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Lipopolysaccharide Sensitizes Neonatal Hypoxic-Ischemic Brain Injury in a MyD88-Dependent Manner

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Neurological deficits in children, including cerebral palsy, are associated with prior infection during the perinatal period. Experimentally, we have shown that pre-exposure to the Gram-negative component LPS potentiates hypoxic-ischemic (HI) brain injury in newborn animals. LPS effects are mediated by binding to TLR4, which requires recruitment of the MyD88 adaptor protein or Toll/IL-1R domain-containing adapter inducing IFN-β for signal transduction. In this study, we investigated the role of MyD88 in neonatal brain injury. MyD88 knockout (MyD88 KO) and wild-type mice were subjected to left carotid artery ligation and 10% O₂ for 50 min on postnatal day 9. LPS or saline were administered i.p. at 14 h before HI. At 5 days after HI in wild-type mice, LPS in combination with HI caused a significant increase in gray and white matter tissue loss compared with the saline-HI group. By contrast, in the MyD88 KO mice there was no potentiation of brain injury with LPS-HI. MyD88 KO mice exhibited reduced NFκB activation and proinflammatory cytokine-chemokine expression in response to LPS. The number of microglia and caspase-3 activation was increased in the brain of MyD88 KO mice after LPS exposure. Collectively, these findings indicate that MyD88 plays an essential role in LPS-sensitized HI neonatal brain injury, which involves both inflammatory and caspase-dependent pathways. The Journal of Immunology, 2009, 183: 7471–7477.

Inflammation has emerged as a key contributing factor in developmental brain injury, particularly in infants born prematurely (1). This concept is supported by experimental data showing specific damage to the cerebral white matter in LPS-exposed fetuses (2, 3), in a pattern similar to that seen in preterm human infants. Both clinical and experimental evidence also suggests that although systemic infection/inflammation alone may not always induce observable CNS lesions, it can affect cerebral vulnerability to a later insult (sensitization or preconditioning) such as hypoxia-ischemia (HI) (4), both acutely and more long term (5–8).

The effects of LPS are mediated through its interaction with a member of the TLR family, TLR4 (9), and the downstream adaptor protein or Toll/IL-1R domain-containing adapter inducing IFN-β (10). TLR4 signaling is important for LPS-sensitized HI brain injury (11); however, the downstream adaptors responsible for TLR4-mediated LPS-sensitized HI brain injury are unknown. We hypothesized that LPS-sensitized brain damage is dependent on activation of the MyD88 pathway. Thus, in this study, we investigated neuropathology, inflammatory responses, and mechanisms of cell death following LPS-sensitized HI brain damage in neonatal mice deficient for the MyD88 gene. We induced LPS-sensitized HI in postnatal day 8/9 mice (neurodevelopment approximately equivalent to term human infant) to mimic a clinical situation of inflammation-induced vulnerability to hypoxic-ischemic brain injury in the newborn.

Materials and Methods

Animals

C57BL/6 mice lacking the gene for MyD88 (MyD88 KO) and C57BL/6 wild-type (WT) mice were bred at Experimental Biomedicine (University of Gothenburg, Göteborg, Sweden) and were crossed to produce heterozygotes (HET), which were bred further to produce littermate animals with mixed genotypes including MyD88 homozygous, HET mutant mice, and WT mice. Mice were maintained under specific pathogen-free conditions and housed with a 12-h light-dark cycle. Free access to a standard laboratory chow diet (B&K) and drinking water was provided. All animal experimentation was approved by the Ethical Committee of Göteborg (No. 314-2005).

Genotyping

The genotype of mice was determined by PCR of genomic DNA obtained from mouse tails, as previously described (12, 13). The WT allele was detected using the forward primer (5′-TGGCATGCTCCTCATCATGTAACC-3′) and the reverse primer (5′-GTCAGAAACACCCACCATGTCG-3′), and the mutant allele detected using the forward primer (5′-ATCGCTTTCAGAGCTGAGATGAATGTC-3′) and the reverse primer (5′-ATCGCTTTCATCGCTTTCCTGAGC-3′). WT mice were identified by a single 550-bp band, MyD88 KO mice by the presence of a single 650-bp DNA band, and HET mice were identified by the presence of both bands.
Induction of LPS-sensitized HI in neonatal mice and drug administration

