Role of the pattern recognition receptors Toll-like receptor 2 and CD14 in murine pneumococcal meningitis

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General summary

Streptococcus pneumoniae is the major pathogen causing meningitis in adults. Despite antimicrobial therapy and critical care medicine, mortality remains high and about 50% of the survivors suffer from neurological sequelae. In the present study, the function of the pattern recognition receptors TLR2 and CD14 in mediating host innate immune response towards *S. pneumoniae*, was investigated in a mouse model of pneumococcal meningitis using mice with a targeted deletion of the corresponding genes. The role of TLR2 and CD14 are the topics of sections I and II. Modulation of outcome in wt, $TLR2^{-/-}$ and $CD14^{-/-}$ mice with meningitis by antibiotic and/or with anti-inflammatory treatment with TNF-alpha converting enzyme (TACE) inhibitor treatment is evaluated in section III.

$TLR2^{-/-}$ mice were found to have more severe clinical symptoms than did wt mice and subsequently showed earlier death during meningitis. This accelerated death was not due to sepsis, but rather to reduced brain bacterial clearing, followed by increased intrathecal inflammation. While the lack of TLR2 delayed bacterial clearance, leukocyte infiltration and enhanced inflammation, the lack or blockade of CD14 had no effect on bacterial clearance, but was associated with a stronger neutrophil recruitment into CSF, leading to excessive meningeal inflammation and aggravated disease after *S. pneumoniae* infection. In addition, this stronger neutrophil migration correlated with MIP-2 concentrations in brain and with enhanced migratory capacity of CD14-deficient PMN. In view of our observations, that different host effector functions were modulated by TLR2 and CD14 during meningitis, we asked the question whether response to therapy was also modulated by these two pattern recognition receptors. We found, that antibiotic treatment

was efficient to rescue wt and $CD14^{-/-}$ mice, whereas in $TLR2^{-/-}$ strain, combination with TACE inhibitor was required. This study reveals the different effects mediated by TLR2 and CD14 respectively in meningitis and illustrates the requirement and success of adjuvant therapy, when bacterial load is high and inflammation is strong in $TLR2^{-/-}$ mice.

General introduction

Bacterial meningitis

Bacterial meningitis is a central nervous system disease, which is characterized by inflammation of the meninges (membranes around the brain) and affects the arachnoid and the subarachnoid space. The major symptoms, including headache, fever and signs of cerebral dysfunction ranging from confusion to coma are found in 85% of patients with acute bacterial meningitis [1]. Nausea, vomiting and photophobia may also appear. Bacterial meningitis remains a leading cause of infection-related death worldwide, with high mortality and morbidity [2]. About 50% of the survivors present with neurological sequelae [3]. A study, including 248 cases of bacterial meningitis, revealed that group B *streptococcus* was the predominant pathogen among newborns, *Neisseria meningitidis* among children 2 to 18 years old and *Streptococcus pneumoniae* among adults [4]. In this study, pneumococcal meningitis had the highest fatality rate (21%). In patients with pneumococcal meningitis, the high case fatality (which can reach 34%) has remained almost unchanged for several years despite antimicrobial therapy [5]. The diagnosis of bacterial meningitis requires in all cases a lumbar puncture. The fluid collected, called cerebrospinal fluid (CSF), is completely clear in healthy people. CSF is cloudy in patients with meningitis, protein concentration and leukocyte numbers are markedly increased (pleocytosis) and the majority of the cells are polymorphonuclear leukocytes (PMNs). In addition, glucose is reduced and lactate is increased in CSF [6]. Bacteria can be identified from CSF culture.

Two distinct but related pathophysiological events are involved in acute bacterial meningitis, these are bacterial invasion of the subarachnoid space and the host inflammatory response to this infection. Bacterial meningitis usually occurs through several steps of bacteria-host interactions. To cause disease, the pathogen has to adhere to and colonize the nasopharynx, invade the intravascular space, and survive or multiply in the bloodstream. Bacteria then cross the blood-brain barrier (BBB) or blood-CSF barrier, survive and replicate in the subarachnoid space, eliciting the host inflammatory response. Adherence to and colonization of the mucosal epithelium of the nasopharynx is a prerequisite for the development of the disease. Colonization involves pneumococcal surface proteins which bind to carbohydrate receptors expressed by epithelial cells such as GalNAc(ß1-3)Gal, GalNAc(ß1-4)Gal [7]. Colonization of the nasopharynx is enhanced by other pneumococcal proteins such as neuraminidases, which decrease the viscosity of the mucus. After mucosal colonization, invasion of the intravascular space is facilitated by pneumococcal hyaluronidase, which degrades the extracellular matrix. To survive within the bloodstream, pneumococci must evade host defenses such as complement attack. Among all pneumococcal virulence factors known and identified, the capsular polysaccharide is the most important in protecting pneumococci against complement. In addition to the capsule, pneumococci release the pneumolysin toxin which also helps survival of the pathogen within the bloodstream. To invade the subarachnoid space, pneumococci have to cross either the BBB or the blood-CSF barrier. The BBB is formed by brain microvascular endothelial cells, which are maintained by tight junctions [8]. This barrier protects the brain from any pathogen present in blood. The blood-CSF barrier is located at the choroid plexus, the site where the CSF is

produced in adult brain. In contrast to brain endothelium, the plexus contains fenestrated capillaries and venules. For most meningeal pathogens, the precise site of entry into the CSF is not yet clear. However, several studies suggest that the brain endothelium rather than the choroid plexus is the primary site of pneumococcal entry into the CSF [9]. It has been shown that pneumococci enter into the cerebral compartment through the BBB via binding of their cell-wall component phosphorylcholine to platelet-activating factor (PAF) receptors expressed on activated cells [10] [11]. Once pneumococci enter the CSF, they multiply easily due to the fact that the CSF contains low levels of immunoglobulins and complement components, such as C3 [12] and usually no PMNs. Bacterial multiplication within the CSF induces the release of proinflammatory cytokines and chemokines, which lead to pleocytosis and therefore increased BBB permeability. Proinflammatory cytokines, such as TNFα, are involved in the pathophysiology of bacterial meningitis [13]. Chemokines are effective for PMN recruitment to inflammatory sites [14]. The CXC chemokine growth-related oncogene-α (GRO-α) was found in CSF of patients with bacterial meningitis and was shown to mediate chemotaxis *in vitro* [15]. Besides chemokines, PMN transmigration requires molecular interactions between receptors expressed on neutrophils and their endothelial counter-ligands. The adhesion molecules, L-selectin, the $ß_2$-integrins and intracellular adhesion molecule (ICAM)-1 play a major role in PMN rolling, adherence and transmigration, respectively [16]. Indeed, CD11/CD18 was shown to be particularly important in migration of PMNs into CSF during pneumococcal meningitis, since inflammation was markedly attenuated by anti-CD18 antibody directed against $ß_2$-integrins [17].

Streptococcus pneumoniae

Streptococcus pneumoniae, a Gram-positive encapsulated diplococcus also named pneumococcus, is a member of the family Lactobacillaceae. Individual cells are between 0.5 and 1.25 μm in diameter, they occur in pairs or chains. They are facultatively anaerobes and non motile. When cultured on blood agar, they are alpha hemolytic. They lack catalase enzyme, which is necessary for the breakdown of hydrogen peroxide. Pneumococci are carried asymptomatically in the nasopharynx in up to 60% of the population and are most of the time completely harmless. However, nasopharyngeal colonization can lead to invasion through the mucosal layer and therefore causes serious infections such as pneumonia, middle-ear infections (otitis media), sinusitis and meningitis [18]. The reasons why invasion of pneumococci occurs in some individuals are still largely unknown. However, both host factors, in particular an impaired immune system and bacterial virulence factors play an important role in the selection of pneumococcal serotypes causing invasive infection. Pneumococcal meningitis can affect individuals of any age but is observed most frequently in very young children (younger than 2 years) and in elderly adults (65 years or more), where the immune system is compromised [19]. The most important virulence factors of *S. pneumoniae* are the polysaccharide capsule, surface proteins and toxins (figure 1). Pneumococci are surrounded by a polysaccharide surface coat, called capsule. The capsule is the most important virulence factor. Indeed, all strains isolated from infected patients are encapsulated [20]. Moreover, a study reported the production of a mutant isolate of *S. pneumoniae* serotype 3 lacking detectable polysaccharide capsule by Tn916 transposon-insertion [21]. In this study, this mutant was found to have greatly reduced virulence in a

mouse model of pneumococcal infection. The main role of the capsule in virulence is to protect pneumococci against phagocytosis [22]. Based on the structures of the polysaccharide capsule, 90 serotypes have been identified [23]. However, only a small fraction of these serotypes are associated with pneumococcal infection (figure 2) [20]. The distribution of serotypes associated with invasive pneumococcal diseases can vary according to age and geographic location. For example, serotype 1 was associated with progressive decrease in risk throughout adulthood, whereas the risk of serotype 3 disease increased progressively up to seventy years. In contrast, serogroups 6, 14 and 19 were associated with a rapid reduction in risk beyond the first ten years [24]. In addition to age, it was observed that serotypes associated with higher nasopharyngeal prevalence, such as serotypes 19 and 23, are more common in developed countries, while invasive serotypes 1 and 5 are more common in developing countries [24].

Besides the capsule, pneumococcal surface proteins are important virulence factors [25] [26]. They can be divided into three families: Choline-binding proteins (Cbps), LPXTG-anchored proteins and lipoproteins.

Cbps are anchored to the cell wall by non-covalent interaction of their carboxy-terminal end with the phosphorylcholine of the pneumococcal cell wall. The Cbp family includes the pneumococcal surface protein A (PspA), pneumococcal surface protein C (PspC) and autolysin (LytA). PspA is one of the most variable proteins which is expressed by all clinically isolates of *S. pneumoniae* [27]. PspA inhibits complement activation and phagocytosis, thus contributing to virulence of serotype 3 *S. pneumoniae* strain [28]. It was further shown that PspA exerts its virulence function by interfering with C3b

deposition onto pneumococci [29] [30]. In addition, it was shown that a PspA-deficient strain but not a PspA-expressing strain was cleared from the blood within 6 h after intravenous injection in mouse [29]. In this study, the authors showed that in mice deficient in C3 or factor B, the PspA-deficient strain was fully virulent, suggesting that PspA affects the activation of the alternative pathway. PspC (also know as CbpA) is implicated in cell surface adhesion and plays a major role in nasopharyngeal colonization in rats [31] and in mouse models of colonization and pneumonia [32]. It was also shown to bind the C3 component of complement [33]. The serotype 3 PspC was found to bind the human complement regulatory protein factor H (FH) and was then named as factor H-binding inhibitor of complement (Hic) [34]. It was recently shown that Hic protein binds to short consensus repeats (SCRs) 8-11 in the middle region of factor H and thus, protects the pneumococci from opsonophagocytosis via inhibition of spontaneous C3 activation [35]. The family of Cbps includes also autolysins, which are members of a group of enzymes that degrade the peptidoglycan backbone of bacteria. In *S. pneumoniae*, the major autolysin N-acetylmuramyl-L-alanine amidase, also know as LytA, was shown to be involved in virulence [36]. In this study, the authors showed that the mutagenesis of *lytA* gene in serotype 2 *S. pneumoniae* significantly reduced virulence of this organism compared to wild-type strain in a mouse intraperitoneal challenge model. An indirect implication of LytA involves the release of cytoplasmic virulence factors such as pneumolysin (Ply).

The second group of pneumococcal surface proteins are covalently anchored to the cell wall through a carboxy-terminal motif-LPXTG. This motif is recognized by a sortase enzyme, which links the threonine residue of the motif to the peptidoglycan of the

bacterial cell wall. These proteins include hyaluronidase and neuraminidase. Hyaluronidase disrupts the hyaluronic-acid component of mammalian tissue and extracellular matrix. Neuraminidase cleaves N-acetyl-neuraminic acid from cell surface glycans such as mucin, glycolipids and glycoproteins. However, mutagenesis of either hyaluronidase or neuraminidase genes did not affect the virulence of serotype 2 *S. pneumoniae* in a mouse intraperitoneal challenge model [36]. Nevertheless, the authors observed a significant additive attenuation in virulence when both pneumolysin and hyaluronidase were mutated. The importance of hyaluronidase was further shown in a murine pneumococcal meningitis model, where intranasal application of pneumococci lead to the disease only if the inoculum was combined with hyaluronidase [37].

