Developmental Onset of Bilirubin-induced Neurotoxicity Involves Toll-like Receptor 2-dependent Signaling in Humanized UDP-glucuronosyltransferase1 Mice*

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Background: Neonatal jaundice with dangerously high levels of serum bilirubin leads to neurological toxicity. Results: Toll-like receptor 2 signaling is essential for regulation of glia activation, neuroinflammation, and oxidative stress when neonatal mice experience severe hyperbilirubinemia. Conclusion: Toll-like receptor 2 signaling is linked to a protection mode against serum bilirubin-induced brain toxicity. Significance: Understanding how signaling from innate immunity contributes to bilirubin-induced pathology.

Biological and signaling events that connect developmentally induced hyperbilirubinemia to bilirubin-induced neurological dysfunction (BIND) and CNS toxicity in humans are poorly understood. In mammals, UDP-glucuronosyltransferase 1A1 (UGT1A1) is the sole enzyme responsible for bilirubin glucuronidation, a rate-limiting step necessary for bilirubin metabolism and clearance. Humanized mice that express the entire UGT1 locus (hUGT1) and the UGT1A1 gene, develop neonatal hyperbilirubinemia, with 8–10% of hUGT1 mice succumbing to CNS damage, a phenotype that is presented by uncontrollable seizures. We demonstrate that neuroinflammation and reactive gliosis are prominent features of bilirubin brain toxicity, and a disturbed redox status resulting from activation of NADPH oxidase is an important contributing mechanism found in BIND. Using knock-out mice and primary brain cells, we connect a key pattern recognition receptor, Toll-like receptor 2 (TLR2), to hyperbilirubinemia-induced signaling. We illustrate a requirement for TLR2 signaling in regulating gliosis, proinflammatory mediators, and oxidative stress when neonatal mice encounter severe hyperbilirubinemia. TLR2-mediated gliosis strongly correlates with pronounced neuroinflammation in the CNS with up-regulation of TNFα, IL-1β, and IL-6, creating a pro-inflammatory CNS environment. Gene expression and immunohistochemistry staining show that hUGT1/Tlr2−/− mice fail to activate glial cells, proinflammatory cytokines, and stress response genes. In addition, bilirubin-induced apoptosis was significantly enhanced by blocking TLR2 signaling indicating its anti-apoptotic property. Consequently, a higher neonatal death rate (57.1%) in hUGT1/Tlr2−/− mice was observed when compared with hUGT1 mice (8.7%). These results suggest that TLR2 signaling and microglia neuroinflammation are linked to a repair and/or protection mode against BIND.

Newborn hyperbilirubinemia or jaundice is characterized by elevated levels of total serum bilirubin and results from a burst in red blood cell production immediately after birth followed by accelerated erythrocyte turnover with scavenging of heme by macrophages. Following heme catabolism, bilirubin releases into the blood, where it binds to serum proteins and is transported to the liver, followed by metabolism through glucuronidation by UDP-glucuronosyltransferase 1A1 (UGT1A1) (1). Neonatal jaundice is mostly benign, but dangerously high levels of total serum bilirubin can be produced when newborns inherit inactivating gene polymorphisms in the UGT1A1 gene, as seen in Crigler Najjar diseases (2), which lead to encephalopathy and kernicterus (3). Thus, understanding the molecular and cellular events that lead to bilirubin-induced neurological dysfunction (BIND)2 is essential to effectively prevent the onset of kernicterus in newborns.

Recently, we have demonstrated that mice humanized with the UGT1 locus (hUGT1 mice) develop severe neonatal hyperbilirubinemia (4). Knock-out of the Ugt1 locus is a lethal mutation in Ugt1−/− mice resulting in neonatal death from severe levels of unconjugated bilirubin (5). However, expression of the human UGT1 locus in the Ugt1-null background rescues the lethality. All of the neonatal hUGT1 mice display dramatically elevated levels of total serum bilirubin, a consequence that results from developmentally repressed expression of the liver UGT1A1 gene (6). Approximately 8–10% of the hUGT1 offspring develop signs of BIND, and the affected mice have difficulty in walking, can be induced to seizure by a sterile reflex, and develop dystonia shortly before dying (4). Humanized UGT1 mice represent a unique animal model that can be exploited to examine hyperbilirubinemia toxicity that targets the brain.

