Toll-Like Receptor 2–Deficient Mice Are Highly Susceptible to *Streptococcus pneumoniae* Meningitis because of Reduced Bacterial Clearing and Enhanced Inflammation

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Toll-like receptor–2 (TLR2) mediates host responses to gram-positive bacterial wall components. TLR2 function was investigated in a murine *Streptococcus pneumoniae* meningitis model in wild-type (wt) and TLR2-deficient ($TLR2^{-/-}$) mice. $TLR2^{-/-}$ mice showed earlier time of death than wt mice ($P < .02$). Plasma interleukin-6 levels and bacterial numbers in blood and peripheral organs were similar for both strains. With ceftriaxone therapy, none of the wt but 27% of the $TLR2^{-/-}$ mice died ($P < .04$). Beyond 3 hours after infection, $TLR2^{-/-}$ mice had higher bacterial loads in brain than did wt mice, as assessed with luciferase-tagged *S. pneumoniae* by means of a Xenogen-CCD (charge-coupled device) camera. After 24 h, tumor necrosis factor activity was higher in cerebrospinal fluid of $TLR2^{-/-}$ than wt mice ($P < .05$) and was related to increased blood-brain barrier permeability (Evans blue staining, $P < .02$). In conclusion, the lack of TLR2 was associated with earlier death from meningitis, which was not due to sepsis but to reduced brain bacterial clearing, followed by increased intrathecal inflammation.

Streptococcus pneumoniae is the major cause of meningitis in adults. Despite antimicrobial therapy and critical care medicine, the mortality remains as high as 28% [1]. In addition, 50% of the survivors have neurologic sequelae, indicating postinflammatory damage [2]. In the pathogenesis of meningitis, penetration of bacteria through the blood-brain barrier (BBB) initiates activation of brain endothelia and leads to leukocyte recruitment and release of inflammatory mediators. Subsequently, the subarachnoidal inflammation stimulates astrocytes, microglia, and neurons to produce cytokines and chemokines [3–5]. A bacterial level of ⩾5 log cfu in the brain initiates a harmful inflammatory cascade, which causes development of symptoms and determines the prognosis [6].

Pneumococci enter into the cerebral compartment through the BBB via binding of their cell-wall component phosphorylcholine to the platelet-activating factor receptors expressed on activated cells [7, 8]. In view of the finding that antagonists of the platelet-activating factor receptor do not completely block bacterial invasion [7, 8], other cell-wall components, such as peptidoglycan and lipoteichoic acid, might also induce inflammation via activation of pattern recognition receptors expressed in these cells [9].

Toll was first cloned in *Drosophila* species [10] and was found to be involved in host defense against fungal infection [11]. In humans, 10 homologous genes have been identified encoding Toll-like receptors (TLRs) [12–14]. TLR2 is involved in cell activation by gram-positive bacterial cell wall and membrane components, such as peptidoglycan, lipoteichoic acid, and lipoproteins [15–17]. Accordingly, macrophages isolated from TLR2-deficient ($TLR2^{-/-}$) mice are hyporesponsive to *Staphylococcus aureus* peptidoglycan stimulation [18]. Despite growing evidence implicating TLR2 in recognition of gram-positive bacterial cell-wall components in vitro, its role in bacterial meningitis is still unknown. In this study, we compared disease severity and outcome in $TLR2^{-/-}$ and wild-type (wt) mice in an adult mouse model of *S. pneumoniae* and *Listeria monocytogenes* meningitis. Bacterial numbers in the brain, bacterial counts, and leukocyte recruitment into the subarachnoid space, as well as meningeal inflammation and BBB permeability, were analyzed.

Materials and Methods

Preparation of bacterial inocula. *S. pneumoniae* (clinical isolate of serotype 3) was grown for 7 h in double Mueller-Hinton broth

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Mice were kept in the Animal House of the Department of Research, University Hospitals, Basel, according to the regulations of Swiss veterinary law.

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(Difco Laboratories), subcultured overnight in new Mueller-Hinton broth, and washed in 0.9% sterile saline (12,000 *g* for 6 min) immediately before use. The inoculum size was calculated from optical density determinations (optical density of 0.4 = 1×10^8 cfu) and was retrospectively assessed by counting colony-forming units on blood agar plates. *L. monocytogenes* (strain EGD; provided by R. M. Zinkernagel, University Hospital, Zürich) was grown overnight at 37°C, yielding 1–2 $\times 10^9$ cfu in trypticase soy broth (BBL Microbiology Systems).

Mouse meningitis models. Six- to eight-week-old C57BL/6 wt (RCC) and TLR2$^{-/-}$ mice (provided by William J. Rieflin, Tularik, South San Francisco, CA; mice had been back-crossed for 6 generations on a C57BL/6 background) were kept under specific pathogen–free conditions. Mice were anesthetized via intraperitoneal injection of 100 mg/kg ketamine (Ketalar; Warner-Lambert) and 20 mg/kg xylazinum (Xylapan; Graeub) and subsequently inoculated intracerebrally into the left forebrain with either 0.9% NaCl, live *S. pneumoniae* (2×10^2 or 3×10^3 cfu), or live *L. monocytogenes* (5×10^2 cfu) in a 25-μL volume. In addition to the intracerebral inoculation, selected experiments were done by an intracisternal route of infection, as described elsewhere for experimental meningitis in infant rats [19]. Mice (n = 10 each C57BL/6 wt and TLR2$^{-/-}$) were deeply anesthetized and infected by direct intracisternal injection of 10 μL of saline containing 8.5 $\log_{10}$ cfu/mL *S. pneumoniae* by means of a 32-gauge needle.