The third group of pneumococcal surface proteins is composed by lipoproteins. This family includes the pneumococcal surface antigen A (PsaA). Further analysis showed that PsaA belongs to an ATP-binding cassette (ABC)-type transporter system involved in manganese transport [38]. The ABC-type transport system is composed of three components, an extracytoplasmic protein (involved in solute binding), an integral membrane protein (for the transport of the solute through the cell membrane) and a cytoplasmic protein binding ATP. It was clearly demonstrated that mutagenesis of the *psaA* gene of serotype 2 *S. pneumoniae* significantly reduces its virulence in both intranasal and intraperitoneal challenge mouse models [39]. It was also shown that *psaA* serotype 2 mutant *S. pneumoniae* was highly sensitive to oxidative stress [40].

Besides pneumococcal surface proteins, the toxin pneumolysin (Ply) has been intensively studied. Ply is a cytoplasmic enzyme which is released during autolysis mediated by LytA. Ply is a multifunctional toxin, characterized by its cytolytic and

complement activation activities [41]. Ply is know as cholesterol-dependent cytolysin since it binds to the host cell cytoplasmic membrane cholesterol and therefore induces the formation of pores which leads to cell lysis. Ply was shown to induce disruption of tissue barriers within the respiratory tract [42] and was found a virulence factor in both intranasal and intraperitoneal challenge mouse model [39]. Ply toxin play a role in the pathogenesis of meningitis, since it was shown that Ply-deficient serotype 2 *S. pneumoniae* strain was less virulent in a mouse meningitis model [43] and purified pneumolysin was an important component for damaging the BBB [44]. This toxin was also shown to induce neuronal apoptosis in a rabbit model of pneumococcal meningitis [45].

Among other pneumococcal components, unmethylated CpG bacterial DNA was also found to contribute to meningitis by causing a macrophage infiltration into the CSF of rats and mice [46]. Intracisternal injection of bacterial DNA induced transient accumulation of cytokines in CSF, which could be abolished by nitrate synthase inhibitors and by selectin blockade [46].

Around 1940, all strains of the bacteria pneumococcus were sensitive to the antibiotic penicillin. However, since a penicillin-resistant pneumococcus was first identified in the early 1960s [47], the incidence of penicillin-resistant *S. pneumoniae* strains has been gradually increasing. In addition to penicillin resistance, multidrug-resistant pneumococci have been identified during the last decade. However, a combination of vancomycin and a third generation of cephalosporin is recommended in areas with reported penicillin-resistant or cephalosporin-resistant strains [48].

There are currently two vaccines that protect against pneumococcal disease, a 23-type polysaccharide vaccine for people over the age of two and a newer 7-type conjugate vaccine for children aged two months to two years.

The 23-type polysaccharide vaccine, which is available since more than 20 years can protect most adults for five years or more against the top 23 pneumococcal polysaccharides causing invasive pneumococcal infection. Since non-conjugated polysaccharides are T-cell independent antigens, the antibody response is short and limited, and memory B-lymphocytes can not be generated. Thus, polysaccharide capsule antigens do not elicit protective levels of antibodies in children under two years of age, and in individuals with advanced immunological impairment. Furthermore, polysaccharides do not induce immunological memory, which is required for subsequent booster responses. The limitations of this vaccine have been overcome by conjugating the capsular polysaccharides to protein carriers [20]. The conjugation enhances the immunogenicity of pneumococcal vaccines by inducing T-cell dependent responses to these protein carriers. The newer 7-type conjugate vaccine (Prevnar 7) contains serotypes 4, 6B, 9, 14, 18C, 19F and 23F conjugated to the T-dependent protein carrier, diphtheria CRM_{197} protein. Recent epidemiological studies from Germany, Denmark, Greece, the United Kingdom, Spain, and southern Sweden have suggested that coverage rates for this conjugate vaccine could range from 53% to 83% among children in these countries [49]. The efficacy of Prevnar 7 in preventing invasive pneumococcal disease was demonstrated in a study including a large number of infants, who were given the vaccine at 2, 4, 6 and 12 to 15 months of age [50].

Antibiotic therapy is applied to eradicate the bacterial pathogen; however several animal studies have shown that bacterial lysis, induced by antibiotic therapy, enhances CSF inflammation, which in turn may contribute to an unfavorable outcome [51]. The cell wall components which result from degradation by either antibiotic or the host immune system are potent chemoattractants [22] and induce synthesis of inflammatory mediators such as TNFα and IL-6 by human monocytes [52]. In addition to the pneumococcal cell wall components, the pneumolysin toxin, which is released after bacterial lysis, has been shown to stimulate *in vitro* the production of inflammatory cytokines such TNFα [53]. The exact mechanisms of host immune activation by pneumococcal components are not yet fully understood. However, it was clearly demonstrated that the cell wall component peptidoglycan binds to the pattern recognition receptor CD14 [54]. The transmission of the signal after activation of CD14 receptor occurs through another pattern recognition receptor containing an intracytoplasmic/signaling domain, TLR2 [55]. It is further known, that pneumolysin interacts with TLR4 [56] and bacterial DNA, which is released during bacterial autolysis activates the host via TLR9 [57].

To determine the function of the pattern recognition receptors TLR2 and CD14 in mediating host innate immune response in meningitis induced by *S. pneumoniae*, we investigated their implications in a mouse pneumococcal meningitis model using mice with a targeted deletion of the corresponding genes. The role of TLR2 will be discussed in section I and the role of CD14 in section II. In section III, we further investigated the outcome in wt, $TLR2^{-/-}$ and $CD14^{-/-}$ mice with meningitis under antibiotic treatment and/or anti-inflammatory treatment with TACE inhibitor drug. We also investigated the course of meningitis and the treatment response of $TLR2^{-/-}/CD14^{-/-}$ mice.

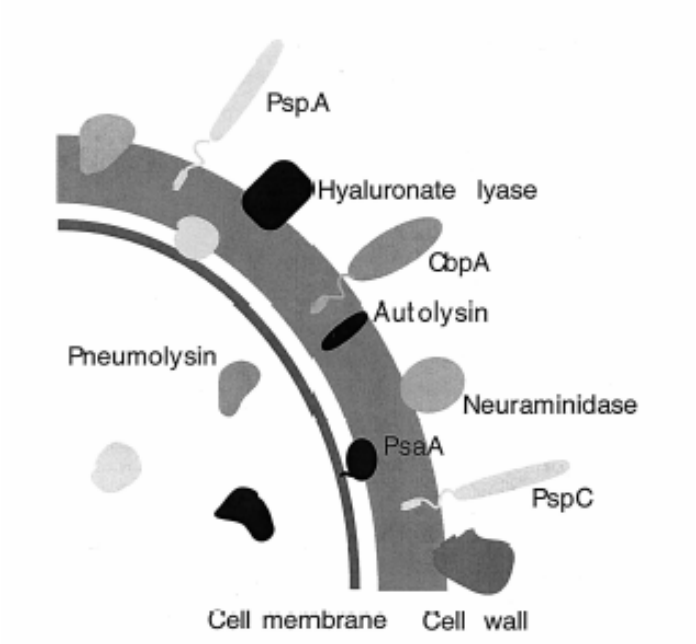

Figure 1. Summary of the main virulence factors of *S. pneumoniae* strains (the capsule is not shown) [26].

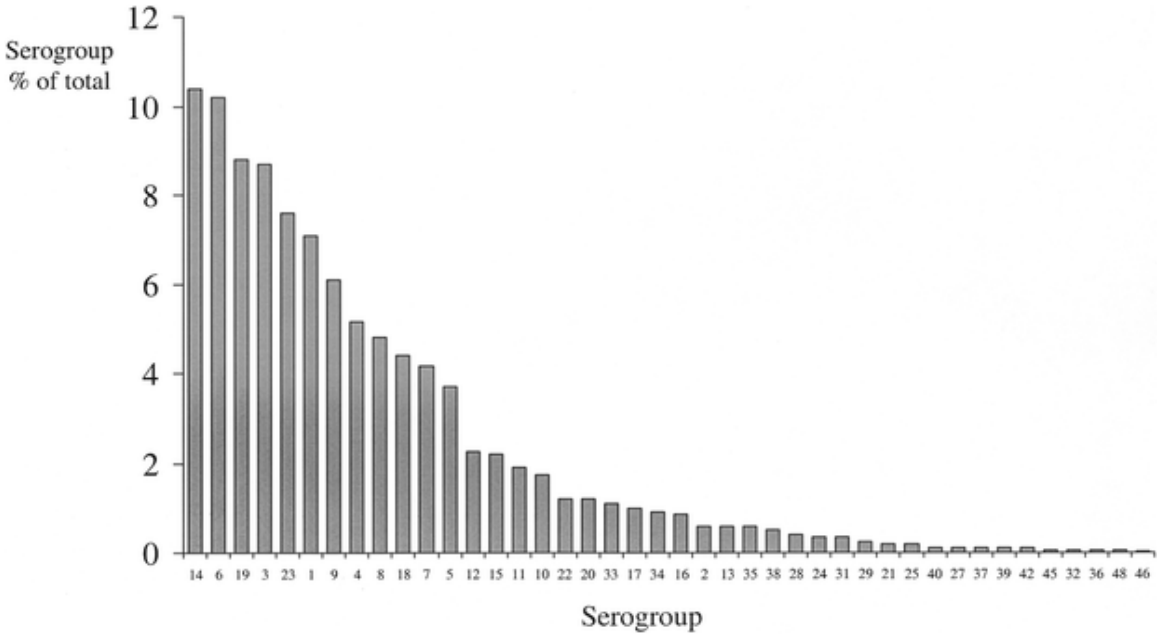

Figure 2. Most common serogroups/types causing invasive pneumococcal disease in Europe and North America [24].

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Section I

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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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Running title: TLR2 in pneumococcal meningitis

The abbreviations used are: **TLR**, Toll-like receptor; **BBB**, blood-brain barrier; **CSF**, cerebrospinal fluid; **CFU**, colony-forming units; **IL-6**, interleukin-6; **TNF**, tumor necrosis factor.

Abstract

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 and bacterial numbers in blood and peripheral organs were similar in 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 presented a 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.

Introduction

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 suffer from neurological sequelae, indicating post-inflammatory 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 inflammatory mediator release. Subsequently, the subarachnoidal inflammation stimulates astrocytes, microglia and neurons to produce cytokines and chemokines [3] [4] [5]. A bacterial level of ≥ 5 log cfu and more 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 block completely 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] [13] [14]. TLR2 is involved in cell activation by gram-positive bacterial cell wall and membrane components, such as peptidoglycan, lipoteichoic acid, and lipoproteins [15] [16] [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 (MHB) (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 x 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 x 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 x 10^2 or 3 x 10^3 cfu), or *L. monocytogenes* (5 x 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 for C57BL/6 wt and TLR2$^{-/-}$) were deeply anesthetized and infected by direct intracisternal injection of 10 μL 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 previously [20]: 1, exhibited normal motor activity and turned upright in <5 s when put on its back; 2, decreased spontaneous activity, but still turned upright in <5 s; 3, turned upright in >5 s; 4, did not turn upright; 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 previously [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 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 using bioluminescent S. pneumoniae transformed with Gram-positive lux transposon. Mice were intracerebrally injected with 3 x 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 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 pro-inflammatory 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 (Sigma, 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 hemisphere was homogenized in 1

mL of 0.1 mol/L PBS and then centrifuged at 1,000 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 1,000 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 in 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 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.

