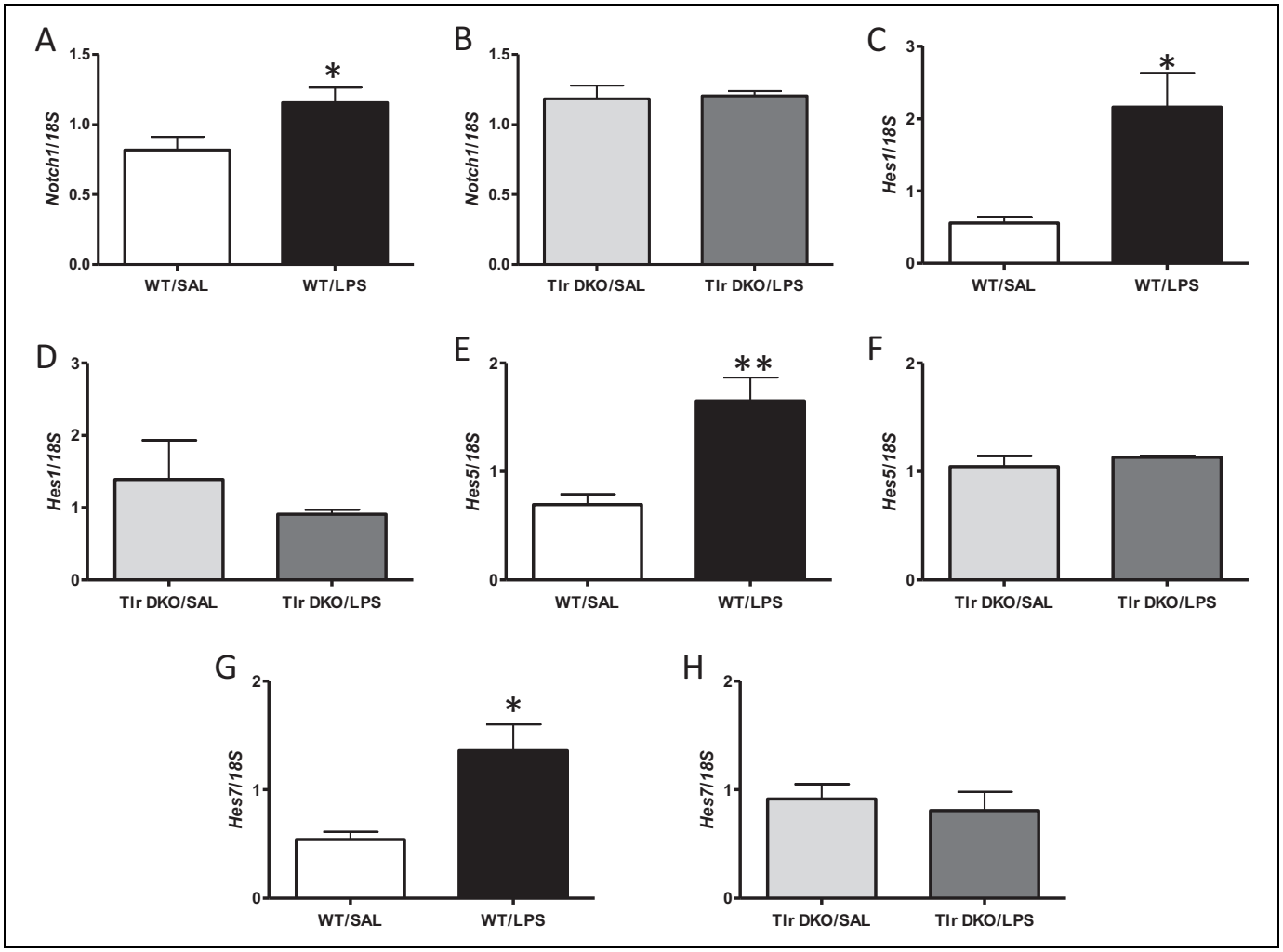

Figure 4. Expression of NOTCH pathway components in fetal brains from wild-type and Toll-like receptor (*Tlr*)2/*4* double knock out (DKO) animals after intrauterine exposure to lipopolysaccharide (LPS). *Notch1* (A and B), *Hes1* (C andD), *Hes5* (E and F), and *Hes7* (G and H) levels were quantified in fetal brains from wild-type (A, C, E, and G) and *Tlr2/4* DKO (B, D, F, and H) animals after in utero treatment with LPS or saline (SAL) using quantitative polymerase chain reaction (qPCR). The levels of these genes were increased in response to exposure to LPS in wild-type, but not *Tlr2/4* DKO, brains. *<0.05, **<0.01 (Student *t* test).

