Toll-Like Receptor 2 Participates in Mediation of Immune Response in Experimental Pneumococcal Meningitis

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Toll-Like Receptor 2 Participates in Mediation of Immune Response in Experimental Pneumococcal Meningitis

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Heterologous expression of Toll-like receptor (TLR)2 and CD14 in Chinese hamster ovary fibroblasts was reported to confer responsiveness to pneumococcal peptidoglycan. The present study characterized the role of TLR2 in the host immune response and clinical course of pneumococcal meningitis. Pneumococcal infection of mice caused a significant increase in brain TLR2 mRNA expression at both 4 and 24 h postchallenge. Mice with a targeted disruption of the TLR2 gene (TLR2−/−) showed a moderate increase in disease severity, as evidenced by an aggravation of meningitis-induced intracranial complications, a more pronounced reduction in body weight and temperature, and a deterioration of motor impairment. These symptoms were associated with significantly higher cerebellar and blood bacterial titers. Brain expression of the complement inhibitor complement receptor-related protein y was significantly higher in infected TLR2−/− than in wild-type mice, while the expression of the meningitis-relevant inflammatory mediators IL-1β, TNF-α, IL-6, macrophage-inflammatory protein (MIP)-2, inducible NO synthase, and C3 was similar in both genotypes. We first ectopically expressed single candidate receptors in HEK293 cells and then applied peritoneal macrophages from mice lacking TLR2 and/or functional TLR4 for further analysis. Overexpression of TLR2 and TLR4/MD-2 conferred activation of NF-κB in response to pneumococcal exposure. However, pneumococci-induced TNF-α release from peritoneal macrophages of wild-type and TLR2/functional TLR4 double-deficient mice did not differ. Thus, while TLR2 plays a significant role in vivo, yet undefined pattern recognition receptors contribute to the recognition of and initiation of the host immune defense toward Streptococcus pneumoniae infection. The Journal of Immunology, 2003, 170: 438–444.
Materials and Methods

Mouse model of pneumococcal meningitis

A well-characterized mouse model of pneumococcal meningitis was used in this study (16, 17). Briefly, meningitis was induced by transthecal injection of 15 μl of a bacterial suspension containing 10^10 CFU/ml S. pneumoniae type 3 into the cisterna magna under short-term anesthesia with halothane. Mice were weighed, put into cages, and allowed to wake up. Four or 24 h after infection, mice were evaluated clinically. The clinical score comprises the following criteria: 1) presence of tremor and pilocerection, 2) vigilance, 3) a beam-balancing test, and 4) a postural reflex test. Thereafter, the body temperature was measured via a rectal probe, and mice were reweighed and anesthetized with ketamine/xylazine. Subsequently, a catheter was inserted into the cisterna magna to measure intracranial pressure (ICP) and to determine CSF leukocyte counts. Afterward, mice were deeply anesthetized with ketamine/xylazine and perfused transcardially with 15 ml of ice-cold PBS containing 10 U/ml heparin. The brains were removed and rapidly frozen. In a subset of experiments, before perfusion, blood samples were taken by transcardial puncture. In addition to brains, spleens and lungs were collected and immediately frozen.

Determination of the blood-brain barrier (BBB) integrity

To assess BBB integrity, mouse brain homogenates were examined for infiltration by both albumin and IgG, abundant serum proteins that are normally excluded from the brain by the intact BBB, using Western immunoblotting (18).

Western blot analysis

Mouse brain protein extracts (20 μg of protein per lane) were separated on a 4–12% gradient NuPage Tris-Bis gel (NOVEX, Frankfurt, Germany), transferred to a polyvinylidene fluoride membrane, and probed with either an anti-mouse albumin peroxidase-conjugated goat polyclonal Ab (1:200); Bethyl Laboratories, Montgomery, TX) or an anti-mouse IgG peroxidase-conjugated rabbit polyclonal Ab (1:200, Sigma-Aldrich, Deisenhofen, Germany). Immunoreactive protein bands were detected using ECL (Amersham Pharmacia Biotech, Freiburg, Germany). X-ray films were digitized and optical densities were determined using a computer imaging analysis system (Visi torn Systems, Puchheim, Germany).