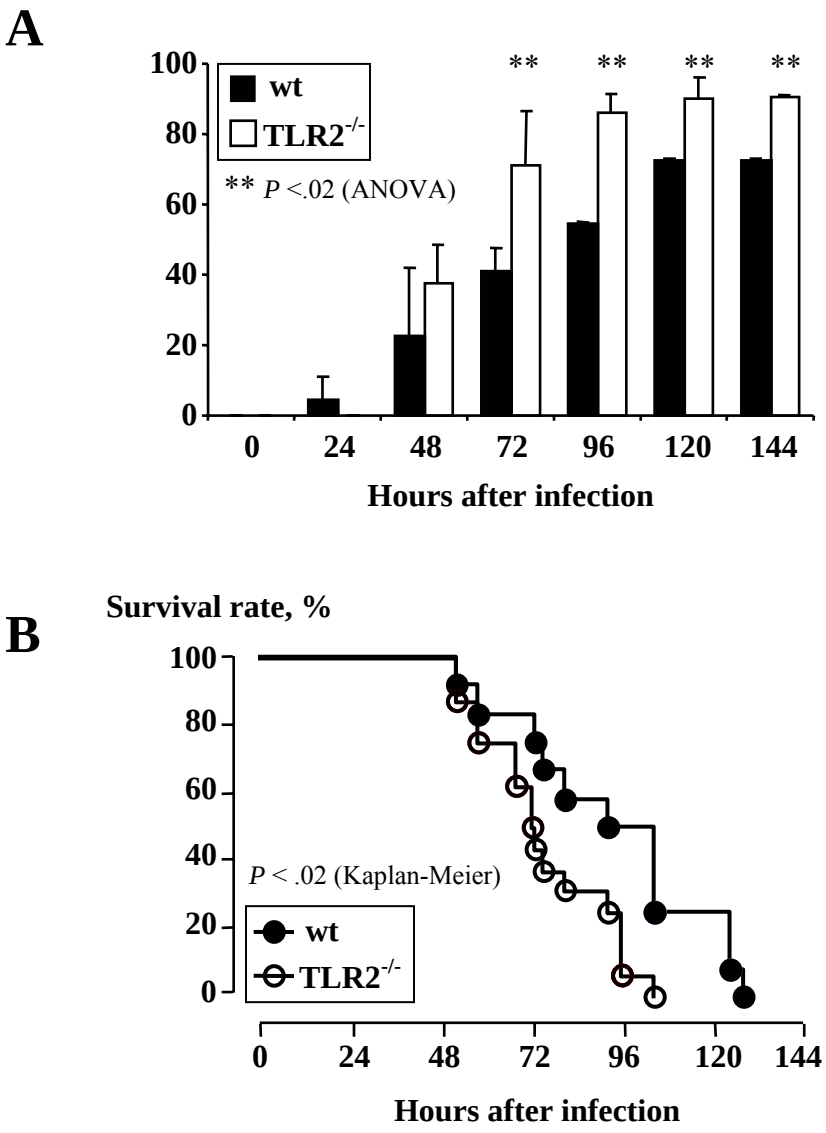

Figure 1. ***A***, Percentage of wild-type (wt; $n = 22$) and Toll-like receptor-2-deficient ($TLR2^{-/-}$; $n = 21$) mice showing high severity score (including score 4 and score 5) after intracerebral injection of 2×10^2 cfu of *Streptococcus pneumoniae*. Data from 3 independent experiments are represented 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*. $P < .02$, in 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 x 10^2 cfu of *L. monocytogenes* in 25 μL of NaCl.

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

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 determined 1, 2 and 3 days after infection with 3 x 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 cultures 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, to cure from 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 rate 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

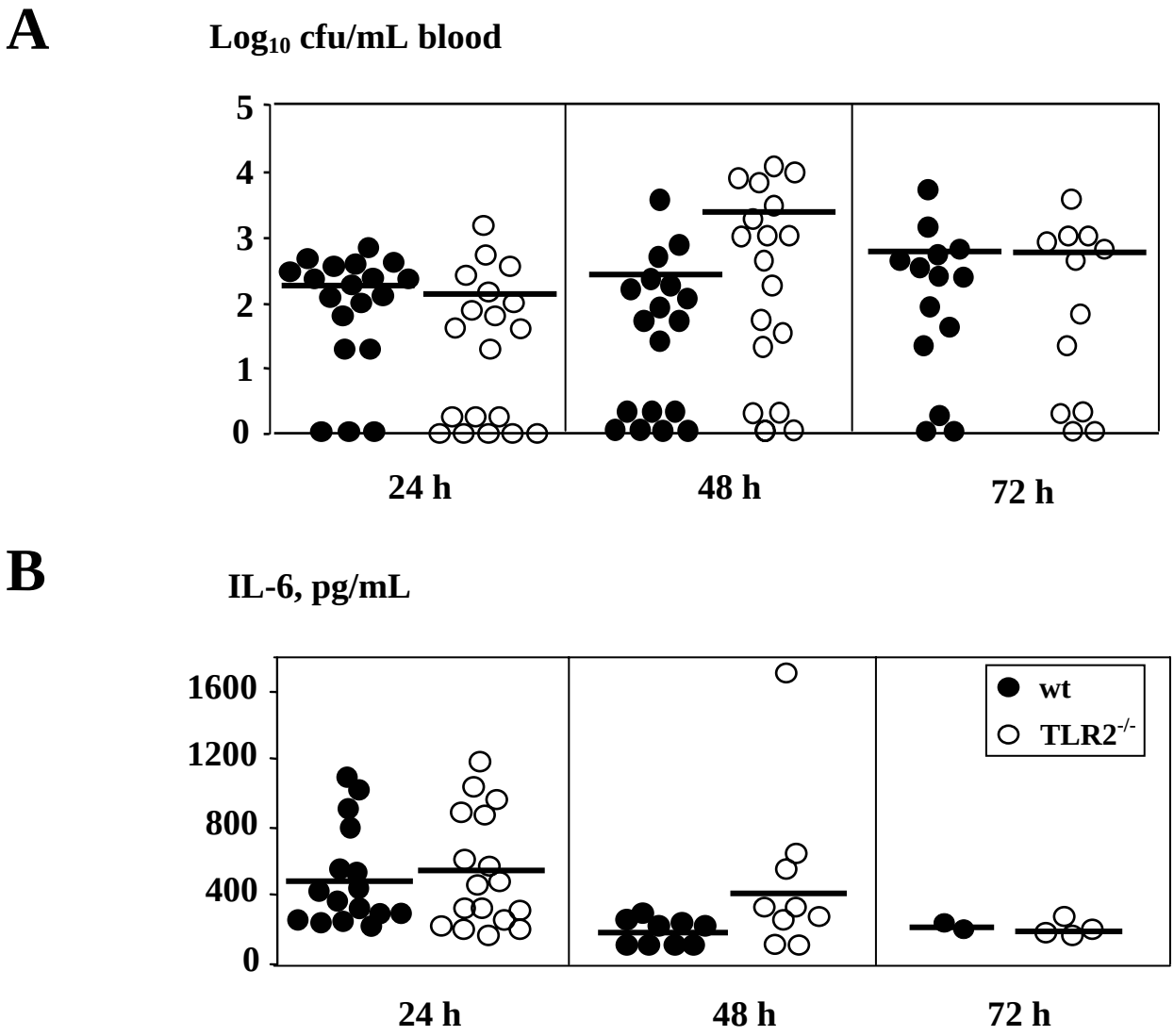

Figure 2. Colony-forming unit counts in blood (***A***) and interleukin (IL)-6 levels in plasma (***B***) in wild-type (wt; n = 22) and Toll-like receptor-2-deficient (TLR2$^{-/-}$; n = 21) mice after intracerebral injection of 3 x 10^3 cfu of *Streptococcus pneumoniae*. Blood was collected 24, 48, and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

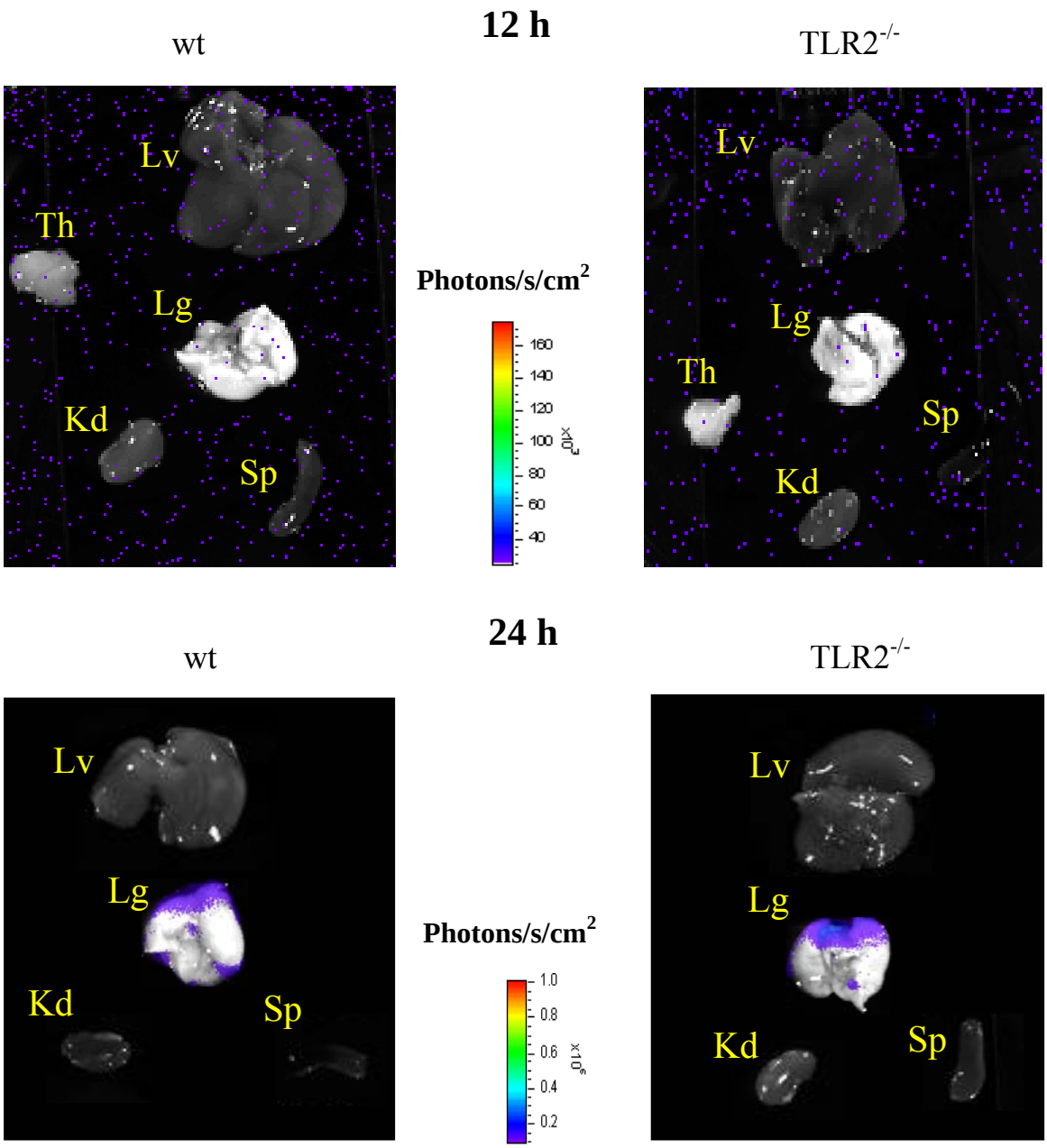

Figure 3. Emitted photons by luciferase-tagged *Streptococcus pneumoniae* in several organs from wild-type (wt) and Toll-like receptor-2-deficient ($TLR2^{-/-}$) mice detected by a highly sensitive CCD (charged-coupled device) camera (IVIS imaging system; Xenogen) 12 and 24 h after intracerebral injection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

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.

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 during 5 days, starting 18 h after intracerebral injection of 3 x 10^3 cfu of *S. pneumoniae*. $P < .04$, $TLR2^{-/-}$ vs. wt mice (log-rank test in Kaplan-Meier analysis).