The health status of the mice was assessed by the following scores, as described elsewhere [20]: 1, exhibited normal motor activity and turned upright in <5 s when put on its back; 2, showed decreased spontaneous activity, but still turned upright in <5 s; 3, turned upright in >5 s; 4, did not turn upright; or 5, did not move. After 6, 12, 24, 48, and 72 h or if they presented with a score of 5, mice were killed by intraperitoneal injection of 100 mg/kg pentobarbital (Abbott Laboratories). Blood was obtained by intracardiac puncture and collected in EDTA. Animals were perfused with Ringer's solution (Braun Medical) into the left cardiac ventricle until the effluent became clear. Cerebrospinal fluid (CSF) was obtained by puncture of the cisterna magna, as described elsewhere [21]. Because of the small volumes (3–6 μL) obtained from each animal, CSF from 4 mice was pooled.

Selected mice were treated with 80 mg/kg ceftriaxone (Rocephin; Hoffmann–La Roche) dissolved in 0.1 mL of saline via intraperitoneal injection twice daily for 5 days. Treatment was started 18 h after infection. For the leukocyte depletion treatment, cyclophosphamide (Sigma) was reconstituted with sterile PBS and injected intraperitoneally (250 mg/kg in 0.2 mL) 48 h before *S. pneumoniae* inoculation.

Real-time in vivo imaging study of meningitis with use of bioluminescent S. pneumoniae *transformed with gram-positive lux transposon.* Mice were intracerebrally injected with 3×10^3 cfu of luciferase-tagged *S. pneumoniae* serotype 3 (Xen10) and subsequently shaved for better imaging. This bacterial strain (provided by L. Chen; Xenogen) was constructed as described elsewhere [22]. After infection, analysis of photons was done repeatedly in mice under isoflurane inhalation anesthesia in an IVIS CCD (charge-coupled device) camera (Xenogen) coupled to the LivingImage software package (Xenogen).

Determination of bacterial counts and inflammatory parameters. Blood samples collected in EDTA and pooled CSF samples were serially diluted in 0.9% NaCl to assess the bacterial load after plating and incubation at 37°C for 24 h. Blood and CSF were centrifuged at 10,000 *g* for 20 min (4°C), and CSF was centrifuged at 800 *g* for 7 min (room temperature), to obtain plasma and cell-free CSF, respectively. Thereafter, they were stored at −20°C until cytokine determinations were done. The pelleted CSF cells were counted and identified via cytospin or were phenotypically analyzed. Brains were removed, and hemispheres were separated and homogenized with a Polytron homogenizer in 1 mL of 0.1 mol/L PBS. Bacterial titers were determined by plating serial 10-fold dilutions in 0.9% NaCl on blood agar plates. The concentration of the proinflammatory cytokine tumor necrosis factor (TNF) in plasma and CSF was determined with a bioassay, measuring the degree of cytotoxicity on WEHI cells in the presence of 1 μg/mL actinomycin D, with use of mouse recombinant TNF as a standard. Interleukin (IL)–6 in plasma was measured by a mouse IL-6 ELISA kit (OptEIA; PharMingen).

Evaluation of BBB integrity. BBB permeability was assessed by measuring Evans blue extravasation, according to a method described elsewhere [23]. Evans blue (0.2 mL, 2% in NaCl; Sigma) was injected into the tail vein of infected (24 or 48 h) mice 60 min before death. Mice were perfused as described above, their brains were removed, and the hemispheres were separated. Each hemi-

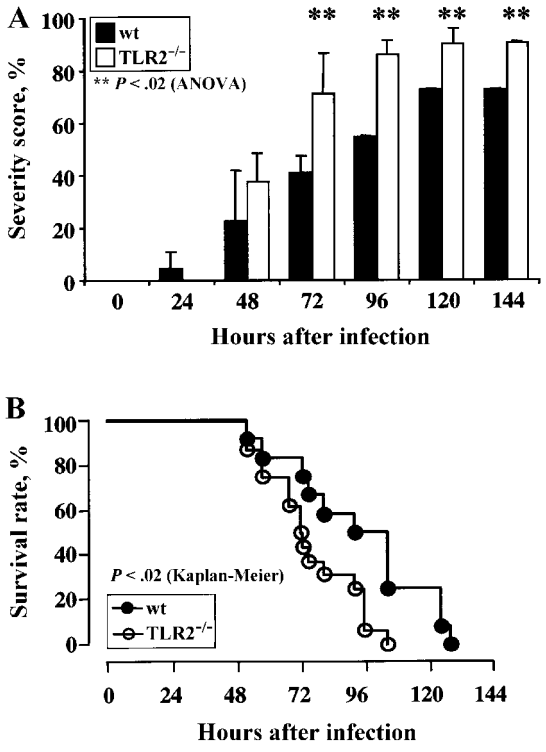

Figure 1. *A,* Percentage of wild-type (wt; n = 22) and Toll-like receptor-2–deficient (TLR2$^{-/-}$; n = 21) mice showing high severity score (including score 4 and score 5) after intracerebral injection of 2×10^2 cfu of *Streptococcus pneumoniae*. Data from 3 independent experiments are represented as the mean percentage of high severity score ± SD. **$P < .02$, repeated-measures analysis of variance (ANOVA). *B,* Survival of TLR2$^{-/-}$ (n = 21) and wt (n = 22) mice after intracerebral injection of 2×10^2 cfu of *S. pneumoniae.* $P < .02$, log-rank test.

Table 1. Mean survival time of *Listeria meningitis* in Toll-like receptor-2–deficient ($TLR2^{-/-}$) and wild-type (wt) mice.