LPS (0.3 mg/kg; LPS 055:B5; Sigma-Aldrich) was administered i.p. in the evening to postnatal day (PND) 8 MyD88 KO and WT mice of both genders. The next morning, 14 h after the LPS injection, mice were anesthetized with halothane (3.0% for induction and 1.5–1.5% for maintenance) in a mixture of nitrous oxide and oxygen (1:1). The left common carotid artery was ligated with prolene sutures. Mice were returned to the cage, allowed to recover for 1 h, and then placed in an incubator perfused with a humidified gas mixture (10.0 ± 0.1% oxygen in nitrogen) at 36°C for 50 min (14–16). After this period of hypoxia, the pups were returned to their dam until sacrifice at 5 days after HI. Control group animals received no surgery, given that we previously demonstrated no difference compared with animals receiving surgery (5 min of anesthesia, cervical incision, and suturing) with sham-ligation with respect to energy and glycolytic metabolites in the brain, acid base/body gas status (unpublished data), expression of immediate early genes (17), or apoptosis (18).

Immunohistochemistry and immunofluorescence staining

Mice were injected with LPS (0.3 mg/kg) at PND8, and brain tissue was collected at 2, 6, and 14 h after LPS exposure for assessment of numbers of microglia or 5 days post-LPS/HI for evaluation of brain injury. Animals were deeply anesthetized and perfused intracardially with saline and 5% buffered formaldehyde (Histofix; Histolab). The brains were rapidly removed and immersion fixed in 5% formaldehyde for 24 h. After dehydration, the brains were embedded in paraffin and cut into 10-μm frontal sections for immunohistochemical staining. Ag recovery was performed by heating the sections in 10 mM boiling sodium citrate buffer (pH 6.0) for 10 min. Nonpecific binding was blocked for 30 min with 4% horse or goat serum (depending on the species used to raise the secondary Ab) in PBS. Sections were incubated in primary Abs against cytochrome c (Cyt C; 1/500; BD Pharmingen) or cytochrome c oxidase (Cox; 1/100; Chemicon International) or ionized calcium-binding adapter molecule 1 (Iba-1; 1/2,000, Wako Chemicals USA) for 60 min at 4°C overnight, followed by the appropriate biotinylated secondary Abs (Vector Laboratories) for 60 min at room temperature. Visualization was performed using Vectorsa Elite with 0.5 mg/ml 3,3′-diaminobenzidine enhanced with 15 mg/ml ammonium nickel sulfate, 2 mg/ml β-glucose, 0.4 mg/ml ammonium chloride, and 0.01 mg/ml β-glucose oxidase (all from Sigma-Aldrich). For immunofluorescence staining, sections were incubated in the primary Abs against antiactive forms of caspase-3 (1/500; BD Pharmingen) with mouse anti-NeuN (1/200; Sternberger Monoclonal) or ionized calcium-binding adapter molecule 1 (Iba-1; 1/2,000, Wako Chemicals USA) for 60 min at 4°C overnight, followed by the appropriate peroxidase-labeled secondary Ab (Vector Laboratories) for 30 min at room temperature. Immunoreactive bands were visualized using the Super Signal Western Dura substrate (Pierce Chemical) and a LAS 1000-cooled charge-coupled device camera (Fujifilm). Immunoreactive bands were quantified using the Image Gauge software (Fujifilm).

Cytokine assay

Cytokine-chemokine expression in brain homogenate supernatants (see: Sample preparation for activity assay, ELISA, and immunoblotting") from WT and MyD88 KO mice were performed using a Linco Research Mouse 22-Plex kit (No. MCYTO-70K-PMX22; Millipore) containing premixed beads capable of detecting a variety of cytokines, according to the manufacturer’s instructions. The microbead array allowed for the simultaneous detection of multiple inflammatory molecules in a single 75-μl brain homogenate or culture medium sample, as previously described (23). Results were analyzed on a Bio-Plex workstaton (Bio-Rad) and normalized to the amount of protein per well. The level of sensitivity for each microbead cytokine standard curve ranged from 1 to 35 pg/ml (23). Protein concentrations were determined using the Bio-Rad DC Protein Assay (Bio-Rad).

Statistics

Data were analyzed by two-way ANOVA with genotype (WT or MyD88 KO) and treatment (vehicle or LPS) as factors or genotype (WT or MyD88 KO) and time after LPS exposure (0, 2, 6, and 14 h) as factors. When overall changes were detected by ANOVA, the Bonferroni post hoc test was used at the 95% confidence interval. Significance was set at p < 0.05. Data are presented as means ± SEM. All statistical analyses were performed using GraphPad Prism 4.0 (GraphPad Software).