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 killed, and the photon emission was stronger and more widely spread in isolated brains of TLR2$^{-/-}$ mice than in those of wt mice (figure 4A). 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 side 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, 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

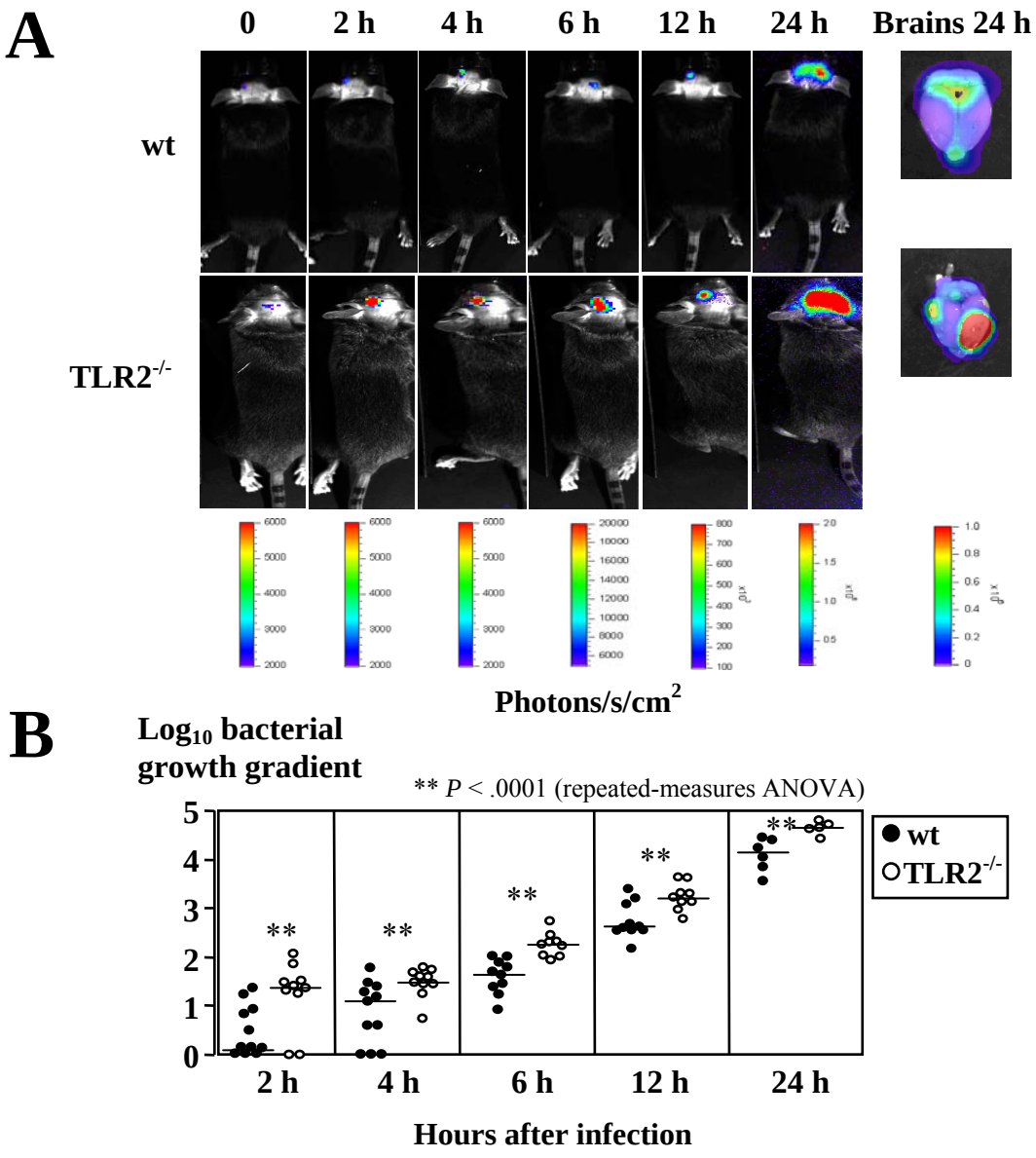

Figure 4. ***A***, Time course of infection in wild-type (wt) and Toll-like receptor-2-deficient (TLR2$^{-/-}$) mice after intracerebral injection of 3 x 10^3 cfu of luciferase-tagged *Streptococcus pneumoniae*. Images from 1 animal in each group at time of infection (0 h) and 2, 4, 6, 12, and 24 h after 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, $n = 6$ and $n = 5$ at 24 h, for wt and TLR2$^{-/-}$ mice, respectively).

used (median, 6.2 x 10^5 cfu and 6.8 x 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; $P < .05$; 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*).

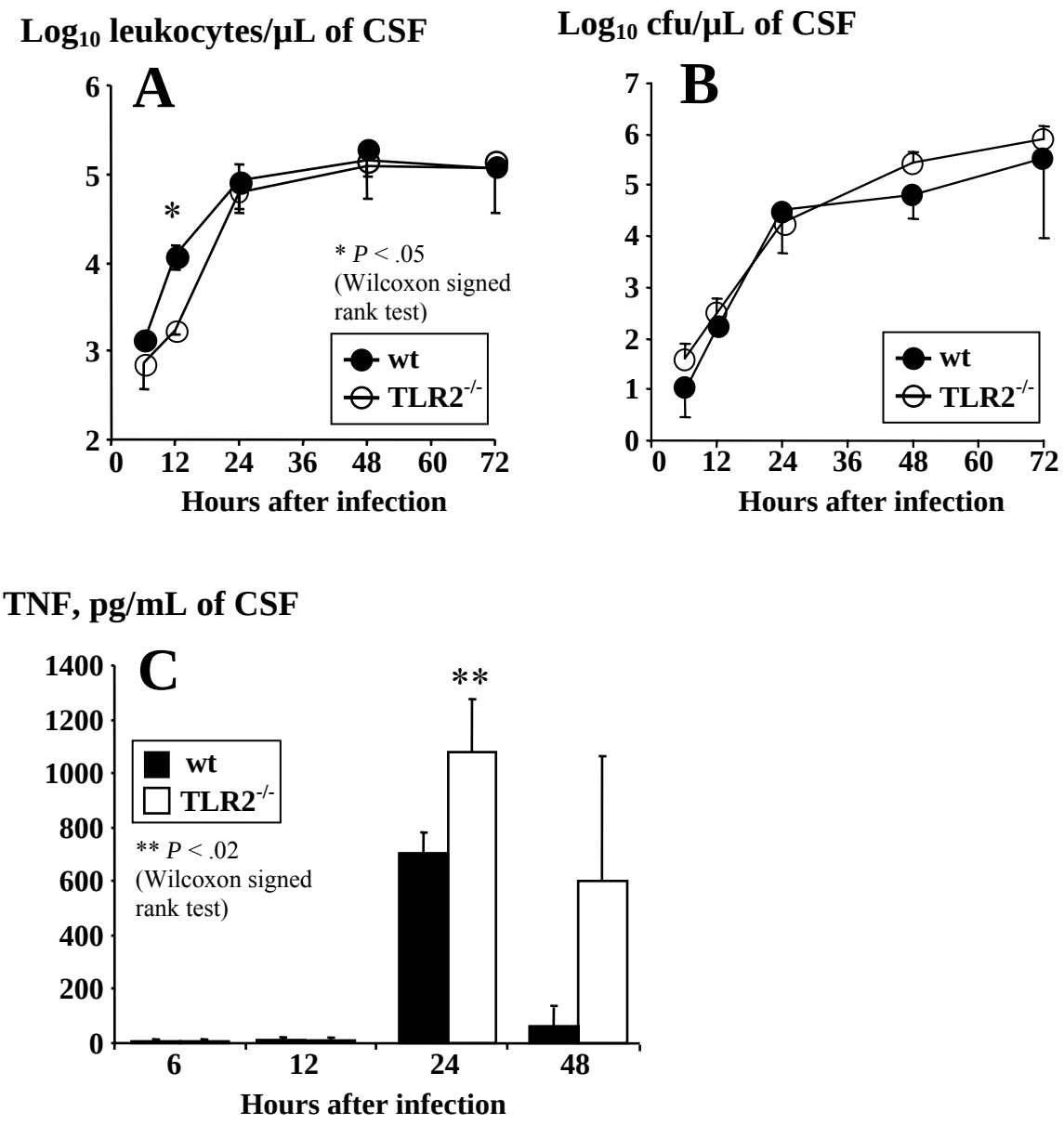

Figure 5. Time course of cerebrospinal fluid (CSF) parameters in wild-type (wt) and Toll-like receptor-2-deficient ($TLR2^{-/-}$) mice (n = 12 at each time point in both groups) after intracerebral injection of 3 x 10^3 cfu of *Streptococcus pneumoniae*. Pooled CSF from 4 mice was collected at indicated 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 the symbol. * $P < .05$ and ** $P < .02$, both by the Wilcoxon signed rank test.

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.

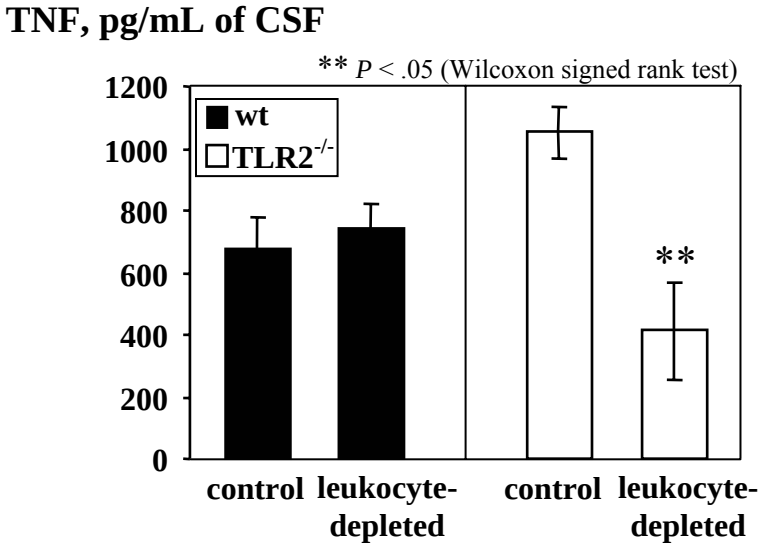

Figure 6. Tumor necrosis factor (TNF) levels in cerebrospinal fluid (CSF) in leukocyte-depleted and immunocompetent control animals after intracerebral injection of 3 x 10^3 cfu of *Streptococcus pneumoniae*. In each experiment, pooled CSF from 4 mice was collected 24 h after infection. Results of 3 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.

TLR2$^{-/-}$ mice have enhanced blood-brain barrier permeability after i.c. 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).

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 x 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 the scoring system.

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

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 in comparison to wt mice after a high but not after a low intravenous 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. 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 hypothetized 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 number 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 lacking 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 the CSF of TLR2$^{-/-}$ than of wt mice. TNF is elevated in CSF in human and experimental meningitis. It has detrimental effects, since 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 than controls 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 characterised 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 downregulation 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 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 as compared to 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 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 than Listeria-infected wt mice (data not shown). This suggests that different mechanisms are involved in *S. pneumoniae* and *L. monocytogenes* meningitis.

As reported elswhere, 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 leukocyte, 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 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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Section II

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CD14 deficiency leads to early death in pneumococcal meningitis due to a high MIP-2, CXCR2, neutrophil transmigration and inflammation

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Abstract

CD14 is a myeloid receptor for the bacterial cell membrane/wall components LPS and LTA, and associates with TLR4 and TLR2 respectively to mediate signal transduction. Its role in infection with live Gram-positive bacteria is controversial. Therefore, the effect of CD14 deficiency and blockade was studied in murine pneumococcal meningitis. 10^3 colony forming units (CFU) of *S. pneumoniae* serotype 3 were inoculated subarachnoidally and mice were followed from 6 h to 5 days. $CD14^{-/-}$ mice and mice pretreated intravenously with 5 mg/kg neutralizing rat anti-CD14 antibodies showed higher disease severity and died earlier ($P < .02$) than C57BL/6 wild type (wt) mice with or without control antibody treatment. Early leukocyte immigration into the cerebrospinal fluid (CSF) was more pronounced $P < .05$) in $CD14^{-/-}$ and wt mice with anti-CD14-treatment than in wt mice without antibody. This was not due to altered adherence molecule expression in either blood or CSF polymorphonuclear leukocytes (PMN) or in brain endothelial cells. MIP-2 and KC were similarly induced in CSF in both mouse strains, but MIP-2 release in brain *in vivo* and in endothelial cells infected *in vitro* was higher in $CD14^{-/-}$ than in wt mice. CD14-deficient PMN showed stronger *in vitro* chemotaxis than wt PMN towards CSF from either wt or $CD14^{-/-}$ injected mice, pneumococcal cell walls (PCW) and recombinant MIP-2. *In vivo* blood leukocyte depletion prevented leukocyte infiltration into the CSF from wt but not from $CD14^{-/-}$ mice. The larger leukocyte numbers in CSF of $CD14^{-/-}$ mice were responsible for a stronger TNF transcription and release from infiltrating cells. They were not able to improve bacterial clearance, since $CD14^{-/-}$ and wt mice showed similar CFU in CSF. TNF

was related to the severity score ($P < .05$) and thus most likely responsible for earlier death. In conclusion, CD14 has a protective effect in pneumococcal meningitis by slowing PMN migration.

1. Introduction

Streptococcus pneumoniae is the major pathogen causing meningitis in adults. Despite antimicrobial therapy and critical care medicine, mortality remains high. Up to 30% of patients die [1] and about 50% of the survivors suffer from neurological sequelae [2]. In the pathogenesis of meningitis, penetration of bacteria through the blood-brain (BBB) or blood-CSF barrier initiates activation of brain endothelia and glia and leads to leukocyte recruitment and inflammatory mediator release. Pneumococcal cell wall (PCW) components such as peptidoglycan (PGN) and lipoteichoic acid (LTA) may induce harmful inflammation via activation of pattern recognition receptors expressed in infiltrating and resident brain cells.