Mouse strain	No. of mice	Survival time, mean h ± SD	*P*
wt	11	91.5 ± 20.4	<.02[a]
$TLR2^{-/-}$	19	71.5 ± 16.5	—

NOTE. Female mice (6–8-weeks old) were infected intracerebrally with 5×10^2 cfu of *L. monocytogenes* in 25 μL of NaCl.

[a] Log-rank test in Kaplan-Meier analysis.

sphere was homogenized in 1 mL of 0.1 mol/L PBS and then centrifuged at 1000 *g* for 15 min; 0.7 mL of 100% trichloroacetic acid was added to 0.7 mL of the supernatant. The mixture was incubated at 4°C for 18 h and then centrifuged at 1000 *g* for 30 min. The amount of Evans blue in the supernatant was measured spectrophotometrically at 610 nm and compared with a serially diluted standard solution. Results were expressed as micrograms per brain hemisphere.

Statistical analysis. Differences in survival between wt and $TLR2^{-/-}$ mice were tested by the Kaplan-Meier analysis log-rank test. Data of disease severity scores were assessed by analysis of variance corrected for repeated measurements, followed by posthoc analysis (Fisher's *P* least-squares difference, Scheffe, and Bonferroni/Dunn tests). Results of the colony-forming unit measurements and IL-6 levels in blood were compared by the Mann-Whitney *U* test. Differences in CSF parameters between wt and $TLR2^{-/-}$ mice were analyzed with the nonparametric Wilcoxon signed rank test. The relationship between 2 parameters (parametric variables) was assessed in a linear regression model with the Spearman rank correlation test. In all statistical tests, $P < .05$ was considered to be statistically significant.

Results

$TLR2^{-/-}$ mice have a higher susceptibility to S. pneumoniae *and* L. monocytogenes *meningitis.* To evaluate the in vivo role of TLR2 during gram-positive bacterial meningitis, wt and $TLR2^{-/-}$ mice were infected intracerebrally with *S. pneumoniae* or *L. monocytogenes*. The severity of the disease was monitored by assessing both the clinical score and the survival rate.

Control animals, injected with 0.9% NaCl, showed no altered health status. Infected mice remained free of clinical signs of meningitis at 6 and 12 h after infection but had gradually reduced spontaneous activity between 12 and 24 h (data not shown). As shown in figure 1*A,* both wt and $TLR2^{-/-}$ mice became severely sick during *S. pneumoniae* meningitis. The percentage of severely ill mice was significantly higher among $TLR2^{-/-}$ than control mice after 72 h ($P < .02$, repeated-measures analysis of variance). Indeed, only 30% of $TLR2^{-/-}$ mice survived at 72 h, and all died at 102 h after infection (figure 1*B*), whereas 60% of the wt mice survived at 72 h and died of *S. pneumoniae* meningitis later (136 h after infection). $TLR2^{-/-}$ mice also showed reduced survival time ($P < .02$, Kaplan-Meier analysis), compared with wt mice with *L. monocytogenes* meningitis (table 1). These results show that $TLR2^{-/-}$ mice had more severe symptoms and earlier death than did wt mice during *S. pneumoniae* and *L. monocytogenes* meningitis.

Higher disease severity in $TLR2^{-/-}$ mice is independent of sepsis. To investigate whether the high susceptibility of $TLR2^{-/-}$ mice to meningitis was due to impaired host defense, colony-forming units in blood were counted 1, 2, and 3 days after infection with 3×10^3 cfu of *S. pneumoniae.* Bacterial counts were similar in wt and $TLR2^{-/-}$ mice at all time points (figure 2*A*). Six and 12 h after infection, <10% of both wt and $TLR2^{-/-}$ mice showed positive blood culture results at low bacterial density (data not shown).

Plasma IL-6, a prognostic marker of sepsis [24], was also analyzed during meningitis. In accordance with bacterial counts, IL-6 levels were not significantly different in wt and $TLR2^{-/-}$ mice 1–3 days after infection (figure 2*B*). Twelve and 24 h after intracerebral infection, emitted photons from luciferase-tagged *S. pneumoniae* were analyzed in peripheral organs (figure 3). Twenty-four hours after infection, fluorescence accumulated only in the lung with a similar intensity in wt and $TLR2^{-/-}$ mice. No other organ showed a detectable level of emitted photons 12 and 24 h after infection. Taken together, these data indicate that wt and $TLR2^{-/-}$ mice had a similar severity of sepsis and therefore an intact systemic host defense during *S. pneumoniae* meningitis.

To differentiate between sepsis and inflammation as a cause of death, wt and $TLR2^{-/-}$ mice received antibiotic treatment

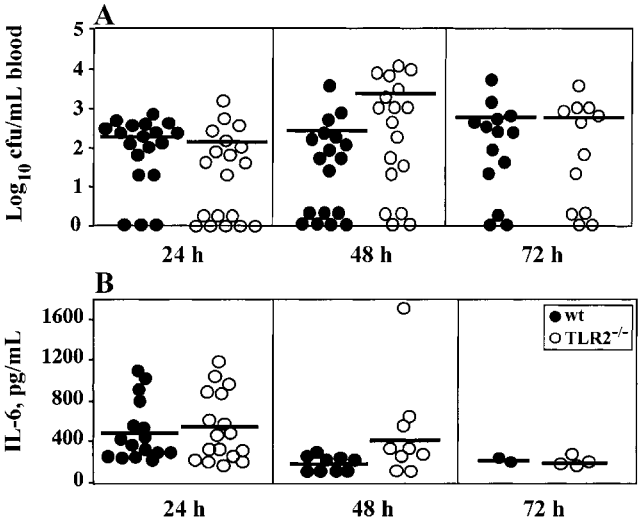

Figure 2. Colony-forming unit counts in blood *(A)* and interleukin (IL)–6 levels in plasma *(B)* in wild-type (wt; $n = 22$) and Toll-like receptor-2–deficient ($TLR2^{-/-}$; $n = 21$) mice after intracerebral injection of 3×10^3 cfu of *Streptococcus pneumoniae.* Blood was collected 24, 48, and 72 h after infection. Results from individual mice are shown. *Horizontal line,* mean. $P > .05$, Mann-Whitney *U* test.