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2 The abbreviations used are: BIND, bilirubin-induced neurological dysfunction; UGT, UDP-glucuronosyltransferase; TLR, Toll-like receptor; GSH, reduced glutathione; GSSG, oxidized glutathione; TUNEL, terminal deoxyribonucleotidyl transferase-mediated deoxyuridine nick-end labeling; CPH, cyclophillin; HO-1, heme oxygenase-1; NOQ-1, NADPH:quinone oxidoreductase 1; GFAP, glial fibrillary acidic protein; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; HSP, heat shock protein.
Glial Cell Activation in Severe Hyperbilirubinemia

Toll-like receptors (TLRs) are among the most well recognized pattern recognition receptors and have been shown to detect an array of pathogen-associated molecular patterns. Recent evidence suggests that TLRs also have pathogen-independent roles by responding to harmful molecules from host-derived, endogenous ligands. For example, tissue injury causing cell death and organelle degradation has been shown to generate endogenous danger molecules that act as TLR ligands (7–9). Moreover, expression of the isoform-specific TLR has been observed in Alzheimer and multiple sclerosis brains (10). Upon ligand binding, TLRs dimerize and undergo conformational changes that lead to the induction of a complex cascade of intracellular signaling events ultimately resulting in regulation of the expression of a wide array of genes involved in inflammatory responses (11). As a result, TLRs are capable of responding to harmful molecules and signaling an immediate response that links the innate immunity to adaptive immune responses. Overactivation of innate inflammatory processes by TLRs is often associated with detrimental physiological consequences as they have been linked to neurodegenerative conditions in numerous CNS diseases (12). However, novel lines of experimentation indicated that TLR signaling may provide a protective role and have beneficial contributions upon CNS injury (13), (14). Most of the TLRs are expressed in the human cerebral tissue and are concentrated particularly in microglia-rich areas in the CNS (12). The fact that TLRs are regulated during the neonatal developmental stage in the brain tissue (15) suggests that they may play a role in CNS homeostasis at the early development stage.

In this study, the initial discovery of heat shock protein (HSP) 27 induction in brain tissue of BIND mice led us to investigate TLR involvement in bilirubin-mediated toxicity as HSPs have been recognized as damage-associated molecular patterns that are associated with TLR activation (16). Among all the TLR isoforms examined, TLR2 is persistently induced in the kernterus brain. We have tested the hypothesis that TLR2 signaling plays a critical role in maintaining homeostasis of the neural environment in the BIND mice. We have determined that TLR2 is directly responsible for activating glia cells and secreting various neurotrophic factors and cytokines in response to severe hyperbilirubinemia. This study sheds an important light on TLR2-dependent neuroinflammation and glial activation as crucial mechanisms in the defensive response to hyperbilirubinemia toxicity.

EXPERIMENTAL PROCEDURES

Animals—Transgenic mice expressing the human UGT1 locus (17) in a Lgt1-null background (5) were generated as previously described (4). The Tg(UGT1A1*28)Lgt11−/− mice used in these studies express the UGT1A1*28 allele and will be referred to as humanized UGT1 (hUGT1) mice. TLR2-deficient mice (Tlr2−/−) were generously provided by Dr. Ekihiro Seki (University of California San Diego) who purchased them from Jackson Laboratories (Bar Harbor, ME). Tlr2−/− mice were crossed with hUGT1 mice to generate hUGT1/Tlr2−/− mice, and backcrossing was continued for at least 5 generations to obtain the same genetic background between hUGT1 and hUGT1/Tlr2−/− or hUGT1/Tlr2−/− mice. All animals were housed at the University of California San Diego (UCSD) Animal Care Facility, and received food and water ad libitum. All animal use protocols, mouse handling, and experimental procedures were approved by the UCSD Animal Care and Use Committee (IACUC), and these protocols were conducted in accordance with federal regulations.

GSH and GSSG Measurements—Oxidative stress was monitored by detecting levels of reduced glutathione (GSH) and oxidized glutathione (GSSG) with the BIOXYTECH GSH/GSSG-412 kit (OxisResearch, OR) using the thiol-scavenging reagent, 1-methyl-2-vinylpyridinium trifluoromethanesulfonate and Ellman’s reagent (5,5’-dithiobis-2-nitrobenzoic acid, DTNB); the latter reacts with GSH to form a spectrophotometrically detectable product at 412 nm. Whole blood samples were collected from wild type and hUGT1 mice, and the GSH and GSSG levels were measured according to the manufacturer’s instructions.