Results

MyD88 deficiency blocks LPS-sensitized HI brain injury

Neuropathological analysis at 5 days after HI demonstrated an increase in total tissue loss in LPS-pretreated WT mice (t(22) = 4.8, p < 0.001; Fig. 1, A and C). In contrast, LPS did not increase damage in HI MyD88 KO mice (t (3.4) = 0.77, p > 0.05; Fig. 1, A and C). Two-way ANOVA revealed a significant interaction (F (1, 43) = 8.5, p < 0.006) interaction between genotype and treatment, suggesting the influence of genotype on LPS-induced tissue loss. Similarly, evaluation of regional neuropathology showed that brain injury was significantly increased in LPS-pretreated WT mice by
112% in the cortex, 76% in the thalamus, 62% in the striatum, and 51% in the hippocampus (Fig. 1B).

Two-way ANOVA revealed a significant interaction between genotype and treatment for each brain region (interaction for respective region: cortex, F(1, 43) = 11.95, p < 0.0012; thalamus, F(1, 43) = 26.29, p < 0.0001; striatum, F(1, 43) = 26.57, p < 0.0001; hippocampus, F(1, 43) = 16.10, p < 0.0002; Fig. 1B).

There was a significant increase in loss of subcortical white matter area positive for MBP at 5 days after HI in LPS-pretreated WT mice (74.50 ± 2.60%, n = 10) compared with saline-pretreated WT mice (31.00 ± 8.22%, n = 11, t(13.6) = 2.84, p < 0.05; Fig. 2). In contrast, in MyD88 KO animals there were no significant differences between the two groups (LPS/HI, 27.27 ± 6.58%, n = 11 vs saline/HI, 22.50 ± 6.06%, n = 12; t(5.14) = 1.09, p > 0.05; Fig. 2).

There was a significant interaction of genotype and treatment (F(1, 43) = 7.81, p = 0.0077), suggesting that as for gray matter damage, genotype contributed to increased LPS-induced white matter injury.

These results are in agreement with our previous findings that LPS pre-exposure sensitizes the brain to HI injury in neonatal rats (6). We now demonstrate for the first time a LPS-induced sensitizing effect in neonatal mice and that this increased vulnerability to damage is regulated in an MyD88-dependent manner.

Increased number of microglia in MyD88-deficient mice

Numbers of microglia were assessed by Iba-1 immunostaining on brain sections collected at 2, 6, and 14 h after LPS exposure (without HI) and in naive age-matched control mice. At 2 h (t(99,000) = 3.11, p < 0.05) and 6 h (t(136,400) = 3.91, p < 0.01) after LPS injection, the number of Iba-1-positive cells was significantly increased in MyD88 KO animals compared with WT mice (Bonferroni post hoc test; Fig. 3). Two-way ANOVA revealed that both genotype (F(1, 35) = 24.74, p < 0.0001) and time after LPS exposure (F(3, 35) = 17.65, p < 0.0001) contributed to the number of Iba-1-positive cells. There was, however, no interaction between genotype and time (F(3, 35) = 1.84, p = 0.1577), indicating that LPS induces microglia activation in both genotypes, but with a stronger response in MyD88 KO mice.

FIGURE 2. Effect of MyD88 deficiency on white matter injury. A. Loss of MBP immunoreactive staining in the subcortical area of the ipsilateral hemisphere (expressed as percent loss vs contralateral hemisphere) after LPS plus HI at PND14. Changes between saline- and LPS-treated WT mice, *, p < 0.05. Two-way ANOVA with Bonferroni post hoc test. Data are means ± SEM. B, Representative pictures of MBP immunohistochemistry staining in WT and KO mice after LPS plus HI and in normal control WT mice (Cont) at low (top) and high (bottom) magnification. Insets represent the area that is depicted at higher magnification. Bar, 50 μm.
MyD88 deficiency attenuates cytokine-chemokine production in neonatal brain

To examine the role of MyD88 in mediating inflammatory mediator release in the brain in response to systemic LPS exposure, we used a multiplex ELISA of multiple inflammatory molecules to determine their expression in brain homogenate. The inflammatory molecules analyzed in the array were broadly divided into proinflammatory (Fig. 4), anti-inflammatory (Fig. 5) cytokines, and chemokines-chemotaxic molecules (Fig. 6). The expression of IL-1β, IL-2, IL-4, IL-12p70, GM-CSF, IFN-γ, and RANTES were below the detection limits of the array at all time points in all groups (data not shown).