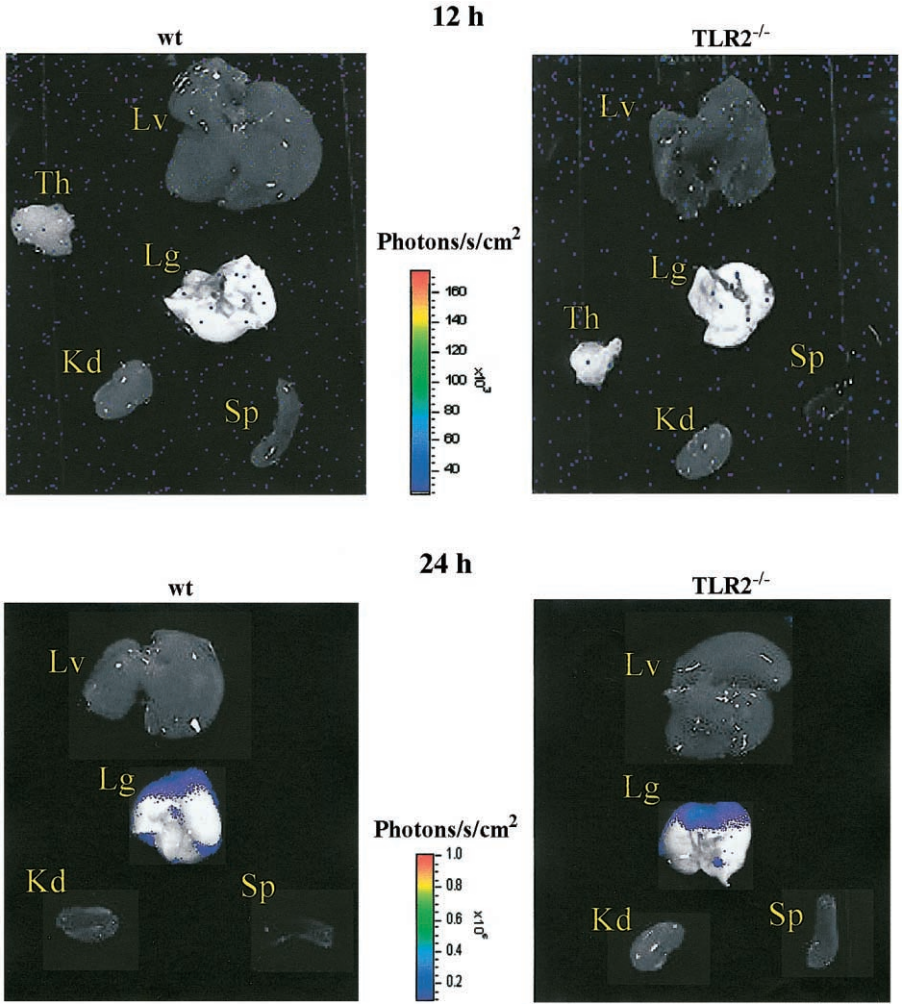

Figure 3. Emitted photons by luciferase-tagged *Streptococcus pneumoniae* in several organs from wild-type (wt) and Toll-like receptor-2–deficient ($TLR2^{-/-}$) mice detected by a highly sensitive CCD (charge-coupled device) camera (IVIS imaging system; Xenogen) 12 and 24 h after intracerebral injection of 3×10^3 cfu of *S. pneumoniae*. Results from 1 wt and 1 $TLR2^{-/-}$ mouse are shown. Kd, kidney; Lg, lung; Lv, liver; Sp, spleen; Th, thymus.

to cure the sepsis. It is known that wt mice treated in the first 24 h after infection with ceftriaxone (80 mg/kg every 12 h for 5 days) survive without apparent signs of meningitis [25]. Delay of treatment beyond 30 h leads to death in >50% of the infected mice [25]. We treated infected mice with ceftriaxone after *S. pneumoniae* infection, and the obtained clinical scores and survival rates of both ceftriaxone-treated and control mice are shown in table 2. With ceftriaxone treatment, all wt mice survived without any clinical signs of disease after 6 days, whereas 4 (27%) of 15 $TLR2^{-/-}$ mice died despite treatment ($P < .04$, Kaplan-Meier analysis). As described above, all untreated wt and $TLR2^{-/-}$ mice died within 4–6 days (data not shown). Treatment failure in $TLR2^{-/-}$ mice could be explained by the fact that at the initiation of the treatment (18 h after infection), 7% of $TLR2^{-/-}$ mice but none of the wt mice were severely sick. By day 6, 27% of the treated $TLR2^{-/-}$ mice remained severely ill and subsequently died, whereas all wt mice recovered during treatment.

$TLR2^{-/-}$ mice have a higher bacterial load in brain and stronger meningeal inflammation after intracerebral infection with S. pneumoniae *than do wt mice.* To further explain the high susceptibility of $TLR2^{-/-}$ mice to *S. pneumoniae* meningitis, we examined bacterial counts, leukocyte recruitment, and TNF release in the CSF as well as bacterial densities in the brain. Monitoring of meningitis in animals intracerebrally injected with luciferase-tagged *S. pneumoniae* revealed a higher fluorescence intensity in $TLR2^{-/-}$ brains than in wt brains between 2 and 24 h after infection (figure 4*A*). Twenty-four hours after infection, mice were

Table 2. Effect of ceftriaxone treatment on survival during *Streptococcus pneumoniae* meningitis in wild-type (wt) and Toll-like receptor-2–deficient ($TLR2^{-/-}$) mice.