CD14 was initially described as a receptor for lipopolysaccharide (LPS), the major component of the outer membrane of Gram-negative bacteria [3]. Later, CD14 has been shown to bind Gram-positive [4] [5] [6] or mycobacterial [7] cell wall components. It was therefore postulated that CD14 functions as a pattern recognition receptor of the innate immune system [7]. CD14 is a 55-kDa glycosylphosphatidylinositol (GPI)-anchored glycoprotein expressed mainly in monocytes and macrophages, but also at low levels in neutrophils [8]. A single mRNA transcript is translated and processed in the endoplasmatic reticulum, where a GPI anchor is attached. As a GPI-linked protein without transmembrane and intracytoplasmic domains, mCD14 most likely does not transmit a signal on its own. It was therefore postulated that in presence of Gram-positive bacteria, CD14-TLR2 complexes may function to initiate signal transduction, since

cellular response to heat-killed *Staphylococcus aureus* and *S. pneumoniae* [9] or purified LTA [10] have been shown to be mediated by CD14 and TLR2.

In addition to membrane bound CD14 (mCD14), two soluble isoforms of CD14 (sCD14) are generated. A 55-kDa isoform is liberated by escaping from GPI anchoring and the 49-kDa isoform is derived from the cell membrane by proteolytic cleavage through a serine protease. Soluble CD14 can mediate activation of cells that lack mCD14 expression, such as human endothelial, epithelial and smooth-muscle cells [11] [12] [13]. This suggests that sCD14 binds a transmembrane receptor, although it has not been shown that sCD14 binds TLR2 and thereby transmits signal in the same way as mCD14 does. Some studies reported elevated levels of sCD14 in human cerebrospinal fluid (CSF) during neuroinflammatory diseases, such as bacterial meningitis [14] [15]. This observation was also confirmed in a mouse model of experimental bacterial meningitis [14].

CD14 is known to be crucial in endotoxin-induced shock as shown by the resistance of CD14-deficient mice to septic shock induced by i.v. or i.p. injection of *Escherichia coli* or LPS [16]. Opposed to that finding, several *in vivo* studies have shown a deleterious effect of CD14 blockade in mice or rabbits injected with *Klebsiella pneumoniae*, *E. coli* or *Shigella flexneri* [17] [18] [19]. To date, only one *in vivo* study has investigated the function of CD14 in a Gram-positive sepsis model. In that study, survival as well as bacterial load in lymphoid organs were similar in CD14-deficient ($CD14^{-/-}$) and wild-type (wt) mice after i.v. infection with *S. aureus* [20]. Despite growing evidence implicating CD14 in recognition of Gram-positive bacterial cell wall components *in vitro*, its pathophysiologic role in Gram-positive bacterial infection is still

unknown. We therefore, investigated the *in vivo* role of CD14 in the pathogenesis of pneumococcal meningitis.

To that aim, disease severity and outcome in mice with a targeted deletion of the CD14 gene and in wt mice treated with neutralizing anti-CD14 specific antibody was compared to wt mice following subarachnoidal infection with *S. pneumoniae*. Bacterial numbers in brain and bloodstream, bacterial counts and leukocyte recruitment into the subarachnoid space, as well as meningeal inflammation were analyzed.

2. Results

2.1 CD14$^{-/-}$ mice show earlier death during pneumococcal meningitis

To investigate the *in vivo* function of CD14 during Gram-positive bacterial meningitis, wt and CD14$^{-/-}$ mice were subarachnoidally infected with *S. pneumoniae.* The severity of disease was assessed by clinical score and survival rate.

S. pneumoniae meningitis gradually induced lethargy in both wt and CD14$^{-/-}$ mice, but the percentage of severely sick mice was significantly higher among CD14$^{-/-}$ than among wt mice from 48 h after infection and this difference was maintained during the whole observation period of the disease (data not shown). The higher severity of disease observed in CD14$^{-/-}$ mice was followed by an earlier mortality in these mice. Indeed, the median survival time was 84 h in CD14$^{-/-}$ mice and 122 h in wt mice (figure 1*A*). Moreover, *in vivo* blockade of CD14 significantly accelerated the mortality after *S. pneumoniae* infection. Indeed, animals treated with anti-CD14 mAb (4C1) showed significantly earlier death as compared to animals receiving isotype-matched control mAb (fFigure 1*B*). Since the levels of soluble CD14 were similarly increased in CSF (3627.5 ± 1862.6 vs 3209.0 ± 3540.4 ng/mL in anti-CD14 vs isotype control treated mice, respectively) and serum (170.3 ± 215.7 vs 44.1 ± 41.8 ng/mL in anti-CD14 vs isotype control treated mice, respectively) of mice, which were treated with either anti-CD14 or isotype control mAb, a role of sCD14 can be excluded. These results indicate that CD14 is directly implicated in the host response to pneumococcal meningitis.

2.2 CD14 deficiency does not play a role in early bacterial clearance and sepsis

Accelerated death in $CD14^{-/-}$ mice could be due to earlier sepsis or enhanced inflammation in the brain. To test whether sepsis explained earlier death, we assessed bacterial load in blood and CSF of *S. pneumoniae* infected mice. Colony-forming units (CFU) in blood were detected only 24 h after infection, and were not significantly different in $CD14^{-/-}$ and wt mice (table I). In addition, CFU in CSF were also similar in $CD14^{-/-}$ and wt mice, 6, 12 and 24 h after infection (table I). Furthermore, brain bacterial load, which mirrors bacteria adherent to or taken up by infiltrating and resident cells, was followed by quantitative analysis of emitted fluorescence in a coupled-charged digital (CCD) camera in luciferase-tagged *S. pneumoniae* infected animals *in vivo* (figure 2*A*). Bacterial load was similar in both strains in the early hours of infection, but was higher in $CD14^{-/-}$ mice than wt mice after 24 h (figure 2*B*). CFU determination in brain homogenates, which were harvested 24 h after infection, confirmed that at 24 h after infection brain bacterial numbers were higher in $CD14^{-/-}$ than wt mice (figure 2*B*). Taken together, these data suggest that CD14 does not affect early bacterial clearance and development of sepsis during meningitis, but that it modulates brain bacterial load after 24 h of infection.

2.3 $CD14^{-/-}$ mice show a stronger leukocyte infiltration in CSF

To further understand earlier death of $CD14^{-/-}$ mice during *S. pneumoniae* meningitis, we analysed leukocyte recruitment into CSF. $CD14^{-/-}$ mice showed a two fold stronger infiltration of PMN in CSF than wt mice 6 and 12 h after infection (figure 3*A*), without an alteration of subpopulation distribution. However, PMN numbers were similar in both mouse strains 24 h after infection (figure 3*A*). We then investigated, whether this

stronger neutrophil infiltration was induced by live *S. pneumoniae* and its cell wall components. We therefore monitored CSF pleocytosis in mice subarachnoidally injected with PCW. Interestingly in both mouse strains, leukocyte numbers in CSF were 10-fold higher 6 h after injection of PCW than after infection with *S. pneumoniae*, yet values in $CD14^{-/-}$ mice were again significantly higher than those in wt mice (figure 3*B*). This suggested that the stronger neutrophil influx in CSF of $CD14^{-/-}$ mice was not dependent on phagocytosis of *S. pneumoniae*, but was rather induced by pino- or endocytosis of small molecules. Importantly, leukocyte influx was the only consequence of PCW injection, neither clinical signs nor TNF were induced by the bacterial preparation (data not shown). To further characterise the *in vivo* role of CD14 in CSF pleocytosis, we analysed CSF neutrophil infiltration in mice treated with anti-CD14 mAb. Mice injected with anti-CD14 at the time of infection, showed a higher CSF infiltration ($3.26 \times 10^4 \pm 1.0 \times 10^4$) 12 h after infection than infected animals which received isotype-matched control Ab ($1.53 \times 10^4 \pm 3.5 \times 10^3$). Indeed, anti-CD14 was detectable on the surface of PMN in the CSF of mice, which had been treated with the specific antibody, but not in those treated with the control antibody (data not shown). Together, these results indicate that either the lack or blockade of CD14 allowed enhanced neutrophil immigration in CSF of infected mice or of mice stimulated with bacterial components.

2.4 The lack of CD14 does not alter adherence molecule expression, but leads to increased MIP-2 levels

We then investigated, whether the higher leukocyte numbers in $CD14^{-/-}$ mice were due to an alteration of leukocytes or of brain endothelial cells. We analysed expression of

the leukocyte adherence molecules CD11a/CD18 (LFA-1), CD11b/CD18 (Mac-1), CD11c/CD18 (gp150, 95) and CD54 (ICAM-1) in CSF cells. All adherence molecules tested were similarly expressed in $CD14^{-/-}$ and wt mice, 6, 12 and 24 h after infection (data not shown). CD11c/CD18 and CD54 were weakly expressed in PMN, with mean fluorescence intensities (MFI) of 10 and 17 respectively. CD11a/CD18 showed MFI of 286.8 and 277.4 for $CD14^{-/-}$ and wt mice, respectively and CD11b/CD18 increased to the same extent in $CD14^{-/-}$ and wt mice (MFI 1577 and 1367 respectively) 24 h after infection.

Moreover, expression of leukocyte adherence molecule CD11a/CD18, CD11b/CD18, CD11c/CD18, CD31 (PECAM-1), CD54 and CD62-L (L-Selectin) was similar in peripheral blood PMN of $CD14^{-/-}$ and wt mice (data not shown). This indicates that the penetration of leukocytes through the blood-brain barrier (BBB) or the plexus was not facilitated by leukocyte adherence molecules and suggests an alteration of the endothelium in $CD14^{-/-}$ mice. Therefore, brain endothelia were stained by immunohistochemistry with antibodies against CD54, CD106 (VCAM-1) and CD62-E (E-Selectin). CD54 and CD106 were equally expressed in plexus choroideus epithelia of $CD14^{-/-}$ mice and wt mice 6 and 12 h after *S. pneumoniae* infection (data not shown). At the early time points these molecules were not found to be upregulated in endothelium. Yet later after pneumococcal infection (24 and 48 h), they were equally expressed in brain endothelium of both mouse strains. Moreover, immunohistochemical analysis of brain endothelium of mice treated with anti-CD14 mAb revealed similar CD54 expression as isotype-control Ab injected mice 12 h after infection, despite a stronger CSF infiltration of neutrophils (data not shown). Taken together, these data indicated that

the early CSF leukocyte influx observed in $CD14^{-/-}$ was not due to an alteration of adherence molecule expression in leukocytes or brain endothelial cells.

Alternatively, neutrophil migration from blood into the subarachnoid space could be affected due to increased chemokine levels in CSF. We therefore measured the neutrophil chemoattractants macrophage inflammatory protein (MIP)-2 and keratinocyte-derived chemokine (KC). MIP-2 and KC levels in CSF increased between 6 and 12 h after *S. pneumoniae* infection and were found to be higher in $CD14^{-/-}$ mice. However the differences in cytokine levels were not statistically significant (table II). Soluble intercellular adhesion molecule (ICAM)-1, which could also play a role in neutrophil transmigration [21], also showed similar levels in $CD14^{-/-}$ and wt mice 6 and 12 h after infection (table II). For IL-8, the human homologue of MIP-2, a strong association with PMN was shown to occur [22]. Furthermore, microglial cells, astrocytes and endothelial cells are all known to be a source of chemokines during inflammation or after bacterial infection [23] [24] [25]. Therefore, MIP-2 was also measured in brain lysates, which contain adherent PMN and resident cells. MIP-2 was significantly higher in brains of $CD14^{-/-}$ than of wt mice (figure 4*A*). To address a potential role of endothelial cells during pneumococcal infection, we performed *in vitro* experiments with brain-derived microvascular endothelial cells (MVECs). Monolayers of MVECs from both genotypes of mice were infected with live *S. pneumoniae* at two different MOI (2 and 20) and MIP-2 levels in the culture supernatant determined 8 and 24 h later. Higher MIP-2 levels were found in $CD14^{-/-}$ MVEC supernatants at both time points and irrespective of the MOI used (figure 4*B*). Similar results were found with the other neutrophil chemoattractants KC (data not shown).