Blood and Tissue Bilirubin Analysis—Blood was collected by cheek bleeding (submandibular vein) from neonatal mice at 11–13 days after birth, and serum was collected following centrifugation at 1000 × g for 10 min. Serum bilirubin levels were measured using a BNISTAT Bilirubinometer (Reichert Technologies, USA). Tissue extraction and bilirubin quantification for bilirubin measurement in the brain tissue were modified based on the assay published previously (18). In brief, the brain tissue (100 mg) was disintegrated to a fine suspension by grinding and subsequently diluted with extraction solvent: methanol/chloroform/n-hexane (63:31:6, v/v). Following centrifugation, the organic phase containing bilirubin was transferred and measured by a spectrophotometer at 420 nm.

Western Blot Analysis—The extracted cell lysate (30 μg) was loaded on a pre-cast BisTris gel (NuPAGE, Novex), and electrophoresis was performed following determination of protein concentrations. The resolved protein was transferred onto a nitrocellulose membrane (Millipore), and the blocking of the membrane and incubation with the appropriate primary antibody and horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology) were described previously (19). Protein was detected by the Renaissance Western blot chemiluminescence reagent (PerkinElmer Life Sciences) and visualized using the Bio-Rad ChemiDoc imaging system.

TNFα Measurement by ELISA—TNFα levels in serum were measured by using a DuoSet ELISA Development Kit (R&D System, DY410). Briefly, the capture antibody was prepared to coat 96-well microplates and was followed by the incubation of serum or the TNFα standard and detection with streptavidin-HPR and the substrate, which were based on the manufacturer’s instructions.

Isolation of Primary Astrocytes and Microglia Cells from Brain—The isolation of primary glial cells was performed with modification as described previously (20). In brief, 1–3-day-old mice were decapitated and the forebrain was placed in the cold DMEM buffer (Invitrogen). The brain tissue was mechanically pulverized with a Pasteur pipette in DMEM. After centrifugation, the cell suspension in DMEM containing 10% FBS (Cellgro) was plated in a flask coated with poly-l-lysine (Sigma). These flasks were maintained in a humidified atmosphere of 95% air and 5% CO2, at 37 °C for 14 days, and the medium was changed every 3 or 4 days. The flasks were shaken for 3 h at 37 °C using an orbital shaker, and floating cells in the superna-
**RESULTS**

**hUGT1 Mice Exhibited Elevated Serum Bilirubin Levels and a Small Percentage Displayed Characteristics Resembling Clinical Kernicterus**—Blood chemistry analysis revealed that neonatal hUGT1 mice, aged 14 days, displayed elevated bilirubin levels (8.8–22.9 mg/dl) compared with ~0.5 mg/dl for the same age wild type mice. Among these mice, a notable behavioral change occurred in a small percentage of neonates. Initially, they showed awkwardness in gait abnormality and muscle tone. Gradually increasing hypotonia followed by tonic seizures and a startle reflex was then observed. After a few hours, these mice developed hypotonia with consequent paralysis that finalized in a coma and then death. Because this behavioral status resembled characteristics of clinical kernicterus in neonates (21), they are referred to as kernicterus mice. The seizure patterns can be observed in the supplemental data from Fujiwara et al. (4). In eight litters, ~8.7% of hUGT1 mice died from this neurological disorder with higher levels of bilirubin at 14 days of age, ranging from 17.8 to 22.9 mg/dl (mean 20.8 ± 2.3 mg/dl) compared with 8.8 to 17.4 in healthy littermates (mean 12.8 ± 2.1 mg/dl), suggesting that higher bilirubin levels in kernicterus mice account for the neurological condition resulting in sustained locomotion deficits in these mice (Fig. 1A). Bilirubin analysis indicates higher bilirubin concentrations in brains from hUGT1 mice that developed seizures (Fig. 1B), confirming patterns of bilirubin accumulation as observed previously (4).

Alteration of Redox Status and Activation of Stress-response Genes, NADPH-oxidase Complex, and NF-κB in Hyperbilirubinemia Mice—Bilirubin is considered to be a potent antioxidant (1); we first evaluated the redox status in mice with severe hyperbilirubinemia (kernicterus mice) by measuring blood glucose, and then measured the levels of stress response genes, NADPH-oxidase complex, and NF-κB to understand their involvement in the disease process.