Mouse strain, days after infection	No. (%) of mice showing severe lethargy	Survivors, %
wt ($n = 15$)		
1	0	100
2	1 (7)	100
3	1 (7)	100
4	1 (7)	100
6	0	100
$TLR2^{-/-}$ ($n = 15$)		
1	1 (7)	100
2	3 (20)	100
3	3 (20)	100
4	4 (27)	87
6	4 (27)	73

NOTE. Ceftriaxone treatment (80 mg/kg) was administered intraperitoneally every 12 h for 5 days, starting 18 h after intracerebral injection of 3×10^3 cfu of *S. pneumoniae*. $P < .04$, $TLR2^{-/-}$ vs. wt mice (log-rank test in Kaplan-Meier analysis).

killed, and the photon emission was stronger and more widely spread in isolated brains of $TLR2^{-/-}$ mice than in those of wt mice (figure 4*A*). In particular, signals from bacteria were differently distributed in $TLR2^{-/-}$ and wt mice. In contrast to the picture in wt mice, bacteria were not accumulated in the ventricles of $TLR2^{-/-}$ mice but concentrated on the ipsilateral and contralateral sides of the injection site. Furthermore, a quantitative time course analysis of emitted fluorescence from infected animals showed a more rapid bacterial growth in $TLR2^{-/-}$ mice, compared with wt mice, which was most pronounced already 2 h after *S. pneumoniae* inoculation (figure 4*B*). Counting of colony-forming units in ipsilateral and contralateral brain hemispheres 24 h after infection of the left side of the brain confirmed that, in both parts, bacterial numbers were higher in $TLR2^{-/-}$ than wt mice (median, 1.8×10^7 cfu [left] and 1.2×10^7 cfu [right] in $TLR2^{-/-}$ mice, and 7.4×10^5 cfu [left] and 8.8×10^5 cfu [right] in wt mice; $P < .04$). Counting of colony-forming units in brain homogenates 24 h after intracisternal infection with *S. pneumoniae* confirmed that bacterial numbers were also significantly higher in $TLR2^{-/-}$ than wt mice when the intracisternal route of infection was used (median, 6.2×10^5 cfu and 6.8×10^4 cfu in $TLR2^{-/-}$ and wt mice, respectively; $P < .04$, Mann-Whitney *U* test).

Twelve hours after intracerebral infection, $TLR2^{-/-}$ mice showed a lower influx of leukocytes, compared with wt mice (mean ± SD, 1703 ± 186 and 12,110 ± 3691 leukocytes/μL of CSF, respectively; $P < .05$; figure 5*A*). After this time point, leukocyte numbers and bacterial counts in the CSF of the 2 groups were almost identical (figure 5*B*). As expected, infiltrating polymorphonuclear cell numbers and bacterial counts in the CSF were similar in $TLR2^{-/-}$ and wt mice after intracisternal infection, as we had observed in the intracerebral infection model.

Meningeal inflammation, as assessed by TNF activity in the CSF, was significantly higher in $TLR2^{-/-}$ than in wt mice 24 h after infection with *S. pneumoniae* (1077 ± 201 and 705 ± 74 pg/mL of CSF, respectively; $P < .02$; figure 5*C*). Early after infection, at 6 and 12 h, TNF activity was low or below detection (<20 pg/mL), and, after 48 h, the difference between $TLR2^{-/-}$ mice and wt mice was no longer statistically significant, because of high variability (figure 5*C*).

An association between disease severity and meningeal inflammation was documented by a significant relationship between TNF activity in the CSF and severity score 24 h after infection ($r = 0.589$; $P < .001$; data not shown). Thus, the level of TNF in CSF might reflect the intensity of clinical signs during the first day of infection.

To investigate whether infiltrating leukocytes contribute to meningeal inflammation during *S. pneumoniae* meningitis, we analyzed TNF activity in the CSF in infected mice rendered leukopenic by cyclophosphamide. As shown in figure 6, TNF activity was significantly decreased in leukopenic $TLR2^{-/-}$ mice, compared with immunocompetent mice 24 h after infection ($P < .05$). In contrast, no difference in TNF activity was observed in wt mice with or without cyclophosphamide pretreatment.

$TLR2^{-/-}$ mice have enhanced BBB permeability after intracerebral infection with S. pneumoniae. Alteration of the BBB is a consequence of meningeal inflammation. Therefore, we analyzed changes in BBB permeability during *S. pneumoniae* meningitis with the Evans blue extravasation method.

Measures of BBB permeability and severity of the disease in wt and $TLR2^{-/-}$ mice are presented in table 3. Infection with *S. pneumoniae* induced alteration of the BBB in both wt and $TLR2^{-/-}$ mice, as evidenced by higher Evans blue concentrations in brains of $TLR2^{-/-}$ mice, compared with control mice. This BBB disruption occurred early in disease (24 h) and was stronger in $TLR2^{-/-}$ mice than in wt mice. At the same time, $TLR2^{-/-}$ mice showed more severe symptoms of meningitis than did wt mice (table 3). In addition, meningeal inflammation, assessed by TNF in the CSF, was significantly related to BBB permeability ($r = 0.768$; $P < .02$; data not shown).