TLR4 receptors,[22] and therefore, we sought to determine whether inactivating MYD88-dependent signaling would be sufficient to prevent a fetal inflammatory response. While wild-type dams exposed to in utero LPS demonstrated increases in IL6 and CCL5 content in the placenta (Figures 5A and C, respectively) and amniotic fluid (Figures 6A and C, respectively) and showed enhanced levels of *Il6* and *Il1*β mRNA in fetal brains (Figures 7A and C, respectively), *Myd88* KO mice had no significant change in *Il6* and *Il1*β levels in all tissues examined (Figures 5B, 6B, 7B, and 7D). In contrast, TRIF-dependent CCL5 expression[25] was still elevated in this line in the placenta and amniotic fluid (Figures 5D and 6D). Thus, as expected, the inactivation of the MYD88-dependent downstream signaling cascade prevents an induction of cytokine production regulated by the MYD88, but not TRIF, pathway.

Inactivation of MYD88-Dependent Signal Transduction is Sufficient to Prevent an LPS-Induced Increase in NOTCH Pathway Activity in the Fetal Brain

To determine whether the activation of the NOTCH signaling pathway in the fetal brain following in utero exposure to LPS is mediated by the MYD88- or TRIF-mediated downstream signaling cascades, we compared NOTCH signaling levels in LPS- and saline-exposed *Myd88* KO mice. As shown in Figure 8, while LPS administration significantly increased the levels of *Hes1* and *Hes5* genes in wild-type fetuses (Figures 8C and E, respectively), the same treatment did not alter the expression of these genes in the *Myd88* KO animals (Figures 8D and F, respectively). The levels of *Notch1* and *Hes7* transcripts in LPS-treated wild-type brains were not different from those in the saline control (Figures 8A and G, respectively).