**TABLE 1**

The list and sequence of primers used for real-time PCR

| Primer | Sequence |
|--------|----------|
| CPH fwd | 5'-CAGAGCCACGTGTCGTT-3' |
| CPH rev | 5'-TGCTTGAGAATTTGCTGCAA-3' |
| HO-1 fwd | 5'-CAGGTCTCCAGAGAAGGTTT-3' |
| HO-1 rev | 5'-TCCTCAGGGCCGGTGTGAT-3' |
| NQO1 fwd | 5'-GGGTGATTTCTGCTTCCATTG-3' |
| NQO1 rev | 5'-CAGAGATCCACTGTCACACAC-3' |
| TLR2 fwd | 5'-CCAGACACGGTGGGTAACATC-3' |
| TLR2 rev | 5'-CGGATGCATTTAGCTTGGG-3' |
| TLR3 fwd | 5'-GGGGTCACAGGGAGAACC-3' |
| TLR3 rev | 5'-CCGGGGAACACTTTAAGTGG-3' |
| TLR4 fwd | 5'-GCCCTTCCAGGAATTAAGCCTC-3' |
| TLR4 rev | 5'-AGATCAACGGTAGGACCTGTA-3' |
| TLR5 fwd | 5'-TCAGAGGCGAGGATAGCTT-3' |
| TLR5 rev | 5'-ATGGTGCAATTGACTGACCTG-3' |
| TLR7 fwd | 5'-CTTTACCTTACATCAACACAC-3' |
| TLR7 rev | 5'-CCCCGTAGAAACAGTGATAC-3' |
| TLR9 fwd | 5'-GCTCGGCTTACGTGAA-3' |
| TLR9 rev | 5'-GGTCCCCATACAGTCCC-3' |
| Cd11b fwd | 5'-ATGGACCGTCATGCGCCAATACC-3' |
| Cd11b rev | 5'-TCCCCCCATACAGTCCC-3' |
| TNFα fwd | 5'-TGCGGTGAGAAATTCGAGTACCA-3' |
| TNFα rev | 5'-TGGGAGTAGACAAGGTACACA-3' |
| IL-1β fwd | 5'-GGCACTGTCGATACCTGACC-3' |
| IL-1β rev | 5'-ATCTTTCGGGGGTCCTGCAATCT-3' |
| IL-6 fwd | 5'-GAGGATACCCATCCCAACAGGACC-3' |
| IL-6 rev | 5'-AAGTGCAATCTGGTTCTCATA-3' |
| COX-2 fwd | 5'-GCGGGATCCACTGACCT-3' |
| COX-2 rev | 5'-GCTCGGCTTACGATTTGAG-3' |
| NQO1 fwd | 5'-TCTTCAGCTCCTAGGGTTTC-3' |
| NQO1 rev | 5'-GGTGGATATTTCAGTTCCCATTGC-3' |
| CPH fwd | 5'-CAGAGCCACGTGTCGTT-3' |
| CPH rev | 5'-TGCTTGAGAATTTGCTGCAA-3' |
| COX-2 rev | 5'-GCTCGGCTTACGATTTGAG-3' |
| COX-2 fwd | 5'-GCGGGATCCACTGACCT-3' |
| HO-1 fwd | 5'-CAGGTCTCCAGAGAAGGTTT-3' |
| HO-1 rev | 5'-TCCTCAGGGCCGGTGTGAT-3' |
| NQO1 fwd | 5'-GGGTGATTTCTGCTTCCATTG-3' |
| NQO1 rev | 5'-CAGAGATCCACTGTCACACAC-3' |

**Statistical Analysis**—Prism software (version 5, GraphPad) was used to analyze the data, and are presented as mean ± S.D. Differences between 2 groups were compared using the two-tailed unpaired Student’s t test. Differences among multiple groups were compared using one-way analysis of variance. p values <0.05 were considered statistically significant, and statistically significant differences are indicated with *, p < 0.05; **, p < 0.005; ***, p < 0.0005. For overall survival analysis, Kaplan-Meier curves were analyzed by the log rank test.
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**FIGURE 1. Bilirubin levels in hUGT1 mice.** Fourteen-day-old hUGT1 mice from 8 litters were divided into 2 groups based on their behavior status. WT, healthy hUGT1, and hUGT1-kernicterus mice. Bilirubin levels were then measured using a Bilirubinometer. 

**A** Serum bilirubin levels (mg/dL) were monitored over a 14-day period, with significant differences observed between the groups.

**B** Brain bilirubin concentrations were measured using Real-time PCR and exhibited a significant increase in the kernicteric brain compared to the healthy group.