Discussion

We have studied the in vivo role of TLR2 in an adult mouse model of *S. pneumoniae* meningitis. $TLR2^{-/-}$ mice had more severe clinical symptoms than did wt mice and subsequently showed earlier death. Similar results were obtained with *L. monocytogenes* as the infecting agent (table 1). This result is in agreement with a previous report on increased mortality of $TLR2^{-/-}$ mice from *S. aureus* sepsis [26]. In that model, $TLR2^{-/-}$ mice showed reduced systemic bacterial clearance, compared with wt mice, after a high but not after a low intra-

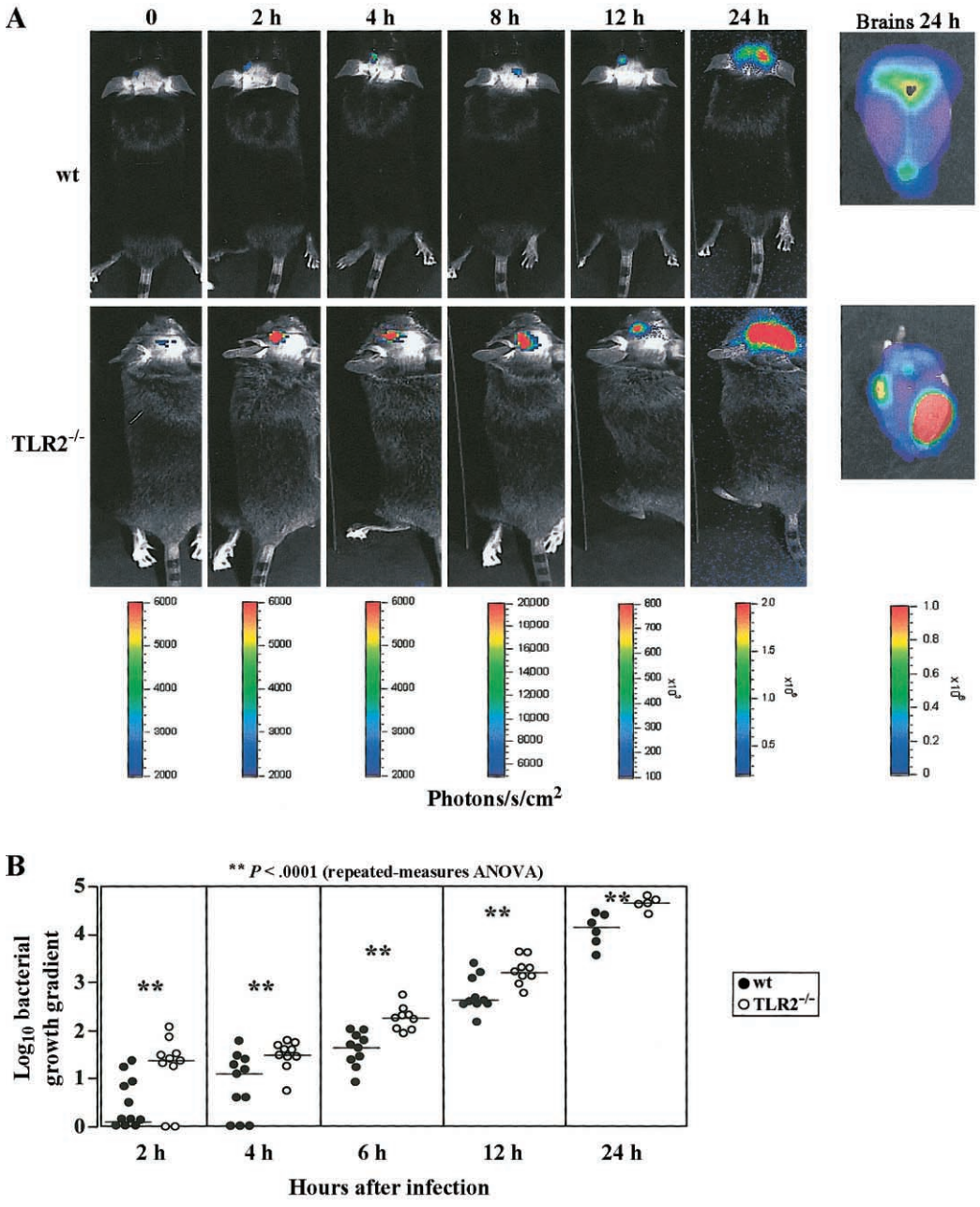

Figure 4. *A,* Time course of infection in wild-type (wt) and Toll-like receptor-2–deficient ($TLR2^{-/-}$) mice after intracerebral injection of 3×10^3 cfu of luciferase-tagged *Streptococcus pneumoniae.* Images from 1 animal in each group at time of infection (0 h) and 2, 4, 6, 12, and 24 h after infection and isolated brains 24 h after infection are shown. *B,* Emitted light was also quantified by LivingImage software (Xenogen) and shown as bacterial growth gradient (calculated as ratio between emitted photons and time elapsed after infection). Results from individual mice are shown, with median *(horizontal line)*. $^{**}P < .0001$, repeated-measures analysis of variance (ANOVA) ($n = 11$ and $n = 10$ at 2 and 4 h, $n = 10$ and $n = 9$ at 6 and 12 h, and $n = 6$ and $n = 5$ at 24 h, for wt and $TLR2^{-/-}$ mice, respectively).

venous inoculum. According to the authors, this was due to failure to recognize invading bacteria [26]. Instead, in our model with a low inoculum in the brain, systemic host defense was not impaired in the absence of TLR2, because similar plasma levels of IL-6 and numbers of bacteria were observed in blood and organs of $TLR2^{-/-}$ and wt mice. Moreover, in ceftriaxone-injected animals, in which the infection is successfully treated, 27% of the $TLR2^{-/-}$ mice died, whereas all wt mice survived.