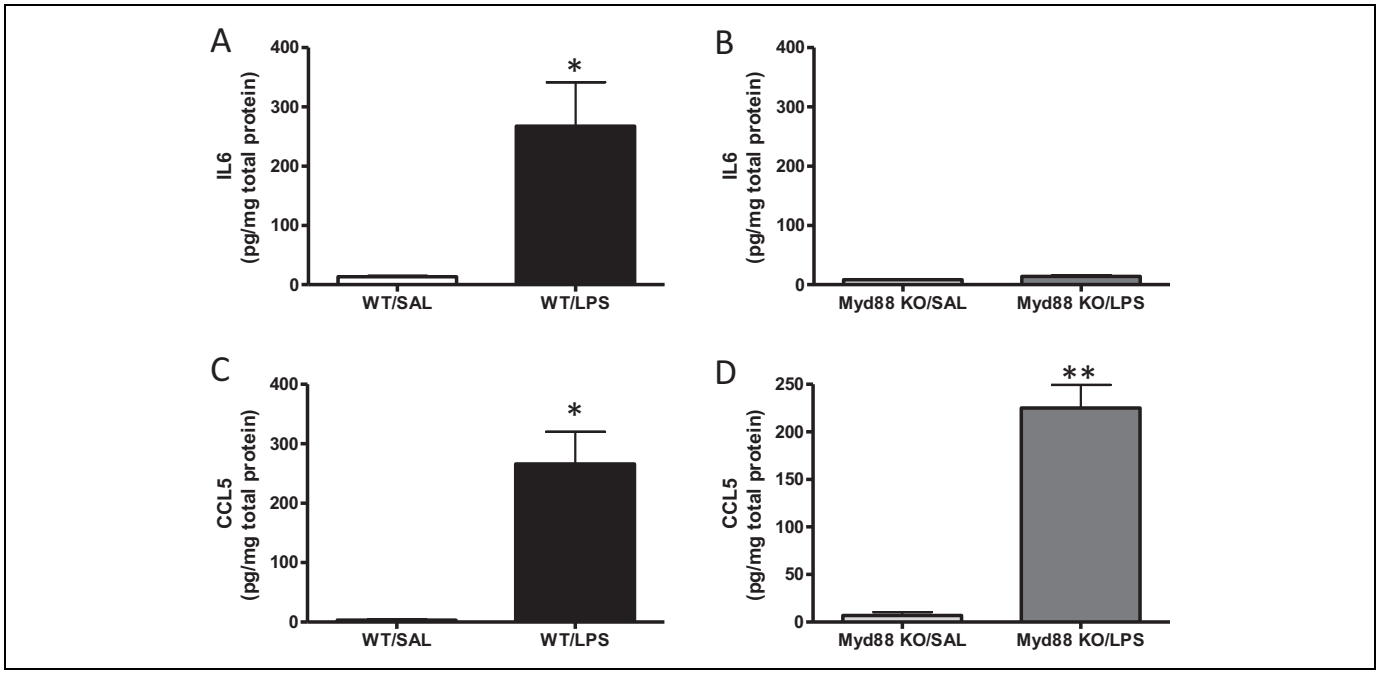

Figure 5. An increase in CCL5, but not interleukin (IL) 6, protein level is observed in the placentas from both wild-type and Myd88 KO animals following intrauterine exposure to lipopolysaccharide (LPS). The IL6 (A and B) and CCL5 (C and D) content was measured in the placentas from wild-type (A and C) and *Myd88* KO (B and D) animals after exposure to LPS or saline (SAL) using enzyme-linked immunosorbent assay (ELISA). The level of IL6 was increased in the placentas from LPS-treated wild-type, but not *Myd88* KO, animals. The CCL5 was elevated in both wild-type and *Myd88* KO Mice. *<0.05, **<0.01 (Student *t* test).