Unexpectedly, blood samples collected from kernicterus mice exhibited significantly decreased levels of the reduced form of glutathione, GSH, and higher levels of the oxidized form of glutathione, GSSG, compared with those from hyperbilirubinemia littermates but healthy mice (Fig. 2A). Oxidative stress potentially signaled by GSH depletion from severe hyperbilirubinemia results in activation of several stress response genes, including heme oxygenase-1 (Ho-1) and NADPH:quinone oxidoreductase 1 (Nqo-1), which are compatible between healthy and wild type mice without statistical significance, indicating that toxic levels of bilirubin in kernicterus mice predispose the brain tissue to oxidative stress. Although it is unclear whether oxidative stress occurring in hyperbilirubinemia is a cause or a result of BIND, these results indicate that oxidative stress in hyperbilirubinemia is manifested by the impaired antioxidant system with a compelling lower ratio of [GSH]/[GSSG].

Activation of the NADPH oxidase has been linked as a major contributor to ROS generation resulting in oxidative stress produced by hyperbilirubinemia. While examining stress response genes, we found that HSP27 is significantly induced in kernicteric brain tissue (Fig. 3A). HSPs are predominantly intracellular molecules and are rapidly released following cell injury and innate immunity prompted us to examine the role of TLR in response to bilirubin toxicity during neonatal brain development. Among TLR isotypes expressed in the CNS, we discovered that TLR2 expression levels were significantly and consistently elevated in the kernicteric brain (Fig. 3B). In addition, expression levels of the Cd11b gene, which is associated with TLR2-triggered T cell adaptive immunity (31), were found to be higher in the kernicteric brain than in the control brain (Fig. 3C). Many independent studies pointed out that TLR signaling may have pathogen-independent functions through host-derived, endogenous ligands, in addition to playing important roles in the defense mechanism by detecting conserved components of invading microbes (10). To further address whether the innate immune response through TLR2 is associated with pathogenic outcomes that take place in the CNS induced by hyperbilirubinemia, Tlr-2−/− mice were cross-bred with hUGT1 mice to obtain hUGT1/Tlr2−/− or hUGT1/Tlr2−/− mice. To achieve the same genetic background, hUGT1/Tlr2−/− were continued to backcross with hUGT1 for at least 5 generations. hUGT1/Tlr2−/− mice were normal in physical appearance and reproductive ability. A small percentage (~8%) died a few days after birth. Many of hUGT1/Tlr2−/− mice, ranging from 12 to 18 days old, went through the irreversible sequelae, including a startle reflex, dystonia, locomotor abnormality, then became paralyzed and died (~1 day later (Fig. 3D). We separated those hUGT1/Tlr2−/− mice that experienced such an episode from other hUGT1/Tlr2−/− mice whose behavior was normal. Similar to kernicterus mice that exhibited extremely high levels of bilirubin and eventually died, all hUGT1/Tlr2−/− mice with abnormal neurological symptoms died at 12–20 days of age. hUGT1/Tlr2−/− mice displayed...
serum bilirubin levels similar to those in healthy hUGT1 mice; however, they were significantly more susceptible to high levels of serum bilirubin that accumulated during neonatal development when compared with hUGT1 mice. At the developmental stage, 57.1% of hUGT1/Tlr2/K11002/K11002 mice died with bilirubin levels of 15.0 ± 3.10 mg/dl (Fig. 3D) compared with an 8.7% mortality rate in hUGT1 mice with bilirubin levels of 20.8 ± 2.32 mg/dl (Fig. 1A and left side of Fig. 3E). These results suggest that the lack of TLR2 signaling results in a neurologically deteriorating impact over bilirubin toxicity. Although the lack of TLR2 signaling was a whole body TLR2 deficiency, the mice progressed into seizures, indicating that elevated bilirubin levels stimulate neurological deterioration.