In summary, brain-derived chemokines, but not altered adherence molecules might be the origin of enhanced leukocyte migration in $CD14^{-/-}$ mice.

2.5 CSF of PCW injected mice and PCW induce a stronger chemotactic activity towards PMN from $CD14^{-/-}$ mice

CSF of patients with bacterial meningitis is known to induce chemotactic activity in human PMN *in vitro* [26]. We therefore tested, whether CSF from infected $CD14^{-/-}$ and wt mice differed in the chemotactic activity towards mouse blood PMN *in vitro*. CSF from infected $CD14^{-/-}$ and wt mice had similar chemotactic activity, since the origin of CSF did not influence the migration capacity of PMN cells of either mouse strain (data not shown). However the percentage of PMN, which migrated towards CSF was significantly higher if cells were derived from $CD14^{-/-}$ than from wt mice (figure 5*A*). To assess the effect of chemokines in the CSF on PMN, chemotaxis was performed with CSF which was pretreated with either neutralizing anti-MIP-2 alone or combined with anti-KC mAb. For this migration assay, PMN from $CD14^{-/-}$ mice were selected because of a higher percentage of migrated cells. As shown in figure 5*A*, preincubation of CSF with anti-MIP-2 mAb did not reduce the number of migrated PMN as compared to CSF preincubated with control rat IgG. Similar results were observed with neutralization of both MIP-2 and KC (data not shown). This was expected since MIP-2 and KC levels did not significantly differ in CSF from $CD14^{-/-}$ and wt mice. A stronger migration was also observed with PMN from $CD14^{-/-}$ than wt mice when a low dose of PCW (30 ng) was used as chemoattractant instead of infected CSF (figure 5*A*). This difference between $CD14^{-/-}$ and wt PMN cells was abolished when a high dose of PCW (3 µg) was applied (data not shown). Interestingly however, PMN from $CD14^{-/-}$ mice also migrated more

strongly towards rMIP-2 and this effect was neutralized by anti-MIP-2 mAb (figure 5*B*). Thus in conclusion, recombinant MIP-2 was more effective on CD14-deficient than on wt PMNs. However the stronger effect of CSF was not mediated by MIP-2, but rather by cell wall products. These results indicate, that in $CD14^{-/-}$ mice not only increased chemokine levels in brain but also strongly migrating PMN contributed to the early CSF pleocytosis.

2.6 $CD14^{-/-}$ mice show a stronger meningeal inflammation in CSF

Intrathecal leukocytes are beneficial for bacterial killing and unfavourable mediators of inflammation. Since we found that early excess infiltration of leukocytes in CSF did not contribute to accelerated bacterial killing in $CD14^{-/-}$ mice, but on the contrary, allowed bacteria to accumulate in brain, we investigated the consequence of leukocyte infiltration on meningeal inflammation.

TNF activity in CSF was higher in $CD14^{-/-}$ than wt mice 24 h after *S. pneumoniae* infection (figure 6*A*). Early after infection, at 6 and 12 h, TNF levels were below 10 pg/mL, they peaked at 24 h and decreased after this time point. To investigate the cellular source of TNF *in vivo*, immunohistochemical stainings were performed in CSF cells 24 h after infection. Infiltrating cells were mainly PMN (80-90%), as identified by cytospin or flow cytometry using the granulocyte specific marker anti-Gr1 (data not shown). Nearly all PMN from both mouse strains contained intracellular TNF. Staining intensity was similar in $CD14^{-/-}$ and wt mice, when it was determined by immunoperoxidase (wt mice, a, and $CD14^{-/-}$, b; figure 6*B*) or flow cytometry (data not shown). These results indicate that the high TNF levels in the absence of CD14 were not due to enrichment in TNF producing cells or to an increased production of intracellular TNF per cell. Most likely

the early higher leukocyte numbers was sufficient to explain the increased TNF concentration after 24 h.

2.7 Infiltrating leukocytes are the major cellular source of TNF and TNF contributes to outcome

To confirm that the excess number of infiltrating leukocytes were the cellular source of increased TNF in $CD14^{-/-}$ mice, we analyzed TNF mRNA expression in brains of infected mice which were depleted in leukocytes by cyclophosphamide pretreatment. Leukocytes from both mouse strains could be depleted in blood (mean ± SD, 87.7 ± 5.0% vs 76.7 ± 10.3% of depletion in $CD14^{-/-}$ and wt mice, respectively; $P < .01$, figure 7*A*), but infiltration into the CSF was not reduced by cyclophosphamide pretreatment in $CD14^{-/-}$ mice (mean ± SD, 0.2 ± 0.3% in $CD14^{-/-}$ vs 89.1 ± 15.9% of depletion in wt mice; figure 7*B*). Analysis of TNF mRNA expression by *in situ* hybridization in non-treated brains 24 h after infection, revealed a higher signal intensity in $CD14^{-/-}$ (figure 8*A*, b) than in wt mice (figure 8*A*, a). In leukocyte-depleted animals, the TNF mRNA signal was clearly reduced in $CD14^{-/-}$ (figure 8*A*, d) and completely abolished in wt mice (figure 8*A*, c). Moreover, combined *in situ* hybridization and immunohistochemistry of brain sections of mice showed a localization of TNF mRNA in CD45-positive leukocytes (mean ± SD, 91.5 ± 7.0%, and figure 8*B*, a). Among these TNF-positive infiltrating cells, F4/80-positive macrophages represented 86.4 ± 14.4% (figure 8*B*, b) and Gr1-positive granulocytes only 17.0 ± 8.1% (figure 8*B*, c). These results suggested that TNF was mainly derived from infiltrating cells. The distribution of TNF mRNA in brain cell subpopulations was similar in $CD14^{-/-}$ and wt mice.

Less than 10% of TNF-positive cells expressed the microglia marker Iba1 (figure 8*B*, e). In fact TNF-/Iba1-double positive cells were small and weakly Iba1-stained, thus probably representing Iba1-positive macrophages [27], while strongly Iba1-positive ramified cells resembled morphologically microglia and were never TNF-positive. Besides, the expression of CD45 by the large majority of TNF-positive cells, about 10% of the TNF-positive cells also stained for GFAP (figure 8*B*, d). Accordingly, CD45 and GFAP markers overlapped in a small fraction of cells; either anti-CD45 Ab stained astrocytes or some GFAP-positive cells expressed CD45.

The TNF level in CSF was significantly correlated to the severity of disease 24 h after *S. pneumoniae* infection ($r = 0.402$; $P < .01$; figure 9). This suggested that the excess leukocytes in $CD14^{-/-}$ mice led to enhanced TNF and this most likely accelerated the course of the disease.

3. Discussion

In the present study, we investigated the *in vivo* function of CD14 in murine pneumococcal meningitis. We found that $CD14^{-/-}$ mice or wt animals pretreated with neutralizing anti-CD14 mAb were more susceptible to *S. pneumoniae* meningitis and died earlier. Our observations are in contrast to a previous study of *Staphylococcus aureus* sepsis [20]. In that mouse model, mortality was not significantly different in $CD14^{-/-}$ and wt mice. Possible explanations to these contradictory results might be that the authors used mice with an incomplete deletion of the CD14 gene, a different Gram-positive bacterial strain and a systemic infection model, while we investigated the role of CD14 in a local infection model with late sepsis. In agreement with the present study, others have shown a deleterious effect of CD14 blockade in mice after infection with *Klebsiella pneumoniae* [17] and rabbits infected with *Escherichia coli* K-1 [18] or *Shigella flexneri* [19].

The lack of CD14 did not impair systemic host defense, since similar bacterial counts were observed in blood and CSF of $CD14^{-/-}$ and wt mice. Moreover, brain bacterial load was not significantly different in $CD14^{-/-}$ and wt mice early in infection; This suggests that CD14 did not directly affect clearance of *S. pneumoniae* by host cells. Similar observations were made after in vitro infection of peritoneal macrophages [28], where *E. coli* binding and uptake did not differ in $CD14^{-/-}$ and wt cells. Our data in $CD14^{-/-}$ mice are in sharp contrast to those observed in $TLR2^{-/-}$ mice, which showed a slower bacterial clearing than wt mice during meningitis [29] and implies that CD14 is not involved in the TLR2-modulated bacterial phagocytosis [30] and killing.

In meningitis death occurs as a consequence of sepsis or of brain inflammation with edema. If $CD14^{-/-}$ mice did not die earlier from sepsis, death was most likely due to inflammation conferred by the early excess leukocyte infiltration.

A strong influx of PMN into CSF is required for elimination of bacteria in meningitis, but an excessive number also contributes to brain inflammation and injury [31] [14] [32]. In an earlier study we found CD14 induced in PMN and in soluble form in CSF of pneumococcus-infected wt mice [33]. We did not know its function, now we showed that CD14 deficiency was associated with excess infiltrating PMN early in infection. Our findings are in agreement with a previous study, which described more infiltrating PMN in peritoneum of $CD14^{-/-}$ than of wt mice after i.p injection of *E. coli* [34]. This process of transmigration requires molecular interactions between receptors expressed on neutrophils and their endothelial counter-ligands. The adhesion molecules, L-selectin, the three $ß_2$-integrins (LFA-1, Mac-1 and gp 150) and ICAM-1 play a major role in PMN rolling, adherence and transmigration, respectively [35]. CD11/CD18 was shown to be particularly important in migration of PMNs into CSF during bacterial meningitis, since inflammation was markedly attenuated by anti CD11/CD18 treatment. [36] [37]. Since CD14 can associate with CD11b/CD18, the lack of CD14 may lead to a modulated CD11b/CD18 expression. [38]. We therefore investigated PMN adhesion molecules in blood and CSF. Our data demonstrated no differences in integrin or selectin cell surface expression in wt and $CD14^{-/-}$ mice. Further analysis of their counter-ligands in brain endothelia, including CD62-E (E-selectin), CD54 and vascular cell adhesion molecule-1 (VCAM-1; CD106) revealed similar increased expression in brains of wt and $CD14^{-/-}$ mice after *S. pneumoniae* infection. In a recent *in vitro* study, PMN migration induced by

S. pneumoniae was not found dependent of ICAM-1, VCAM-1 or E-selectin expression in endothelial cells, since none of these adhesion molecules was upregulated in response to *S. pneumoniae*. Moreover, neutralizing Ab to CD54 did not prevent PMN migration [39]. This *in vitro* result contrasted with those results obtained *in vivo* in rat and rabbit bacterial meningitis, where intravenous treatment with anti-ICAM-1and anti CD18 mAb respectively reduced the influx of leukocytes into CSF [40] [36] [41] [37]. In agreement with these findings, our *in vivo* data revealed an up-regulation of integrins in PMN and ICAM-1, VCAM-1 in brain endothelial cells in pneumococcal meningitis. However, the stronger neutrophil migration observed in $CD14^{-/-}$ mice, following *S. pneumoniae* infection, did not correlate with up-regulation of any of these adhesion molecules.