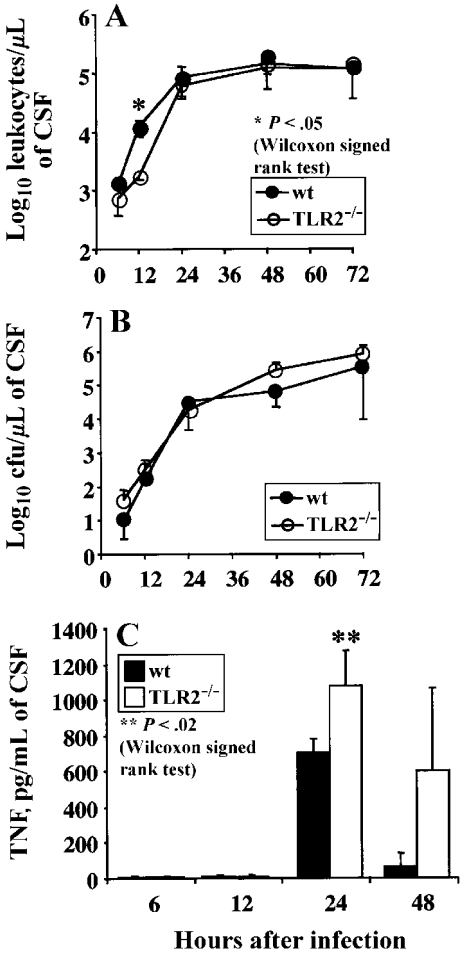

Figure 5. Time course of cerebrospinal fluid (CSF) parameters in wild-type (wt) and Toll-like receptor-2–deficient ($TLR2^{-/-}$) mice ($n = 12$ at each time point in both groups) after intracerebral injection of 3×10^3 cfu of *Streptococcus pneumoniae.* Pooled CSF from 4 mice was collected at indicated times after infection. Infiltrating leukocytes *(A),* bacterial numbers *(B),* and tumor necrosis factor (TNF) levels *(C)* were analyzed in CSF. Results of 3 independent experiments are shown as mean ± SD. When errors bars are not seen, they fall within symbol. $*P < .05$ and $**P < .02$, both by the Wilcoxon signed rank test.

This indicates that the higher mortality in $TLR2^{-/-}$ mice was not due to defective bacterial clearance in the periphery.

We observed more bacteria in the brains, but not in the CSF, of $TLR2^{-/-}$ mice. More rapid growth, which was detectable as early as 2 h after infection, was associated with a protracted accumulation of the bacteria around the site of injection in $TLR2^{-/-}$ mice. This was in contrast to wt mice, in which the bacteria were mainly in the ventricles after 24 h. It is known that TLR2 is constitutively expressed in the choroid plexus and the lateral ventricle lining [27]. It can be hypothesized that the lack of TLR2 in the choroidal epithelia and/or endothelia and in the ependymal cells contributed to the particular distribution pattern of the bacteria by altering the CSF flow. The different localization of the bacteria in brains of wt and $TLR2^{-/-}$ mice probably also explains why the numbers of colony-forming units in CSF were similar in both strains.

Leukocyte infiltration following bacterial invasion into the central nervous system is correlated with brain edema and injury in bacterial meningitis [28, 29]. The rapid increased bacterial growth in brain homogenates of $TLR2^{-/-}$ mice may, in turn, have contributed to the enhanced inflammation and to the earlier aggravation of the symptoms in these animals. Despite higher bacterial numbers in brain, pleocytosis was not significantly different in wt and $TLR2^{-/-}$ mice, except for a transient delay in leukocyte recruitment 12 h after infection in $TLR2^{-/-}$ mice. Perhaps the delayed leukocyte influx into the CSF could be explained by a lack of TLR2 action on integrin or intercellular adhesion molecule (ICAM) expression, because it has been shown that blocking of the integrin CD18 and of the adhesion molecule ICAM attenuated meningeal inflammation and tissue damage [30, 31]. However, it is also possible that the recruitment of leukocytes may have been delayed in $TLR2^{-/-}$ mice because of the lack of TLR2 activity on IL-8 [32]. Indeed, a previous study showed that intravenous treatment with antibody against IL-8 attenuates the neutrophil pleocytosis during experimental pneumococcal meningitis [33].

Despite the slower early influx of leukocytes, we observed higher TNF activity during *S. pneumoniae* meningitis in CSF of $TLR2^{-/-}$ than of wt mice. TNF is elevated in CSF in human and experimental meningitis. It has detrimental effects, because its neutralization decreases meningeal inflammation, as shown in a rabbit pneumococcal meningitis model [34, 35]. However, the total lack of TNF also adversely effects host defense in meningitis. Indeed, mice with a targeted deletion of TNF die earlier

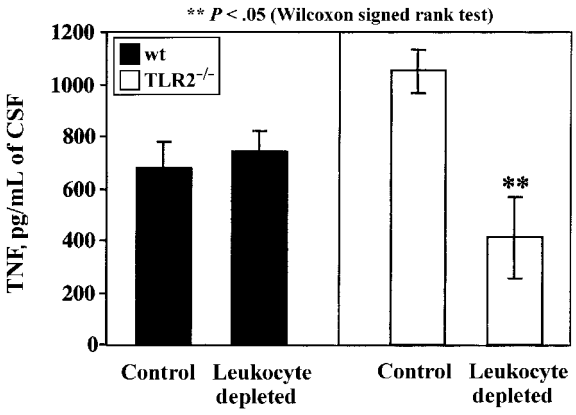

Figure 6. Tumor necrosis factor (TNF) levels in cerebrospinal fluid (CSF) in leukocyte-depleted and immunocompetent control mice after intracerebral injection of 3×10^3 cfu of *Streptococcus pneumoniae.* In each experiment, pooled CSF from 4 mice was collected 24 h after infection. Results of 3 independent experiments are shown as mean ± SD. $**P < .05$, nonparametric Wilcoxon signed rank test. Leukocyte depletion in CSF was 81.2% ± 20.1% in Toll-like receptor-2–deficient ($TLR2^{-/-}$) mice and 76.2% ± 10.5% in wild-type (wt) mice.