**TLR2 Signaling Is Associated with Activation of Glial Cells**—Based on the compelling result of TLR2 deficiency mice that are more sensitive to succumbing to hyperbilirubinemia toxicity, we conducted experiments to compare biological events among hUGT1/Tlr2/+ (referred to as control), hUGT1 mice with extreme hyperbilirubinemia (referred to as hUGT1/K11002/K11002), and TLR2 knock-out mice that experienced a dystonia/seizure episode (referred to as hUGT1/K11002/K11002). Although its functional significance remains largely undefined, the signaling pathway involving TLRs in the CNS has been linked to reactive gliosis, particularly activation of microglia and astrocytes (11). To determine the role of extreme hyperbilirubinemia in glia activation, gliosis of microglia and astrocytes was examined using anti-ionized calcium-binding adapter molecule (Iba-1, Wako) and anti-GFAP (CHEMICON International), respectively. The results with brain sections revealed that, compared with control mice that exhibited moderate levels of bilirubin, the reactive state of glial...
Cells in the extreme hyperbilirubinemia brain of the kernicterus mouse was signified by an increase in the number of cells labeled by Iba-1 and GFAP, hallmarks of reactive microglia and astrocytes, respectively (Fig. 4A and B). When bilirubin levels accumulated in brain tissue, activated microglia cells were recruited to the response sites surrounding the brain stem area (Fig. 4A, a coronal section as shown with ×10 magnification). In addition, increased levels of intermediate filament GFAP were observed around the hippocampal region (Fig. 4B, as shown with ×10 magnification) suggest the occurrence of astrogliosis in bilirubin-directed brain damage. However, deletion of TLR2 suppresses activation of microglia and astrocytes as lower levels of Iba-1 and GFAP were detected in the brain of hUGT1/Tlr2−/− (Fig. 4A and B). Consistent with immunostaining results, Iba-1 and GFAP mRNA levels significantly increased in the hyperbilirubinemia brain and the increase was abolished in the hUGT1/Tlr2−/− brain (Fig. 4C). These results suggest that TLR2 signaling is closely linked to glia cell activation.

**TNFα and Other Proinflammatory Cytokines Are Activated in Microglia through TLR2 Signaling in Response to Bilirubin**—To further determine the role of TLR2 in bilirubin-induced signaling that is associated with glia activation, we examined proinflammatory cytokines in hyperbilirubinemia brains. TLR2 signaling was accompanied by pronounced neuroinflammation in the CNS with up-regulation of Tnfα, Il-1β, and Il-6 gene expression, creating a pro-inflammatory environment in the brain tissue. Deletion of TLR2 in hUGT1 mice blocked the induction of these inflammatory cytokine genes. Furthermore, TLR2 signaling also impacts the redox status as the induction of the gp91phox gene expression was abolished in TLR2 deficiency. Similarly, induction of the oxidative stress response gene Ho-1 by hyperbilirubinemia was inhibited in the absence of TLR2 (Fig. 5). These results suggest that TLR2 signaling contributes to the inflammatory state and disturbed redox status in response to the bilirubin insult.

To explore the potential relationship of the activation of glial cells, induction of proinflammatory cytokines, and oxidative stress response with regard to TLR2 signaling, we conducted *in vitro* experiments by isolating primary microglia and astrocytes from wild type and Tlr2−/− mice. The specificity of microglia and astrocytes was verified by expression levels of Iba-1 (a microglia marker) and GFAP (an astrocyte marker), as shown by real time-PCR (Fig. 6A). Newborn mice expressed markedly enhanced levels of TLR2 and pro-inflammatory cytokines Tnfα and IL-6 in response to bilirubin (50 μM) stimulation, particularly in microglial cells (Fig. 6B). By contrast, oxidative stress response to the Ho-1 gene was induced by bilirubin in both microglia and astrocytes. When TLR2 was absent in brain cells, microglia lost their ability to respond to bilirubin in up-regulating Tnfα and IL-6. Similarly, Ho-1 induction in response to bilirubin treatment was abolished in TLR2-deficient cells indicating that bilirubin can promote proinflammatory cytokine production, particularly in microglia cells, and TLR2 plays a critical role in regulating these downstream signaling molecules (Fig. 6C).

**Deletion of TLR2 Results in Programmed Cell Death and COX-2 Induction**—To explore the possibility that up-regulation of proinflammatory mediators induced by bilirubin affects neural cell death, we performed the TUNEL assay in both cortex and cerebellum brain sections among hUGT1/Tlr2+/− (control), hUGT1_kernicterus, and hUGT1/Tlr2−/−_kernicterus mice. No apoptosis was found in healthy hUGT1 or hUGT1/Tlr2−/−_mice (data not shown). A low level of apoptosis was detected in the brain section of hUGT1_kernicterus mice; interestingly, brain sections in the cortex and cerebellum from kernicterus mice that lack TLR2 functionality displayed...
pronounced apoptotic cell death suggesting that TLR2 signaling may have an anti-apoptotic function (Fig. 7A). A great increase of neuronal programmed cell death in the TLR2 knock-out brain coincides with intensified expression of Cox-2. In hUGT1\_kernicterus mice, high bilirubin levels gave rise to approximately a 20-fold induction of Cox-2; deletion of TLR2 in kernicterus mice potentiated bilirubin-induced Cox-2 gene expression to over 100-fold (Fig. 7B). These results indicate that TLR2 signaling is involved in establishing an anti-apoptotic property responding to severe hyperbilirubinemia and implicates a deleterious role for hyperactive-Cox-2.