In addition to adhesion molecules, PMN require chemoattractants to direct their migration into the subarachnoid space. Chemokines and bacterial components are the best know candidates for leukocyte recruitment to inflammatory sites [42]. The CXC chemokine growth-related oncogene-α (GRO-α) was found in CSF of patients with bacterial meningitis and was shown to mediate chemotaxis *in vitro* [26]. In mice, MIP-2 - the murine homolog of GRO-α - was found released in CSF of mice infected with *Listeria monocytogenes* and similarly induced chemotactic activity *in vitro* [43]. The blockade of the keratinocyte-derived chemokine (KC; murine homolog of GRO-α) as well as MIP-2 or its receptor CXCR2 [44], has been demonstrated to reduced neutrophil migration in several mouse infection models [45] [46] [47] [48]. On the other hand, intracerebral injection of KC and MIP-2 resulted in neutrophil recruitment into the CSF [49]. In the present study, MIP-2 and KC concentrations in CSF, were similar in wt and $CD14^{-/-}$ mice, which is consistent with a previous observation excluding any correlation

between neutrophil counts and chemokine concentrations in CSF during bacterial meningitis [50]. Moreover, soluble ICAM-1 (sICAM-1), which has been previously found in high concentrations in CSF of patients with bacterial meningitis [21] did not differ in the two mouse strains. However, MIP-2 concentration in brain homogenates, which is derived from PMN-bound MIP-2 [22] and from resident cells, was 10-fold higher in $CD14^{-/-}$ than in wt mice. Thus, the absence of CD14 leads to an upregulation of chemokine release, which was not detectable in CSF. Besides the increased chemotactic ligand concentrations, we assessed the chemotaxis of mouse blood PMN towards the CSF of infected mice. In agreement with earlier studies, which demonstrate a chemotactic activity in CSF from mice [43] and patients [26] with bacterial meningitis, CSF from *S. pneumoniae* infected mice showed a chemotactic activity *in vitro*. However, the fact that neutralizing anti-MIP-2 and anti-KC mAbs failed to inhibit, even partially, CSF mediated chemotaxis, suggested the presence of additional, bacterial chemotactic factors or of very low chemokine concentrations in vivo. Some authors, using a rabbit model of pneumococcal meningitis, suggested that bacterial cell wall components contribute to the chemotaxis of CSF [51]. Indeed, peptidoglycan, lipoteichoic acid (LTA) and teichoic acid (TA), which are the most active components of pneumococcal cell wall (PCW), induced leukocyte pleocytosis *in vivo* after intracisternal injection into rabbits [52]. In our model, subarachnoidal injection of PCW induced a stronger neutrophil migration in CSF of $CD14^{-/-}$ than wt mice *in vivo*. Moreover, PMN from $CD14^{-/-}$ mice migrated more than wt PMN cells upon PCW *in vitro.* The fact that CSF from wt and $CD14^{-/-}$ mice showed similar chemotactic activity despite a stronger migration capacity of $CD14^{-/-}$ PMN, supports the conclusion that the chemotaxin was similar in both strains, but that CD14–

deficient PMN had altered chemokine receptor expression chemotactic receptors or a stronger signalling. MIP-2 and KC are known to bind the same CXCR2 receptor on PMN, even if KC was 10-fold less potent than MIP-2 [53]. In addition, mice which lack CXCR2 receptor showed a severe impairment of neutrophil recruitment [44]. Recently, it has been shown that LPS, acts through Toll-like receptor 4 (TLR4) to downregulate expression of two specific kinases (GRK2 and GRK5) interacting with G-protein coupled receptor in response to MIP-2. This downregulation of GRK2 and GRK5 resulted in decreased CXCR2 receptor internalization and increased PMN migration [54]. Thus, it is tempting to hypothesize that a similar mechanism could be involved in our model of Gram-positive meningitis. i.e. CXCR2 might be upregulated in the absence of CD14 and TLR2 [55] [56] [57] [10].

Our analysis of CD14/TLR2 in wt CSF cells showed that about 50% of the cells carry both TLR2 and CD14 and 35% have only TLR2, few have only CD14. We found earlier that $TLR2^{-/-}$ mice had a delay in leukocyte infiltration [29]. It is postulated that in these mice the presence of CD14 alone is insufficient to get the appropriate migration and chemokine release. On the other hand the presence of TLR2 alone in $CD14^{-/-}$ mice, signals faster upon chemotaxins and induces more chemokines. This points to a newly recognized mutual role of CD14 and TLR2 in leukocyte migration.

The stronger recruitment of PMN in CSF of $CD14^{-/-}$ mice was most likely associated with increased TNF release in CSF. Two observations supported this hypothesis. First, we found that TNF increased after neutrophil pleocytosis had started. Second, depletion of leukocytes by cyclophosphamide before infection showed downregulated TNF mRNA transcripts. Moreover, we could show by combined *in situ* hybridization and

immunohistochemistry that the TNF mRNA signal mainly colocalized with CD45+ infiltrating leukocytes. These data indicated that TNF was mainly derived from infiltrating cells. As reported previously, the consequence of meningeal inflammation is a disruption of the blood-brain barrier and therefore aggravation of the disease [29].

In conclusion, the present study clearly demonstrated that the lack or blockade of CD14 was associated with a stronger neutrophil recruitment into CSF, leading to excessive meningeal inflammation and aggravated disease after *S. pneumoniae* infection. This stronger neutrophil migration correlated with chemokine concentrations in brain and with an enhanced migratory capacity of CD14-deficient PMN. Our results show for the first time a direct implication of CD14 in regulation of neutrophil migration in mouse pneumococcal meningitis.

4. Materials and Methods

4.1 Bacteria

Streptococcus pneumoniae (clinical isolate of serotype 3) was grown for 7 h in double Mueller Hinton broth (MHB) (DIFCO Laboratories Detroit, USA), then subcultured overnight in new MHB, and washed in 0.9% sterile saline (12,000 x g for 6 min) immediately before use. The inoculum size was calculated from optical density (OD) determinations at 620 nm (OD 0.4 = 1 x 10^8 CFU/mL) and was retrospectively assessed by CFU counting on blood agar plates.

The unencapsulated *Streptococcus pneumoniae* derivative R6 strain (a kind gift from Dr. S. Leib, University of Bern, Switzerland) was grown in Brain-heart infusion (BHI) medium for 6 to 8 h at 35.2°C in 5.5% CO_2, followed by subculture in fresh BHI medium to mid-logarithmic phase (optical density at 620 nm of 0.5). After washing, bacteria were killed by heating for 20 min at 80°C. Serial dilution were plated on blood agar plate and incubated at 37°C for 24 h to confirm the absence of growth.

Pneumococcal cell wall (PCW) was prepared as described earlier with minor modification [52] [58] [59]. *Streptococcus pneumoniae* (clinical isolate of serotype 3) was grown to an OD of 0.5 at 620 nm (1.5 × 10^8 cfu/mL), chilled on ice, to prevent autolysis and centrifuged at 4°C at 4000 x g. After washing the pellet was resuspended in cold 50 mM Tris-HCl (pH 7.5) and placed in boiling 5% SDS solution for 15 min. After centrifugation at 10000 x g for 10 min, pellet was washed twice in 1 M NaCl and three times in distilled water to remove remaining SDS. The pellet was resuspended in water, added to an equal volume of sterile glass beads, and vortexed strongly for 5 min. After centrifugation at 700 x g for 10 min, the supernatant containing cell walls was

centrifuged again at 38000 x g for 15 min. The pellet was resuspended in 100 mM Tris-HCl (pH 8.0) containing 20 mM $MgSO_4$. DNase (10 µg/mL) and RNase (50 µg/mL) were added and incubated at 37°C for 2 h. Proteins were degraded overnight at 37°C in 100 µg/mL trypsin containing 10 mM $CaCl_2$. SDS was added to a final concentration of 1% and incubated at 60°C for 15 min. The solution containing cell walls was centrifuged at 38000 x g for 15 min and washed three times in distilled water to remove remaining SDS. The pellet was centrifuged again at 38000 x g for 15 min and resuspended to a final concentration of 1 mg/ml in saline solution.

4.2 Mouse meningitis models

Six to eight week old C57BL/6 wt (RCC, Ittingen, Switzerland) and $CD14^{-/-}$ mice (kindly provided by MW. Freeman**;** Lipid Metabolism Unit, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts, USA), which had been backcrossed for 6 generations on a C57BL/6 background) were kept under specific pathogen free conditions in the Animal House of the Department of Research, University Hospitals Basel according to the regulations of the Swiss veterinary law. Mice were anesthetized via intraperitoneal (i.p.) injection of 100 mg/kg Ketamine (Ketalar©; Warner-Lambert AG, Baar, Switzerland) and 20 mg/kg Xylazinum (Xylapan©; Graeub AG, Bern, Switzerland), and subsequently subarachnoidally inoculated into the left forebrain with either 0.9% NaCl, live *Streptococcus pneumoniae* (3×10^3 CFU), PCW (300 µg/kg) or heat-killed R6 strain (3×10^6 CFU).

In vivo CD14 blockade experiments were performed by pretreatment with i.v. injection of 4C1 or isotype-matched control rat IgG2b mAbs 5 min prior to i.c. injection of 3 x 10^3 CFU of live *S. pneumoniae*. The health status of the mice was assessed by the following scores as described previously [60] (1) normal motor activity and turned upright in < 5s when put on their back; (2) decreased spontaneous activity, but still turned up in < 5s; (3) turned up in > 5s; (4) did not turn up; (5) did not move. After 6, 12, 24, 48 and 72 h or if they presented score 5, mice were sacrificed by i.p. injection of 100 mg/kg pentobarbital (Abbott Laboratories, North Chicago, IL). Blood was obtained by intracardiac puncture and collected in EDTA. Animals were perfused with Ringer's solution (Braun Medical AG, Emmenbrücke, Switzerland) into the left cardiac ventricle until the effluent became clear. Cerebrospinal fluid (CSF) was obtained by puncture of the cisterna magna as described previously [61]. Due to the small volumes (3-6 μL) obtained from each animal; CSF from 2 to 3 mice was pooled in some experiments.

For the leukocyte depletion treatment, cyclophosphamide (Sigma) was reconstituted with sterile PBS and injected i.p. (300 mg/kg in 0.2 mL) 48 h before *S. pneumoniae* inoculation.

4.3 Real-time in vivo imaging study of meningitis using bioluminescent *S. pneumoniae* transformed with Gram-positive lux transposon

Mice were subarachnoidally injected with 3 x 10^3 CFU luciferase-tagged *S. pneumoniae* serotype 3 (Xen10) and subsequently shaved for better imaging. This bacterial strain, kindly provided by L. Chen (Xenogen Corporation, Alameda, CA) was constructed as described previously [62].

After infection, analysis of photons was performed repeatedly in mice under isoflurane inhalation anesthesia in an IVIS CCD (charge-coupled device) camera (Xenogen Corporation, Alameda, CA) coupled to the LivingImage software package (Xenogen Corporation, Alameda, CA).

4.4 Determination of bacterial counts and inflammatory parameters

Blood samples collected in EDTA and pooled CSF samples were serially diluted in 0.9% saline to assess the bacterial load after plating and incubation at 37°C for 24 h. CSF samples were centrifuged at 800 x g for 7 min (room temperature) to obtain cell free CSF. Thereafter, they were stored at -20°C until cytokine and chemokine determinations. The pelleted CSF cells were counted and identified via cytospin or phenotypically analyzed. Brains were removed 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% saline on blood agar plates. For *in situ* hybridization, brains were fixed in 4% paraformaldehyde (Aldrich) and embedded in paraffin blocks.

The concentration of the pro-inflammatory cytokine TNF in CSF was determined with a bioassay, measuring the degree of cytotoxicity on WEHI cells in the presence of 1 μg/mL actinomycin D, using mouse rTNF as a standard. Soluble ICAM-1 and the chemokine MIP-2 and KC levels in CSF were measured by ELISA Kits (R&D Systems Europe, Abingdon, UK) according to the instructions of the manufacturers.

4.5 Preparation of peripheral blood-derived polymorphonuclear (PMN) cells for chemotaxis

Mouse blood was harvested by intracardiac puncture and collected in EDTA and PMN were isolated by density gradient centrifugation on a discontinuous Percoll gradient with 59 and 67% Percoll (Percoll, Pharmacia Biotech AB, Uppsala, Sweden) in PBS as previously described [63]. The interface of the 59 and 67% Percoll layers, which contains the PMN, was collected and contaminating erythrocytes were removed by hypotonic lysis in water. By morphologic criteria, the final cell preparation contained > 97% PMN and was used at a concentration of 6.9×10^5 cells/mL in chemotaxis assays. The viability of the cells, as assessed by trypan blue exclusion, was >95%.

4.6 Chemotaxis assay

Migration of mouse PMN was assessed *in vitro* using 48-well microchemotaxis chambers as previously described [43]. Dilutions of samples or CSF diluted ½ (v/v) in 28 µL Hanks Hepes buffer were added to the lower wells in triplicates. A polyvinylpyrrolidone-free polycarbonate filter membrane (3 µm pore size; Nuclepore, Sterico AG, Dietikon, Switzerland) was placed between the lower wells and the upper wells containing 45 µL of cell suspensions (6.9×10^5 cells/mL in Hanks Hepes buffer). The chemotaxis chambers were incubated for 60 min at 37°C in 5% CO_2 in humidified air. After incubation, the filters were removed, fixed and stained with Wright-Giemsa. The number of cells that migrated through the pores of the filter was counted in 6 high-

powered fields (x 63). Cell migration was expressed as the mean percentage of PMN that migrated per well. Hanks Hepes buffer alone was used as a control for random migration, and *N*-formylmethionyl-leucyl-phenylalanine (fMLP, Sigma Chemical Co.) as a positive control at concentration 10^{-6} M.

For neutralization experiments, CSF samples were preincubated with anti-MIP-2 (30 μg/mL) or control rat IgG2b (30 μg/mL) mAbs for 120 min at 37°C.