Table 3. Blood-brain barrier permeability and clinical score in Toll-like receptor-2–deficient ($TLR2^{-/-}$) and wild-type (wt) mice after intracerebral injection of 3×10^3 cfu of *Streptococcus pneumoniae*.

	24 h		48 h	
Mouse strain	Clinical score[a]	Evans blue stain[b]	Clinical score[a]	Evans blue stain[b]
wt	1.3 (1–2.8)	1.1 ± 0.1	3.5 (3–4)	3.4 ± 1.9
$TLR2^{-/-}$	1.9 (1–3)	1.3 ± 0.6	4.9 (4.8–5)	7.0 ± 0.9

[a] Data are median (range) clinical score. See Materials and Methods for a description of the scoring system.

[b] Data are mean ± SD fold increase, compared with uninfected control mice.

than control mice from intracerebral *S. pneumoniae* infection because of the lack of systemic clearance of bacteria [36]. A systemic protective effect of TNF in our model is unlikely, in view of similar signs of sepsis in $TLR2^{-/-}$ and wt mice. An important finding of the present study is that, paradoxically, $TLR2^{-/-}$ mice show an increased inflammation after infection. Thus, it appears that these animals are characterized by inefficient regulation of the inflammatory reaction. Because $TLR2^{-/-}$ mice had more TNF activity in the CSF than did wt mice, it is tempting to speculate that the engagement of TLR2 contributes to a down-regulation of the TNF in the CSF. This is not ascribed to an increased production of IL-10 (data not shown) and might be associated with an altered release of TNF from intracellular stores and the cell surface.

The consequence of meningeal inflammation is an alteration of the BBB integrity. In the present study, the BBB disruption was markedly increased in $TLR2^{-/-}$ mice, compared with control mice, and was related to TNF levels in the CSF. This corroborates the known role of TNF in BBB disruption and consequently in the course of disease [35, 37, 38].

Our observation of a stronger in vivo TNF induction in $TLR2^{-/-}$ than in wt mice is in contrast to the reduced in vitro TNF production of $TLR2^{-/-}$ in macrophages in response to *S. aureus* [26]. However, an important difference in our experimental model is the use of live bacteria, whereas that study [26] was conducted with heat-killed bacteria. We therefore conclude that live *S. pneumoniae* and their turnover products induce TNF differently than do heat-killed bacteria. It is likely that, during infection, other receptors are activated together with TLR2. The nature of these other receptors is not clear. They could be other TLRs, such as TLR1 and TLR6, which are also triggered by bacterial products [39]. In this case, their stimulation led to the release of large amounts of TNF, which are higher that those induced in the presence of TLR2. This might occur because these TLRs show sequence similarities in their intracytoplasmic regions and then share common transducing mechanisms. Other receptors involved in this signaling could be specific for *S. pneumoniae* products, such as platelet-activating factor receptors [7] or C-reactive protein [40]. Finally, another possibility is that triggering of TLR2 on a particular cell type only might lead to the regulation of TNF. However, in this case, it is not clear how the engagement of TLR2 would down-regulate the TNF release.

The same accelerated death rate as for those infected with *S. pneumoniae* was also observed in *Listeria*-infected $TLR2^{-/-}$ mice, yet they and *Listeria*-infected wt mice had similar TNF activity in the CSF (data not shown). This suggests that different mechanisms are involved in *S. pneumoniae* and *L. monocytogenes* meningitis.

As reported elsewhere, in *L. monocytogenes* meningitis, monocytes contributed to the leukocyte infiltration in CSF, whereas *S. pneumoniae* meningitis caused only polymorphonuclear leukocyte accumulation [41]. Together with our recent observation that TLR2 protein is constitutively expressed in mouse polymorphonuclear leukocytes, but not in macrophages (authors' unpublished data), the excess TNF in *S. pneumoniae* meningitis may be polymorphonuclear leukocyte–derived. To confirm this hypothesis, we investigated which cells contributed to the TNF release in the subarachnoid space during *S. pneumoniae* meningitis. Depletion of leukocytes by cyclophosphamide treatment before infection led to a decrease in TNF level in $TLR2^{-/-}$ but not in wt mice. This result validated that leukocytes participated in TNF production but were not the only source of TNF in $TLR2^{-/-}$ mice. Brain microvascular endothelia have been shown to produce TNF in pneumococcal meningitis or after stimulation with cell walls of *S. pneumoniae* [7, 42]. Furthermore, we and others could show that primary cultures of astrocytes, microglial cells, and neurons release TNF after stimulation with live *S. pneumoniae* (authors' unpublished data) and pneumococcal cell wall components [43].

In conclusion, the mouse *S. pneumoniae* meningitis model described here is fatal for both wt and $TLR2^{-/-}$ mice. The clinical course of meningitis, the number of bacteria, the level of meningeal inflammation, and BBB damage are aggravated in mice lacking TLR2. Taken together, these results indicate, for the first time, a contribution of TLR2 in regulation of bacterial clearing and inflammatory response in pneumococcal meningitis.

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