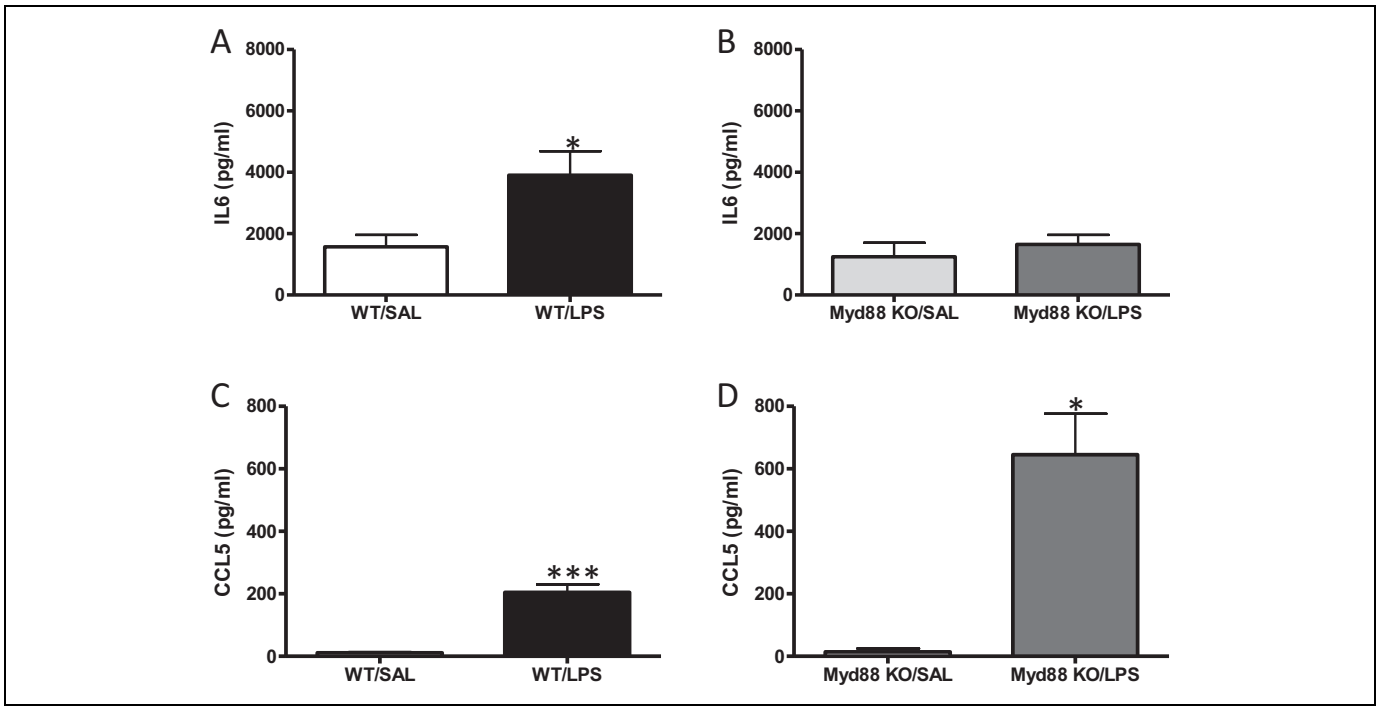

Figure 6. An increase in CCL5, but not interleukin (IL) 6, protein level is observed in the amniotic fluid from both wild-type and Myd88 KO animals following intrauterine exposure to lipopolysaccharide (LPS). The IL6 (A and B) and CCL5 (C and D) content was measured in amniotic fluid samples from wild-Type (A and C) and *Myd88* KO (B and D) animals after exposure to LPS or Saline (SAL) using enzyme-linked immunosorbent assay (ELISA). The IL6 was increased in the samples from LPS-treated wild-type, but not *Myd88* KO, animals. The CCL5 level was elevated in both wild-type and *Myd88* KO animals. *<0.05, ***<0.001 (Student *t* test).