Two-way ANOVA revealed that changes in most proinflammatory cytokines (IL-1β, IL-5, IL-6, IL-7, and TNF-α) were statistically significant with respect to interaction between genotype and time (Table I). The LPS-induced increases in these cytokines were largely inhibited in MyD88 KO mice (Fig. 4). In contrast, there was no significant interaction (Table I) for the proinflammatory cytokines IL-15 and IL-17 and the anti-inflammatory cytokines (IL-9, IL-10, and IL-13) and no genotype-dependent differences in response to LPS (Figs. 4 and 5). Chemokines-chemotaxic molecules demonstrated an expression pattern similar to that of proinflammatory cytokines with a significant interaction with genotype and time (Table I). Post hoc analysis showed a time- and genotype-dependent inhibition in expression of chemokines-chemotaxic molecules in MyD88 KO mice (Fig. 6).

Effect of MyD88 deficiency on NF-κB activation

NF-κB activation plays a key role in cytokine production (24). To examine the activation of NF-κB in response to LPS exposure in WT and MyD88 KO neonatal mouse brains, p-IκB was assessed by Western blotting in brain homogenates after LPS. p-IκB is a prerequisite for NF-κB activation (25). As such, increased expression of cytosolic p-IκB can be used as an indirect marker of activation of the NF-κB pathway. Two-way ANOVA showed a significant interaction between genotype and time after LPS (F(3, 38) = 6.1, p = 0.0016). Post hoc analysis revealed that MyD88 KO animals had a higher initial level of p-IκB at 16,000 (t = 2.7, p < 0.05), but then a diminished response to LPS at 6 h (t = 3.3, p < 0.01; Fig. 7).

LPS-induced caspase-3 activation is increased in MyD88-deficient mice

Apoptosis is particularly important in the developing brain, and caspase-3 is markedly induced after neonatal brain injury (22, 26–28). To further investigate the mechanisms of LPS-induced sensitization to HI, apoptotic changes after LPS pre-exposure were detected by measuring brain caspase-3 activity. Two-way ANOVA showed a significant interaction (F(3, 32) = 4.0, p = 0.0154) of genotype and time on caspase-3 activity. Post hoc analysis revealed an increased caspase-3 activity in LPS-treated MyD88 KO animals compared with LPS-treated WT animals at 2 h after LPS (t = 4.4, p < 0.001, Fig. 8).

Because Cyt C release from the mitochondria to the cytosol initiates an apoptotic protease cascade (29), we also performed fluorescent double-labeling of caspase-3 and Cyt C and found that
caspase-3 coexpressed with Cyt C in the cytosol (Fig. 8B). Further, activated caspase-3 expression was observed in microglia and neurons in both WT and KO mice (Fig. 8B).

Discussion
In this study, we demonstrate that MyD88, a central adaptor in TLR4 signaling, plays an essential role in LPS-sensitized HI brain injury. The cerebral proinflammatory cytokine-chemokine response to LPS was virtually blocked in neonatal MyD88 KO mice, but there was a paradoxical increase in number of microglia. MyD88 KO mice also displayed an early increased activation of caspase-3 in the brain in response to LPS exposure, to levels greater than in LPS-exposed WT mice. These results support our previous findings identifying LPS as an important factor in altering the vulnerability to HI injury in the neonate (5–8) and provide novel evidence that the sensitizing effect of LPS is mediated via MyD88. This is the first report identifying a role for MyD88 regulation in LPS-induced HI brain injury.

Nonbacterial stimuli, such as heat shock protein 60, have been shown to kill cultured neurons via TLR4- and MyD88-dependent mechanisms in microglia (30), whereas both TLR2 and TLR4 deficiency provides neuroprotection following ischemia in adult animals (31). In this study, there was no difference in tissue loss between saline pre-exposed WT and MyD88 KO mice following HI (Fig. 1). These data suggest that MyD88 may not be critical for brain damage following sterile-induced inflammation in the immature brain (32) and imply a potential maturational-dependent role of innate immunity in brain injury.

As well as a decrease in total tissue loss, MyD88 KO mice also exhibited a reduction in white matter damage, as assessed by the area of MBP-positive immunostaining. These data are in agreement with a recent finding that MyD88 KO mice displayed a reduction in CNS inflammation (decreased leukocyte infiltration) and axonal and myelin damage in an animal model of multiple sclerosis (33). This is of particular importance for the immature brain due to the high occurrence of white matter injury during early development.