mRNA isolation and RT-PCR analysis

Total RNA was extracted from frozen sections (brain, spleen, and lung) with TRizol-LS reagent (Life Technologies, Gaithersburg, MD) and reversely transcribed using Superscript II (Life Technologies). The cDNA was amplified by PCR with gene-specific primers of the following sequences: TLR2 sense, 5′-CTCCTCTGAATTTGCTCAGTACCGG-3′; TLR2 antisense, 5′-TCAGCTCTTCAAGCAGAGGAG-3′; IL-β sense, 5′-TCTAGGGATGATGATGATAACCTGCT-3′; IL-β antisense, 5′-CTTCTTCTGAGCTCAGCTTAC-3′; TNF-α sense, 5′-AGTTTGGTGTCAGTGACAC-3′; TNF-α antisense, 5′-GGAGGTGCAGTGAGCTCTTCC3′; inducible NO synthase (iNOS) sense, 5′-ACGCTTCACCTTGTTGTTTCTTAC-3′; iNOS antisense, 5′-CGCTCAGTGGGCTCTGAGTAC-3′; Crry sense, 5′-CATCAGCTCTTCCCTGACC-3′; Crry antisense, 5′-CATCAGCTCTTCCCTGACC-3′; complement receptor-related protein y (Cry) sense, 5′-GCCCTCTCTTCTGTGGC-3′; and Cry antisense, 5′-ATCGTGGCTGCTCTGCTATATA-3′. Mouse β-actin was coamplified as an internal control using the following primer sequences: sense 5′-GGACCTCTTATGGGTCAGGACC-3′ and antisense 5′-GGGAGGAGCCATGCTGCTAG-3′. Linearity of DNA amplification was determined for each primer set in experiments establishing the PCR procedures in terms of DNA amounts and cycle number applied. PCR products were separated on a 1.5% agarose gel, stained with ethidium bromide, visualized by UV illumination, and photographed. Densitometry was performed on the negative image, and the relative absorbances of TLR2, IL-β, IL-6, TNF-α, MIP-2, iNOS, C3, and Cry were normalized by relation to absorbance of β-actin RT-PCR products.

Determination of bacterial titers in blood and organs

Cerebella and spleens were dissected and homogenized in sterile saline. Blood samples and cerebellum and spleen homogenates were diluted serially in sterile saline, plated on blood agar plates, and cultured for 24 h at 37°C with 5% CO₂.

Experimental groups in the mouse model

The following experimental groups were investigated: 1) wild-type (wt) mice injected intracisternally with 10^5 CFU of S. pneumoniae (n = 16), 2) TLR2−/− mice injected intracisternally with S. pneumoniae (n = 16). In these groups, mice were killed 24 h after onset of pneumococcal meningitis. At that time, all mice exhibited obvious symptoms of disease, marked intracranial complications, and pronounced meningeal inflammation (16, 17, 19). To elucidate the impact of TLR2 deficiency on early events associated with pneumococcal meningitis, the following additional subset of experiments was performed: injected wt and TLR2−/− mice (n = 4 for each group; groups 4 and 5) killed 4 h after pneumococcal inoculation. Targeted mice lacking expression of TLR2 were obtained from Tulipar (South San Francisco, CA) and DeltaGen (Menlo Park, CA). A portion of the Cter gene encoding the transmembrane part of the transmembrane aa C54 to aa 593 (residues calculated for immature protein) was replaced by a neomycin cassette through homologous recombination (20). TLR2-deficient 129Sv × C57BL/6 mice were bred back against the genetic C57BL/6 background for four generations. Then two heterozygous pairs were used to establish TLR2−/− homozygous breeding colonies with the corresponding genetic background. Matched groups of mice descending from these breeding colonies were applied synchronically for experiments. All of the experiments were approved by the government of Upper Bavaria.