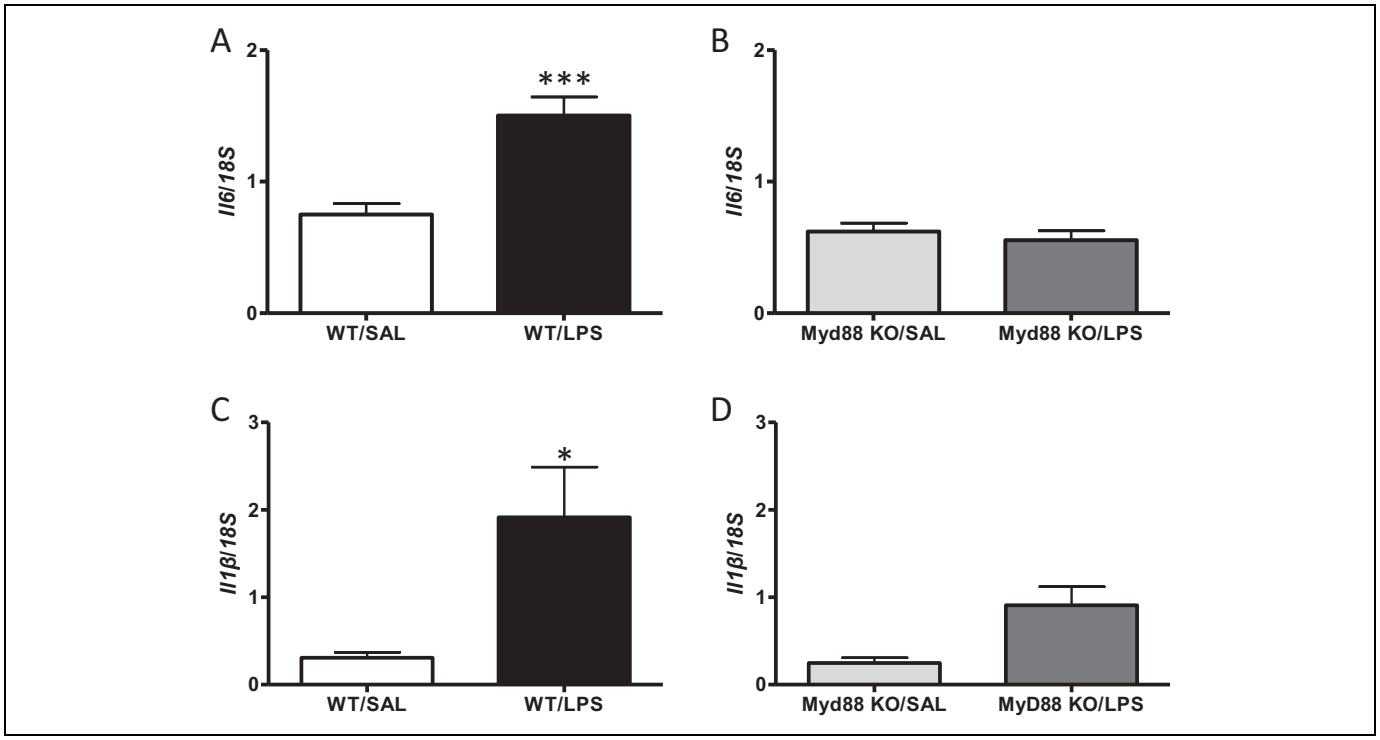

Figure 7. Interleukin (Il) 6 and *Il1*β expression is unaltered in fetal brains from *Myd88* KO animals after intrauterine exposure to lipopolysaccharide (LPS). The *Il6* (A and B) and *Il1*β (C and D) levels were measured in fetal brains from wild type (A and C) and *Myd88* KO (B and D) animals after exposure to LPS or saline (SAL) using quantitative polymerase chain reaction (qPCR). The *Il6* and *Il1*β expression was increased in the brains from LPS-treated wild type, but not *Myd88* KO, animals. *<0.05, ***<0.001 (Student *t* test).

The expression of these genes also did not differ between LPS and saline-exposed brains in *Myd88* KO animals (Figures 8B and H, respectively).

Discussion

Intrauterine infection and inflammation during pregnancy represents a common risk factor for fetal and neonatal brain injury and shows a strong epidemiological association with adverse neurobehavioral outcomes in exposed children. However, the cellular and molecular mechanisms mediating the effect of prenatal inflammation on brain development and function remain poorly understood. An increase in cytokine production in both maternal and fetal tissues following exposure to bacterial infection has been linked to neurological disorders in the offspring and was shown to have a deleterious effect on the developing brain.[6,29,48,49] The data presented in this study demonstrate that the inactivation of the TLR4 signaling pathway in pregnant dams eliminates both the cytokine response in the fetus and inflammation-induced brain injury associated with an increased expression of the NOTCH signaling pathway members *Hes1* and *Hes5*.

Perinatal brain injury linked to prenatal inflammation has been characterized by various molecular and cellular abnormalities detected in the brains of the affected offspring, including white matter damage, neuronal injury and an increased cytokine production in the brain.[3] These changes can be readily assessed using expression analysis of well-described molecular markers.[6,10,29] In the current study, we observed a robust cytokine response in the fetal brain after in utero exposure to LPS, and to further assess the complexity of brain injury, we applied additional molecular markers, *Hes* genes. The expression of *Hes* genes is known to be altered in the presence of brain injury,[34,35,36,37] including that caused by prenatal inflammation.[38,50]

The NOTCH signaling pathway plays a critical role in the formation of the central nervous system and is involved in the regulation of multiple steps during normal brain development, including the specification of neural precursors from neuroectoderm, brain boundary formation, the maintenance of neural progenitor cells, preventing premature neuronal differentiation, establishing a bipotential glial state, the specification of glial cell lineages and neurite branching and outgrowth.[33,51,52] Our data, showing an increase in NOTCH pathway activity in response to in utero inflammation manifested by an elevated expression of known transcriptional effectors of NOTCH, *Hes1* and *Hes5* genes,[53,54] suggest that the aforementioned developmental events may be dysregulated in fetal brains exposed to intrauterine inflammation. Interestingly, certain cellular abnormalities caused by alterations in NOTCH signaling resemble some of the classic phenotypes of perinatal brain injury associated with in utero inflammation, such as an insufficient production of oligodendrocytes leading to the hypomyelination of neuronal fibers[8,13,55] as well as abnormal