We have previously shown that peripheral LPS exposure regulates the expression of a large number of genes in the neonatal brain (34),

Table I. Two-way ANOVA of cytokine/chemokine protein expression in the brain at different time points after LPS exposure in neonatal WT and MyD88 KO mice

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>p (time)</th>
<th>p (genotype)</th>
<th>p (interaction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1α</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>IL-5</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>IL-6</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>IL-7</td>
<td>0.0025</td>
<td>0.7675</td>
<td>0.0327</td>
</tr>
<tr>
<td>IL-9</td>
<td>&lt;0.0001</td>
<td>0.0048</td>
<td>0.6480</td>
</tr>
<tr>
<td>IL-10</td>
<td>&lt;0.0001</td>
<td>0.0025</td>
<td>0.9382</td>
</tr>
<tr>
<td>IL-13</td>
<td>&lt;0.0001</td>
<td>0.0533</td>
<td>0.8639</td>
</tr>
<tr>
<td>IL-15</td>
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<td>0.9614</td>
<td>0.0550</td>
</tr>
<tr>
<td>IL-17</td>
<td>&lt;0.0001</td>
<td>0.0052</td>
<td>0.7608</td>
</tr>
<tr>
<td>TNF-α</td>
<td>&lt;0.0001</td>
<td>0.0084</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>IP-10</td>
<td>&lt;0.0001</td>
<td>0.0115</td>
<td>0.0003</td>
</tr>
<tr>
<td>G-CSF</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
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<tr>
<td>KC</td>
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<tr>
<td>MCP-1</td>
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<td>&lt;0.0001</td>
</tr>
<tr>
<td>MIP-1α</td>
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<td>0.0511</td>
<td>0.0309</td>
</tr>
</tbody>
</table>
including a significant number associated with inflammatory processes, such as cytokines and chemokines. In this study, exposure to systemic LPS increased mainly proinflammatory cytokine-chemokine protein levels in the brain, whereas there were no significant changes in anti-inflammatory cytokines. The proinflammatory response to LPS was largely abolished in MyD88 KO mice, which is supported by previous in vitro studies (23, 35, 36).

Proinflammatory cytokines cause damage if injected directly into the brain (37) and have the capacity to exacerbate brain damage (38), whereas gene deletion-blocking proinflammatory cytokine production reduces brain damage (20, 39, 40). Although the majority of proinflammatory cytokines-chemokines were significantly elevated in the brain of WT mice early after LPS, these proinflammatory responses were largely resolved at 14 h, the time point where HI was induced. Therefore, it is unclear to what extent proinflammatory cytokines contributed to the increased LPS-induced brain vulnerability in the present study. Others have found that inhibition of NF-κB activity attenuates HI brain injury in neonatal mice, independent of cytokine production (41, 42). In this study, we found an increased basal level of NF-κB activation, as indicated by p-IκB, but diminished NF-κB activation in MyD88 KO mice in response to LPS. Therefore, we cannot exclude the possibility that the attenuated brain injury in MyD88 KO mice was due to the inhibition of NF-κB activation observed at 6 h rather than the cytokine production per se.

The diminished cytokine response in MyD88 KO mice occurred despite an increase in number of microglia. Microglia are the resident mononuclear phagocytes in the CNS and are responsible for initial host immune defenses (43–45). In this study, the number of Iba-1-positive cells with the morphology of activated microglia increased at 6–14 h to a greater extent in MyD88 KO mice compared with WT animals, despite the reduction in LPS-HI injury. These data suggest that cerebral cytokine release is not necessarily associated with morphological signs of microglia activation and that microglial activation is not necessarily damaging. In support of these data, the shape of microglia can vary considerably from the resting phenotype to an active ameboid form, without increased cytokine production (45, 46), whereas microglia have been shown to play both damaging and protective roles in CNS injury (47, 48).

Apoptosis, including caspase-3 activation, is associated with developmental and injury-induced cell death. The elevation in caspase-3 activation in the brain after LPS exposure agrees with our previous finding in neonatal rats (34). However, to the best of our knowledge, a role for MyD88 in regulating caspase-3 activation in response to LPS in the brain has not been reported. In this study, we found an approximate doubling in caspase-3 activation in LPS-exposed MyD88 KO mice, although those changes are minimal compared with the at least 50-fold increase observed in conjunction with brain damage following HI in neonatal mice (22, 49). We speculate that the moderate LPS-induced caspase-3 activation before HI in MyD88 KO mice is insufficient to injure the brain, but may acts as a preconditioning stimulus, which desensitizes the brain to the subsequent HI insult. In support of these data, we previously demonstrated that both hypoxia- and inflammation-induced preconditioning in the immature brain is associated with a significant apoptotic/cell death gene response (50–52), whereas a low degree of caspase-3 activation was shown to exert preconditioning protection in vitro (53).
In summary, we demonstrated a novel MyD88-dependent regulatory mechanism in LPS-sensitized HI immature brain injury, which may provide a novel target for neuroprotective therapies.

Disclosures
The authors have no financial conflict of interest.

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