NF-κB reporter gene assay

The human embryonic epithelial kidney cell line HEK293 (American Type Culture Collection, Manassas, VA) was maintained in DMEM supplemented with 10% FCS (Reanal, Budapest, Hungary) and penicillin/streptomycin-free medium, commercially available E. coli O111:B4 (10 ng/ml; Sigma-Aldrich), 2) polyinosine-polycytidylic acid (5 μM; Gentrics cDNA for TLR9 was a kind gift from Drs. Beutler and Bauer (25, 26). Expression of TLRs was confirmed by tag-dependent immunoblot analysis and/or functional analysis by application of specific stimuli. Transfected HEK293 were exposed to either S. pneumoniae (10^6 CFU/ml; applied concomitantly with 100 μg/ml ceftriaxone for the prevention of bacterial overgrowth) or the following bacterial products: 1) Escherichia coli LPS O111:B4 (10 ng/ml; Sigma-Aldrich), 2) polyinosine-polycytidylic acid (5 μM; Gentrics or functional analysis by application of specific stimuli. Transfected HEK293 were exposed to either S. pneumoniae (10^6 CFU/ml; applied concomitantly with 100 μg/ml ceftriaxone for the prevention of bacterial overgrowth) or the following bacterial products: 1) Escherichia coli LPS O111:B4 (10 ng/ml; Sigma-Aldrich), 2) polyinosine-polycytidylic acid (5 μM; Gentrics cDNA for TLR9 was a kind gift from Drs. Beutler and Bauer (25, 26).

TNF-α release from primary macrophages

Peritoneal macrophages were isolated from TLR2−/− mice (5-fold backcrossed toward the C3H inbred strain background; TLR2−/−/TLR4−/−), C3H/HeN mice (wild-type), C3H/HeJ mice (TLR2−/−/TLR4−/−), and CD14−/−CD14−/− mice lacking both TLR2 and functional TLR4 (TLR2−/−/TLR4−/−) (28–30). Briefly, mice were injected i.p. with 2 ml of 4% thioglycolate (Sigma-Aldrich). After 5 days, peritoneal exudate cells were collected by washing the peritoneal cavity with ice-cold PBS supplemented with 2% FCS. Cells (2 × 10^6 cells/well in 96-well dishes) were washed with PBS for removal of nonadherent cells. Adherent macrophages were stimulated in DMEM supplemented with 10% FCS, standard penicillin/streptomycin, 10 μM monothioglycerol, and murine IFN-γ (50 ng/ml; PeproTech, London, U.K.) either with S. pneumoniae (10^4 CFU/ml; applied concomitantly with 100 μg/ml ceftriaxone for the prevention of bacterial overgrowth) in penicillin/streptomycin-free medium, commercially available E. coli LPS (Sigma-Aldrich), or the bacterial lipopolysaccharide analog tripalmitinoyl-cysteinyl-seryl-lysyl-lysine (ECL, Tübingen, Germany). After 24 h, cell culture supernatants were sampled and analyzed for TNF-α content using an ELISA (R&D Systems, Minneapolis, MN).
Statistical analysis
The principal statistical test was unpaired Student’s t test. Differences were considered significant at \( p < 0.05 \). Data are expressed as mean \( \pm \) SD.

Results
Up-regulation of TLR2 mRNA expression in the brain during pneumococcal meningitis
In brain homogenates from PBS-injected control mice, a low basal expression of TLR2 mRNA was detected by RT-PCR. Pneumococcal infection caused a substantial increase in the mRNA expression of TLR2 in brains obtained from wt mice 4 h as well as 24 h after challenge (Fig. 1a). The relatively high constitutive TLR2 expression in the spleen was not affected by intracisternal pneumococcal inoculation (data not shown).

Effect of TLR2 depletion on the clinical status
Within 24 h after pneumococcal inoculation, all infected wt mice exhibited a similar degree of disease as evidenced by a loss in weight, hypothermia, piloerection, lethargy, and impaired motor functions (Fig. 2a). TLR2 deficiency was associated with a moderate increase in disease severity (Fig. 2a). For example, TLR2\(^{-/-}\) mice showed a more pronounced reduction in both body temperature (34.6 \( \pm \) 1.1°C vs 36.0 \( \pm \) 1.7°C in infected wt mice) and body weight (\(-13.5 \pm 3.3\%\) vs \(-9.9 \pm 3.1\%\) in infected wt mice).