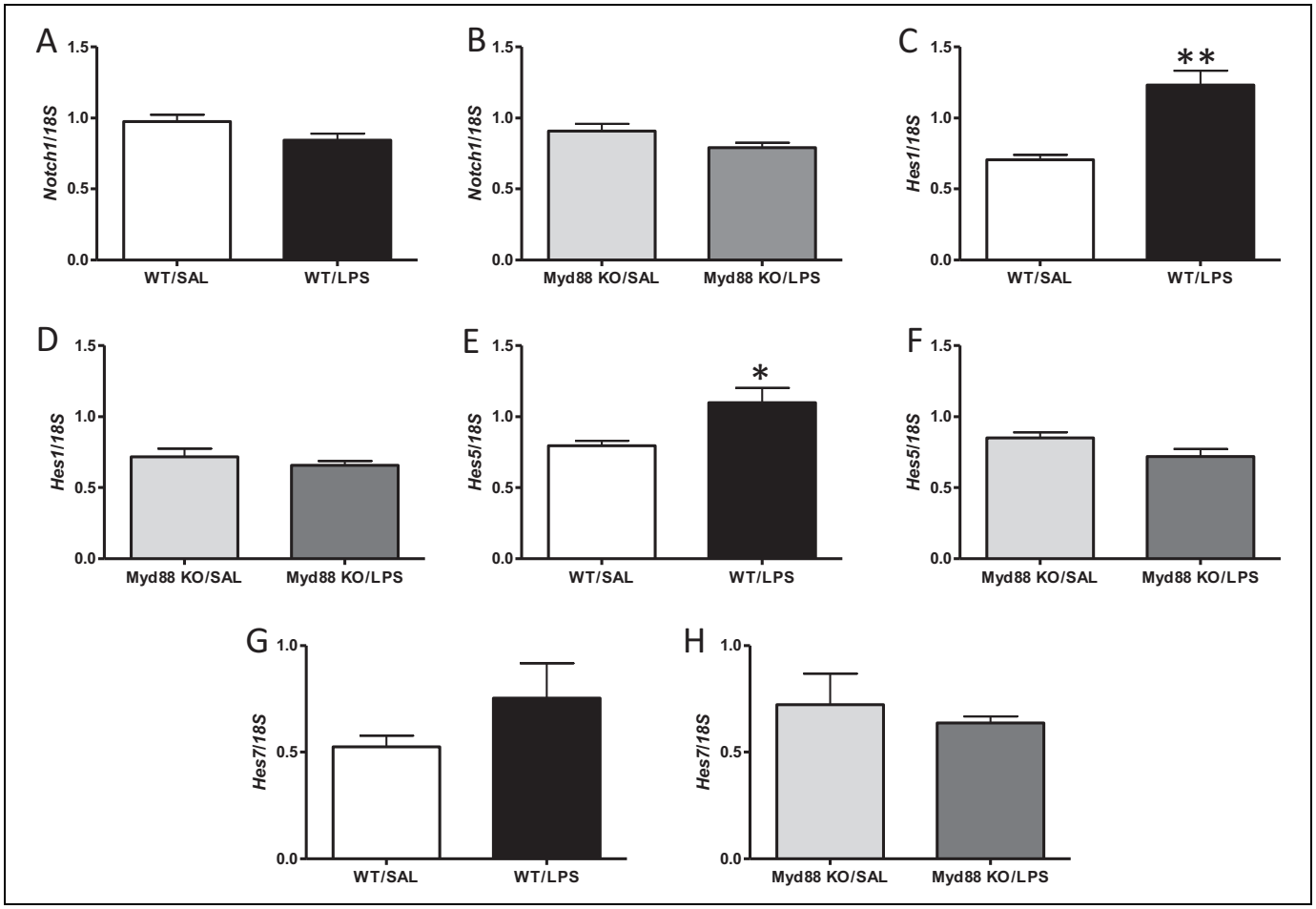

Figure 8. Expression of NOTCH pathway components in fetal brains from wild-type and *Myd88* KO animals after intrauterine exposure to lipopolysaccharide (LPS). *Notch1* (A and B), *Hes1* (C and D), *Hes5* (E and F) and *Hes7* (G and H) levels were quantified in fetal brains from wild-type (A, C, E, and G) and *Myd88* KO (B, D, F, and H) animals after in utero treatment with LPS or saline (SAL) using quantitative polymerase chain reaction (qPCR). The *Hes1* and *Hes5* expression was increased in wild type (C and E), but not *Myd88* KO (D and F, respectively), brains in response to LPS Treatment. The *Notch1* and *Hes7* levels remained unaltered in LPS- and saline-exposed brains in both wild-type (A and G) and *Myd88* KO (B and H) animals. *<0.05, **<0.01 (Student *t* test).

dendritic arborization.[10,14] Thus, our data begin to ascribe a new mechanism by which altered activity of the NOTCH signaling pathway may lead to fetal brain injury from prenatal inflammation.

While we consistently observed an induction in *Hes1* and *Hes5* expression in the brains from LPS exposed wild-type dams, the levels of 2 other NOTCH pathway members, *Hes7* and *Notch1*, were not consistently increased in response to inflammatory stimulation. In particular, LPS treatment elevated the levels of these genes in wild-type fetal brains from the *TLR2/4* DKO experiment but *Hes7* and *Notch1* expression was unaltered in LPS-exposed fetuses in the *Myd88* KO experiment. This inconsistency could be attributed to the required use of antibiotic containing water for *Myd88* KO dams and their respective controls due to increased sensitivity of the *Myd88* KO animals to bacterial infections.[44] In contrast, *Tlr2/4* DKO dams and their wild-type counterparts do not require regular antibiotic administration for fecundity and viability. It is, therefore, possible that the exposure to antibiotic treatment has altered the composition of microbial communities in the offspring from the *Myd88* KO experiment leading to alterations in brain function as described previously.[56] These changes can affect gene expression in the developing brain interfering with the effect of in utero inflammation on *Hes7* and *Notch1* expression in wild-type fetuses exposed to LPS.