**DISCUSSION**

TLRs have traditionally been considered to have a pivotal role in the defense mechanism against infection by detecting conserved components of invading microbes and initiating innate responses (32). However, there is growing appreciation that TLRs can interact with endogenous alarm signals (10, 33). Many lines of evidence suggest that during tissue injury or proteolysis, extracellular matrix components undergo cleavage, gaining the ability to act as TLR ligands, thus initiating TLR signaling without the presence of invading pathogens. For example, HSPs, fibronectin containing extra domain A, fibrinogen, and hyaluronic acid, which are released from stressed cells, may activate the TLR pathway (29, 30, 34–37). We became interested in the role of TLR signaling because the release of HSP27 coincides with its potential as an endogenous ligand for TLR activation. This study exemplifies that the innate immune system is activated by endogenous “danger” signals,

**FIGURE 4. Immunofluorescence analysis and gene expression of Iba-1 and GFAP in the brain.** A and B, paraffin brain coronal sections obtained from control (hUGT1/Trl2\^-/-\) that displayed normal behavior, n = 3) and both hUGT1 kernicterus (n = 3) and hUGT1/Trl2\^-/-\ (n = 3) mice that experienced locomotor abnormality and seizure were prepared, and reactive microglia and astrocytes were visualized by immunofluorescence staining using anti-Iba-1 (A) and anti-GFAP (B) antibodies, respectively. Sections of cerebral cortex and sections of hippocampus were stained for Iba-1 and GFAP immunohistochemical staining, respectively. Following the primary antibody staining, sections were incubated with the fluoro-Alexa 488 secondary antibody and the fluorescence images were analyzed under a microscope at 40- or 10-fold magnification. We repeated these experiments three times and the data are representative images from one mouse of each group. Scale bars, 50 μm. C, gene expression of Iba-1 and Gfap in the brain tissue was compared using real time-PCR.
which originate from stressed and injured cells, although the precise ligand that initiates TLR2 signaling remains the subject of ongoing investigation.

In this study, we show a requirement for TLR2 signaling in regulating gliosis, proinflammatory mediators, and oxidative stress. Reactive gliosis, characterized by activation of microglia...
and astrocytes in the CNS, is a prominent feature of kernicterus mice. Using \( hUGT1/Tlr2^{-/-} \) mice as animal models and primary glial cells, we demonstrate that TLR2-mediated gliosis strongly correlates with induction of proinflammatory cytokines and the occurrence of oxidative stress. The clinical manifestations of severe hyperbilirubinemia are likely caused by these factors, accounting for an 8–10% lethality rate. Our results show that the inflammatory state and disturbed redox status are the targets of TLR2-dependent innate immunity and contribute to the pathological outcome of BIND in \( hUGT1 \) mice. We recognized that increased oxidative stress resulting from activation of NADPH oxidase is an important contributory mechanism: the antioxidant level is reduced in mice with severe hyperbilirubinemia, which did not occur in the littermates with mild hyperbilirubinemia. In line with elevated oxidative stress, the transcription factor NF-\( \kappa \)B is activated along with increased phosphorylation of MAP kinases, including ERK1/2 and JNK, although a cause-effect relationship between these events should be further established.

CNS injury following TLR2 signaling is only one aspect of the TLR2 role in bilirubin toxicity in the brain. Another interesting aspect is when TLR2 is deficient, there is decreased gliosis and inflammatory infiltration in the brain tissue, but paradoxically animals have a decreased survival rate manifested by increased apoptosis. This suggests a repair mode linked to a TLR2-mediated inflammatory signal, which also simultaneously contributes to a detrimental effect with elevated oxidative stress as mentioned earlier. The notion that TLR signaling is necessary for repair of cell injury is exemplified in a recent study showing that mice deficient in TLR4/TLR2 signaling were impaired in their ability to heal after acute lung injury (38), in which the activated TLR2 and TLR4 signaling leads to inflammatory cell transmigration and protection against apoptosis. Another study with LPS preconditioning demonstrated that blocking TNF\( \alpha \) abolished neuroprotection (39). The outcome of TLR activation may depend on the dose of the ligand, the etiology of CNS diseases, and the timing of activation (13). A recent study of a stroke event has shown that stimulating TLRs with a low