None of the control mice injected intracisternally with PBS exhibited signs of infection within the observation period. No significant change in body weight (\(-0.5 \pm 3.1\%\)) could be observed. Body temperatures were within the normal range (37.1 \( \pm \) 0.3°C).

Effect of TLR2 depletion on meningitis-induced intracranial complications
Because CNS complications are major determinants of an unfavorable clinical outcome in bacterial meningitis (2, 3), we investigated the impact of TLR2 deficiency on ICP and BBB permeability in our mouse meningitis model.

Pneumococcal infection induced a significant increase in ICP in wt mice. At 24 h after pneumococcal inoculation, TLR2\(^{-/-}\) mice had significantly higher ICP values than infected wt mice (Fig. 2b). Because vasogenic edema is a predominant cause of the meningitis-associated increase in ICP, we determined the immunoreactivity for the endogenous serum proteins albumin and IgG in mouse brain homogenates by Western blotting. In brain homogenates from PBS-injected control mice, only faint bands for both albumin and IgG were present. Intracisternal injection of pneumococci

\[ \text{FIGURE 1.} \quad \text{Brain mRNA expression of TLR2 and meningitis-relevant inflammatory mediators during pneumococcal meningitis.} \]

\[ \text{FIGURE 2.} \quad \text{Effect of TLR2 deficiency on the clinical status and CNS complications.} \]

\( a, b, c, d, e, f \)
caused an extensive extravasation of albumin (Fig. 2c) and IgG into the brain (data not shown). Infected TLR2<sup>−/−</sup> mice exhibited a significantly more pronounced immunoreactivity for albumin (but not IgG) than infected wt mice. These data hint at a slight deterioration of meningitis-induced BBB disruption in mice with a targeted disruption of the TLR2 gene.

**Effect of TLR2 depletion on bacterial titers in blood and organs**

To test whether this worsening of disease resulted from altered bacterial growth in vivo, the bacterial numbers in the cerebellum, blood, and spleen were determined at 4 and 24 h after intracisternal pneumococcal infection. At both time points, the number of blood, and spleen were determined at 4 and 24 h after intracisternal pneumococcal infection. At 4 and 24 h after intracisternal pneumococcal infection, the number of infected mice was significantly higher than in wild type mice. However, after 4 h of pneumococcal infection, the brain expression of TNF-α and MIP-2 (but not of IL-1β, IL-6, iNOS, and C3) was significantly lower in TLR2<sup>−/−</sup> mice than in wild type mice whereas the Cry mRNA levels were higher. However, in the spleen, infected TLR2<sup>−/−</sup> mice exhibited a higher mRNA expression of both MIP-2 and iNOS (but not the other factors investigated) than infected wt mice (data not shown). In the lung, TLR2 deficiency was associated with a significantly increased expression of TNF-α mRNA, but not of the other mediators determined (data not shown). Combined, this comparative expression analysis revealed rather mild differences in the host immune response of wt and TLR2<sup>−/−</sup> mice that were restricted to the early phase of pneumococcal meningitis.

**Effect of TLR2 depletion on the inflammatory host reaction**

To investigate whether the higher susceptibility to pneumococcal infection is due to a defective immune response, we assessed the impact of TLR2 deficiency on the inflammatory host response in our meningitis model.