The activity of the NOTCH signaling pathway is known to be modulated by different cytokines that are normally expressed in the fetal brain.[31] Thus, it is plausible that an increase in cytokine production in the brains of fetuses exposed to intrauterine infection would alter the function of the NOTCH signaling pathway, thereby causing specific disruptions during brain development. By inactivating the function of the TLR4 signaling pathway, we completely abrogated the cytokine response in LPS-exposed *Tlr2/4* DKO fetuses and found no changes in NOTCH pathway activity in the brains of these animals. These findings demonstrate that preventing a maternal and feto–placental immune response can avert fetal brain injury. Furthermore, the inactivation of the *Myd88* gene whose

function is involved in the downstream TLR signal transduction also eliminates an LPS-induced elevation in NOTCH pathway activity. These data strongly suggest that an increase in NOTCH signaling in the injured brain is mediated by the downstream TLR4/MYD88-, but not TLR4/TRIF-, dependent signaling pathway. It is possible that the action of MYD88-dependent cytokines, such as IL6, may positively regulate the expression of NOTCH pathway members following exposure to an intrauterine inflammatory challenge, as these molecular players have been shown to interact during normal brain development.[31] While we observed no changes in *Hes1* and *Hes5* expression in the *Myd88* KO fetuses, we cannot rule out the possibility that these pups might have other manifestations of brain injury resulting from a TRIF-dependent immune response that was not detected by the outcomes that we measured or the time period in which the injury was assessed.

The involvement of the TLR4 signaling pathway in inflammation-induced brain injury has been studied in some detail using animal models of neonatal brain injury.[57,58] However, the role of these pathways in brain injury during prenatal period has not been thoroughly assessed. An earlier study conducted by our research group utilized the same mouse model of intrauterine inflammation as that applied in this work to show that the TLR4 signaling pathway is involved in initiation, but not progression, of neuronal injury in the fetal brain.[9] Our present findings expand these data by demonstrating that the TLR4/MYD88 signaling pathway is necessary for the induction of a cytokine response and *Hes1* and *Hes5* expression in the fetal brain in response to the prenatal inflammatory stimulation.

Compared to systemic inflammatory stimulation, the use of a localized model of prenatal inflammation better recapitulates what occurs clinically in humans[3,5]; however, this model does have some limitations. Specifically, white matter development in mice is completed postnatally compared to late in gestation in the human.[59] Several other limitations of the study include the assessment of fetal brain injury at a single time point during gestation, the narrow scope of examined cytokines which were selected based on previous studies,[12-14] and the lack of a histological examination of the fetal brain. While we did not determine whether an in utero exposure to LPS leads to cellular abnormalities characteristic for fetal brain injury, we assessed the extent of brain injury by using specific molecular markers (*Hes* genes). A similar approach which relies on the quantification of the markers of white matter and neuronal injury has been successfully implemented before.[14] In addition, this study was not able to assess whether the effect of intrauterine inflammation was modified by fetal sex. Considering recent data on sex differences in response to inflammation,[60,61] this should be an area of future research.

In conclusion, our data demonstrate that in the absence of the TLR4 signaling pathway, both the fetal inflammatory response and brain injury measured based on expression of the NOTCH signaling pathway members, *Hes1* and *Hes5*, are completely abrogated. In addition, the lack of MYD88 function mediating one of the major intracellular signaling cascades downstream from activated TLR4 receptors is also sufficient to suppress NOTCH-associated brain injury caused by exposure to intrauterine inflammation. A better understanding of the molecular mechanisms by which the activated TLR4/MYD88 pathway leads to alterations in brain development may pave a path forward for the prevention of inflammation-induced brain injury and the resulting neurological sequelae.

Authors' Note

Natalia M. Tulina, PhD, and Amy G. Brown, PhD, contributed equally to this work.

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Declaration of Conflicting Interests

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