![FIGURE 7. TUNEL assay and COX-2 expression. A, TUNEL staining of mouse brain sections (left, cortex; right, cerebellum) for recognizing apoptotic cells in \( hUGT1/Tlr2^{-/-} \) control (\( n = 3 \)), \( hUGT1 \) kernicterus (\( n = 3 \)), and \( hUGT1/Tlr2^{-/-} \) kernicterus mice (\( n = 3 \)). The TUNEL assay was performed according to the manufacturer’s instructions (Roche, In-Situ Cell Death Detection TMR, red), and the slides were incubated with 4',6-diamidino-2-phenylindole (DAPI) to stain the nuclei as shown in blue. Red nuclear staining indicates a positive apoptotic cell. B, gene expression levels of Cox-2 by real time-PCR.](image-url)
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dose of ligand prior to stroke leads to a decrease in infarct size and an improved neurological outcome in response to ischemic injury (40), supporting the role of neuroprotection of TLR signaling. The similarities of our results in a model of hyperbilirubinemia brain injury support a common theme of repair linked to TLR2 signaling. Second, TLR2-dependent recruitment of microglia and astrocytes in the brain tissue may also provide protection against cytotoxicity. Reactive astrocytes and microglia are often associated with neurodegenerative diseases in the CNS (41). However, investigators have reported that in the event of spinal cord injury and ischemic stroke, these cells act as the surveillance system to combat inflammatory cells, secrete various neurotrophic factors and anti-inflammatory cytokines (e.g. TGFβ, IL-10), remove cellular debris and oxidized molecules generated from the CNS damage, enhance cell proliferation and differentiation, and eventually maintain the homeostasis of the neural environment (42–47). The mechanisms that dictate the functional outcomes that are related to either destructive neurodegenerative phenotypes or neuroprotective tissue repair phenotypes need to be clarified, especially with regard to the role of glial cell activation. Third, to counteract the oxidative damage from excessive ROS in hyperbilirubinemia, complex antioxidant defense mechanisms exist to remove oxidation-damaged molecules from the brain (48). A redox-sensitive pathway, such as induction of Ho-1 and Nqo-1 as shown in the kernicterus brain, may represent an effective defense mechanism that protects the tissue from oxidative damage and maintains redox homeostasis. Loss of TLR2 signaling impairs this redox-sensitive pathway, and mice lose the ability to combat oxidative stress.

An extreme high level of bilirubin creates a complex microenvironment where pro- and anti-inflammatory factors are simultaneously present. The outcome of bilirubin neurotoxicity critically depends on how brain cells will integrate these opposing signals. Under such circumstances, proinflammatory cytokines represent important molecules that mediate immune activation, and TNFα, IL1β, and IL-6 are the main cytokines able to activate brain cells in a TLR2-dependent pathway. It is well known that systemic production of endogenous mediators (e.g. TGFα) that counterbalance these pro-inflammatory events subsequently occur to resolve the inflammation (49). When TLR2 signaling is absent, the counteracting between pro- and anti-inflammatory responses is likely disregulated. COX-2 also exerts proinflammatory actions, although the role of COX-2 in neurodegenerative disease is controversial. COX-2 has been found to be up-regulated by oxidative stress and TNFα (50), which verifies the marked induction of Cox-2 gene expression in the hUGT1 kernicteric brain. The intriguing super-induction of Cox-2 expression in hUGT1/Tlr2−/− points to a pathological condition. One possible explanation for the extremely high level of Cox-2 gene expression may come from bilirubin- and TLR2-dependent induction of TNFα, which in turn exerts a key role in mounting an appropriate inflammatory response to control downstream genes including Cox-2. When TNFα signaling is disrupted, COX-2 undergoes a hyperactivation through a feedback mechanism, augmenting inflammatory responses. Our findings suggest that the TLR2/TNFα signaling cascade increases inflammation and accentuates bilirubin toxicity. By the same token, brain cells are resistant to apoptosis in the hUGT1 kernicteric brain presumably because the antiapoptotic signal delivered by inflammatory cytokines is dominant over the proapoptotic effect. Importantly, bilirubin-induced apoptosis was enhanced by blocking TLR2. A marked increase in inflammation from COX-2 hyperactivation may subsequently trigger brain cells to undergo apoptosis because in such an environment, brain cells may become more sensitive to bilirubin-induced apoptotic effects. In conclusion, the current study adds to our understanding of how signaling from innate immunity contributes to bilirubin-induced pathology and plays a spontaneous overall protection role.

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