Pneumococcal infection led to a massive leukocyte infiltration into the subarachnoid space in wt mice. Neither at 4 h nor at 24 h after pneumococcal infection, a significant difference in CSF leukocyte counts between TLR2<sup>−/−</sup> mice and wt mice was detectable (e.g., at 24 h postchallenge, 14,373 ± 7,937 cells/μl in TLR2<sup>−/−</sup>, infected mice vs 11,991 ± 6,175 cells/μl in infected wt mice). Moreover, mouse brain, spleen, and lung homogenates were analyzed for their expression of several meningitis-relevant host factors by RT-PCR (31). After 24 h of intracisternal inoculation, differences in mRNA accumulation of the proinflammatory cytokines IL-1β and TNF-α, the neutrophil chemoattractant MIP-2, the cytokine IL-6, the iNOS isoform, or the complement factor C3 in the brain, spleen, and lung were insignificant in wild-type and TLR2<sup>−/−</sup> mice (Fig. 1, b–f; data on IL-1β and IL-6 mRNA expression not shown). Of the mRNA levels analyzed at 24 h upon infection solely, the level of Crry mRNA was significantly higher in infected TLR2<sup>−/−</sup> mice as compared with levels in infected wt mice. However, after 4 h of pneumococcal infection, the brain expression of TNF-α and MIP-2 (but not of IL-1β, IL-6, iNOS, and C3) was significantly lower in TLR2<sup>−/−</sup> mice than in wt mice whereas the Cry mRNA levels were higher. However, in the spleen, infected TLR2<sup>−/−</sup> mice exhibited a higher mRNA expression of both MIP-2 and iNOS (but not the other factors investigated) than infected wt mice (data not shown). In the lung, TLR2 deficiency was associated with a significantly increased expression of TNF-α mRNA, but not of the other mediators determined (data not shown). Combined, this comparative expression analysis revealed rather mild differences in the host immune response of wt and TLR2<sup>−/−</sup> mice that were restricted to the early phase of pneumococcal meningitis.

**Role of TLR-2 and TLR-4 in pneumococci-induced cellular activation in vitro**

Because TLR2 deficiency had only a moderate impact on the inflammatory host response in murine pneumococcal meningitis, we further investigated the mechanisms of cellular activation by *S.
**Discussion**

Recent work provided evidence that TLR2 plays a crucial role in the detection of microbial infection and in the activation of inflammatory and antimicrobial innate immune responses. In vitro, TLR2 was shown to respond to a variety of microbial products, such as lipoteichoic acid specific for Gram-positive bacteria, peptidoglycan specifically from Gram-positive bacteria, lipoprotein from bacteria such as Gram-negatives and particularly spirochetes, as well as from mycoplasma, and mycobacterial products such as lipooligosaccharide (for reviews, see Refs. 11, 12, 32, and 33). The importance of TLR2 in the host defense was further supported by studies from knockout mice showing higher bacterial loads in organs and decreased survival of TLR2−/− mice after i.v. infection with Gram-positive Staphylococcus aureus (34). After intradermal inoculation of atypical Gram-negative Borreli burgdorferi, TLR2−/− mice were also found to harbor up to 100-fold more spirochetes in tissues and displayed greater ankle swelling than wt littermates (35). Although both constitutive as well as de novo expression of TLR2 mRNA upon systemic application of E. coli LPS to mice, but not upon injection of S. aureus peptidoglycan or lipoteichoic acid, were found in different brain regions (14), the function of TLR2 in cerebral innate immunity is still unclear.

We demonstrated in this study that TLR2 participates in sensing and activating the initial immune response to intracranial challenge with *S. pneumoniae*. Parallel molecular detection systems, for which pattern recognition through further TLR family of protein members are good candidates, must be in place because TLR2−/− mice can still sense (cytokine levels) CNS infection with *S. pneumoniae*. Involvement of TLR2 and further pattern recognition receptors was further evidenced by the findings that 1) HEK293 cells expressing TLR4 also respond to *S. pneumoniae* and 2) peritoneal macrophages lacking TLR2 and/or functional TLR4 release TNF-α upon stimulation with pneumococci. Overexpression of the human TLRs 1, 5–8, and 10 did not confer responsiveness of HEK293 cells toward challenge with *S. pneumoniae* reminiscent of TLR3 and TLR9 (Fig. 4 and data not shown). CD14 and MD-2 were coexpressed in all experiments (36), but further yet-unidentified coreceptors present in macrophages and other immune cells but lacking in HEK293 cells might be required for recognition of *S. pneumoniae* through TLRs different from TLR2 and TLR4. Nonetheless, absence of TLR2 significantly increased the severity of intracranial complications and clinical symptoms upon infection in vivo.

The worsening of disease observed in infected TLR2−/− mice was associated with significantly higher bacterial titers in the CNS. Previous studies in animal models of pneumococcal meningitis have shown that the bacterial inoculum size/bacterial titer is an important factor predetermining severity of the disease (37, 38). The pathologic alterations (e.g., BBB disruption or neuronal cell death) in *S. pneumoniae*-induced meningitis are suggested to be a two-pronged problem. On the one hand, the host inflammatory response to the pathogen was found to contribute substantially to the development of CNS complications and the unfavorable clinical outcome (e.g., Refs. 17, 39, and 40). In contrast, pneumococcal toxins such as pneumolysin and hydrogen peroxide were reported to act as direct inducers of cell death and as immune activators in vitro and in vivo (41–43). Our observation that, in established meningitis (24 h postchallenge), the immune response did not differ between TLR2−/− mice and wt littermates, hints at a role for TLR2 in bacterial toxins concentrations as a major factor for deterioration to more severe CNS alterations and clinical symptoms in TLR2−/− mice. Moreover, the increased release of bacterial toxins due to an increased bacterial burden as observed in infected TLR2−/− mice may also contribute to the abrogation of the potential anti-inflammatory properties exerted by TLR2 deficiency (13). Both toxins can nonspecifically induce the production of different inflammatory mediators such as cytokines (44, 45).

Our observation that TLR2 deficiency was associated with higher cerebellar bacterial titers is remarkable considering that, in numerous animal studies, pneumococcal growth in the CNS remained unaltered by “anti-inflammatory” treatment strategies or by targeted disruption of genes involved in the inflammatory cascade. For example, neither mice deficient in TNF-α, both of its receptors, caspase-1, or iNOS, nor animals treated with matrix metalloproteinase inhibitors, iNOS inhibitors, or antioxidants showed any differences in CNS bacterial growth from infected wt littermates or infected, untreated animals (46–50). These observations supported the concept that the subarachnoid space is a localized area of host immunodeficiency allowing unrestrained proliferation of pneumococci which, if untreated, overpowers the host until death occurs. This functional deficit is assumed to be due to the lack of sufficient Ig and complement concentrations to achieve opsonic and bactericidal activity (51, 52). In contrast, Tuomanen et al. (53) reported that complement depletion (by treatment with Egyptian cobra venom factor) resulted in a diminished opsonophagocytosis of encapsulated *S. pneumoniae* and consequently to increased bacterial titers in the CSF of rabbits with pneumococcal meningitis. Thus, complement factors (e.g., C3b) appear to mediate partial killing, although not clearance of *S. pneumoniae* from CSF. According to previous reports by Stahel et al. (54, 55), we observed a marked up-regulation of the brain C3 mRNA expression during meningitis. Brain C3 mRNA levels did not differ between infected TLR2−/− mice and wt littermates. However, TLR2 deficiency was accompanied by an increased brain expression of Crry. Murine Crry is a known inhibitor of the activation of the third component of complement (56). Combined, it seems conceivable that the higher cerebellar bacterial titers in TLR2−/− mice correlate with increased brain Crry expression rather than with the slightly reduced initial host immune response, which was evidenced by decreased brain expression of TNF-α and MIP-2 in infected TLR2−/− mice at 4 h postchallenge.

In our study, we also detected (a slight) increase in both spleen MIP-2 and iNOS mRNA and lung TNF-α expression in TLR2−/− mice during early meningitis. This was associated with a marked increase in blood bacterial titers. Previous studies have clearly demonstrated that secondary bacteremia is a common occurrence
in meningitis, is directly related to the concentration of the microorganisms within the CSF, and is dependent on active bacterial multiplication within the subarachnoid space (57). Therefore, it is conceivable that the higher brain bacterial titers lead to higher concentrations of S. pneumoniae in the blood, which in turn induces a more pronounced initial host immune response in TLR2−/− mice.

In conclusion, our study showed that TLR2 plays a significant role in a murine model of experimental pneumococcal meningitis. Nonetheless, a robust immune response was established in TLR2−/− mice upon infection, pointing at TLR2-independent cellular recognition of S. pneumoniae cell-wall products. Ectopic expression-dependent cell activation implicates TLR4 as another potential candidate for sensing pneumococcal infection. However, its role in vivo remains to be analyzed, and further pattern recognition receptors remain to be implicated in recognition of pneumococcal infection of the host organism.

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