4.7 Microvascular endothelial cell cultures

Brain-derived microvascular endothelial cells (MVEC) were isolated from 6-12 week old C57BL/6 or CD14-deficient mice of either sex according to published methods [64] [65] [66] with slight modifications. Briefly, the brain was aseptically removed and cortical tissue was cleaned of meninges and superficial vessels using fine foreceps under a binocular microscope. The cortices were transferred into cold DMEM (Gibco, Invitrogen AG, Basel Switzerland) supplemented with 5% FCS (PAA Laboratories, Linz, Austria), 0.4 mM N-acetyl-L-alanyl-L-glutamine (Biochrom, Berlin, Germany) and 20 μg/mL gentamycin (Sigma, Fluka AG, Buchs, Switzerland) (DMEM/5%), cut into small pieces and homogenized with a 10 mL-syringe. Dispase II solution (Roche, Rotkreuz, Switzerland) was added to a final concentration of 0.2% (0.096 U/mL) and preparation was allowed to digest for 1 h at 37°C under stirring in a water bath. The homogenate was centrifuged (1000 x g, 10 min, 4°C) and the supernatant removed. The pellet was resuspended in 2 mL DMEM containing 20% dextran (MW = 71327, Sigma), homogenized with a fire-polished pipette and centrifuged (1700 x g, 10 min, 4°C). The top layer containing most of the myelin was discarded and the pellet resuspended in

DMEM/5%, layered onto 13% dextran solution in DMEM and centrifuged (1700 x g, 10 min, 4°C) to remove remaining myelin. Thereafter, the pellet was suspended in DMEM/5% and digested for 30 min at 37°C with collagenase/dispase (Roche) and DNase I (Sigma) at a final concentration of 1 mg/mL and 0.05 mg/mL, respectively. The cells were centrifuged (380 x g, 10 min, 4°C), washed twice with DMEM/5% and plated on collagen I-coated culture dishes (60 mm diameter, BioCoat, Becton Dickinson, Basel, Switzerland). The culture medium was composed of DMEM containing 20% FCS, 20 μg/mL endothelial cell growth supplement (ECGS, Serva, Heidelberg, Germany), 2 mM N-acetyl-L-alanyl-L-glutamine, 20 μg/mL gentamycin and 16 U/mL heparin (Sigma). Cells were grown at 37°C in a humidified 8% CO_2 atmosphere. Two days after seeding, cells were washed with HBSS and fresh medium was added. Thereafter, the medium was replaced every 2 days. MVEC were grown to confluence and passaged between day 6 and 9. Based on their expression of von Willebrand Factor (Dako, Glostrop, Denmark) and uptake of Dil AcLDL (Molecular Probes, Yuro Supply, Lucerne, Switzerland) the MVEC cultures were found to have a purity of at least 90%. Cells were trypsinized (0.25% Trypsin/EDTA, Biological Industries, Kibbutz Beit Haemek, Israel) and seeded either in collagen I-coated culture dishes (0.2 Mio. cells/dish, 35 mm diameter, BioCoat) or 96-well F-plates (2 x 10^4 cells/well, BioCoat). After overnight culture, the medium was replaced with fresh culture medium without gentamycin and infected with viable *S. pneumoniae* at a multiplicity of infection (MOI) of 2, 10 and 20. In some experiments, the bacteria-containing medium was removed 2 h later, the cells were washed twice with PBS and replaced with fresh DMEM culture medium supplemented with 100 μg/mL

gentamycin to prevent extracellular bacterial growth. Supernatants were harvested 8 and 24 h later, centrifuged (2000 x g, 15 min, 4°C) and stored at -20°C until ELISA analysis.

4.8 Immunocytochemistry for TNF

Conventional avidin-biotin system immunocytochemistry was carried out with some modifications as previously described [67]. CSF cells from *S. pneumoniae* infected mice were harvested and collected directly in RPMI 5% FCS containing Brefeldin A (Sigma) at 10 μg/mL and plated in 16-well glass LabTek^{TM} chamber slides (Nalge Nunc, Naperville, IL) for 2 h at 37°C. Cells were washed and fixed with ice cold 4% paraformaldehyde for 5 min. The fixative was removed and cells were washed in wash solution composed by Hanks' balanced salt solution (HBSS, Life Technologies GIBCO), 10 mM Hepes (Calbiochem) and 0.1% Saponin (Sigma). Endogenous peroxidase activity was blocked by incubating cells with 0.3% H_2O_2 in wash solution for 20 min at RT. After washing, cells were incubated overnight at 4°C with rat anti-mouse TNF (BD Pharmingen 554641) or rat IgG1 mAbs at 40 μg/mL in wash solution containing 2% normal rabbit serum. After washing, cells were incubated 1 h at RT with a secondary biotinylated rabbit anti-rat Ab (Vector Laboratories, Burlingame, CA) at a dilution of 1/200, followed by incubation with avidin/biotinylated-peroxidase complexes (Vectastain *Elite* ABC, Vector Laboratories) for 30 min. Cells were then rinsed with 0.05 M Tris-HCl pH 7.6 and incubated with peroxidase substrate 3-amino-9-ethyl carbazole (DAKO Corporation, Carpinteria, CA) for 5 min. After rinsing in distilled water, cells were counterstained with hematoxylin (Medite, Nunningen, Switzerland) for 40 s and mounted.

4.9 cRNA probes and in situ hybridization

Plasmids were linearized and the sense and antisense riboprobes synthesized as described [68]. Radioactive cRNA copies were synthesized by incubation of 250 ng linearized plasmid in $MgCl_2$ 6 mM, Tris (pH 7.9) 40 mM, spermidine 2 mM, NaCl 10 mM, dithiothreitol 10 mM, ATP/GTP/CTP 0.2 mM, 100 µCi of α-^{35}S-UTP (Dupont NEN, #NEG 039H), 20 U RNAsin (Promega, Catalys, San Luis Obispo, USA) and 10 U of either T7, SP6 or T3 RNA polymerase for 60 min at 37 °C. Unincorporated nucleotides were removed using ammonium-acetate precipitation method; 100 µL of DNAse solution (1 µL DNAse, 5 µL of 5 mg/mL tRNA, 94 µL of 10 mM Tris / 10 mM $MgCl_2$) was added, and 10 min later, a phenol-chloroform extraction was performed. The cRNA was precipitated with 80 µL of 5 M ammonium acetate and 500 µL of 100% ethanol for 20 min on dry ice. The pellet was dried and resuspended in 50 µL of 10 mM Tris/1 mM EDTA. A concentration of 10^7 cpm/probe was mixed into 1 mL of hybridization solution (500 µL formamide, 60 µL 5 M NaCl, 10 µL 1 M Tris [pH 8.0], 2 µL 0.5 M EDTA [pH 8.0], 50 µL 20x Denhart's solution, 200 µL 50% dextran sulfate, 50 µL 10 mg/mL tRNA, 10 µL 1 M DTT, [118 µL DEPC water - volume of probe used]). This solution was mixed and heated for 10 min at 65 °C before being spotted on slides. Hybridization histochemical localization of TNF mRNA was carried out on every twelfth sections of the whole rostro-caudal extent of each brain using ^{35}S-labeled cRNA probes as described previously [68]. The sections were exposed at 4 °C to X-ray films (Biomax, Kodak, Rochester, NY) for 1-3 days. The slides were thereafter defatted in xylene, dipped in NTB-2 nuclear emulsion (Kodak; diluted 1:1 with distilled water), exposed for 10 days. The slides were then developed in D19 developer (Kodak) for 3.5 min at 14-

15°C, washed 15 s in water, and fixed in rapid fixer (Kodak) for 5 min. Tissues were thereafter rinsed in running distilled water for 1 h, counterstained with thionin (0.25 %), dehydrated through graded concentrations of alcohol, cleared in xylene, and coverslipped with distrene plasticizer xylene (DPX) mounting medium (Electron Microscopy Science, Washington, PA). Signal intensity was quantified with the NIH Image software.

4.10 Statistical analysis

Differences in survival between wt and $CD14^{-/-}$ mice were tested using the log rank test and Kaplan-Meier analysis. Results of the CFU in brain homogenates, leukocyte numbers in blood and CSF and the chemotactic activity of CSF samples were compared using the Mann-Whitney *U* test. Differences in chemokine and sICAM-1 levels in CSF between wt and $CD14^{-/-}$ mice were analyzed with the non-parametric Wilcoxon Signed rank test.

The relationship between two parameters (parametric variables) was assessed in a linear regression model using Spearman rank correlation test. In all statistical tests, a $P < .05$ was considered statistically significant.

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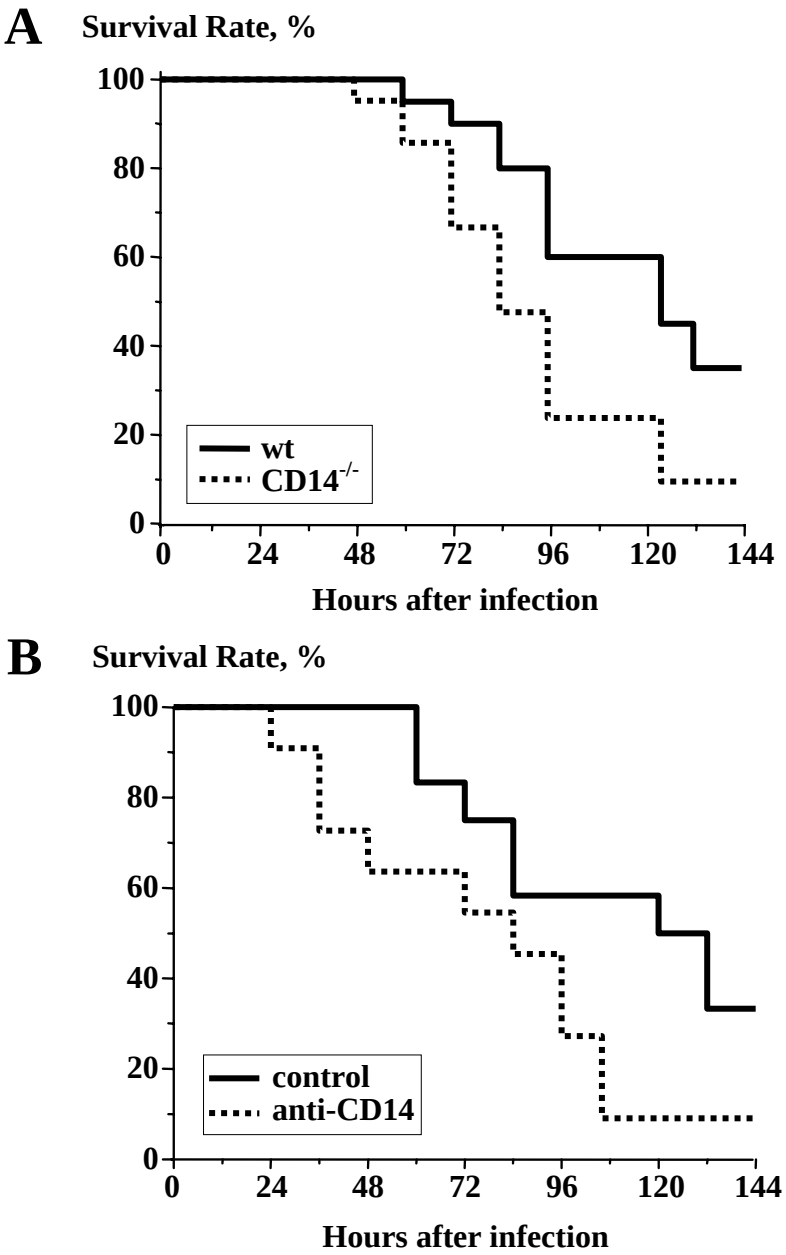

Figure 1. ***A***, Survival of $CD14^{-/-}$ and wt mice after infection with 3 x 10^3 CFU of *S. pneumoniae*. $CD14^{-/-}$ mice (dashed line; n = 22) showed earlier death as compared to wt mice (solid line; n = 21) ($P < .02$).

B, Effect of CD14 blockade on survival of wt mice after infection with *S. pneumoniae*. Mice were treated i.v. with 5 mg/kg of either rat anti-CD14 (4C1) (dashed line; n = 11) or isotype-matched control rat IgG2b (solid line; n = 12) mAbs 5 min prior infection. Anti-CD14 treated mice died earlier than animals receiving isotype-matched control mAb ($P < .02$).

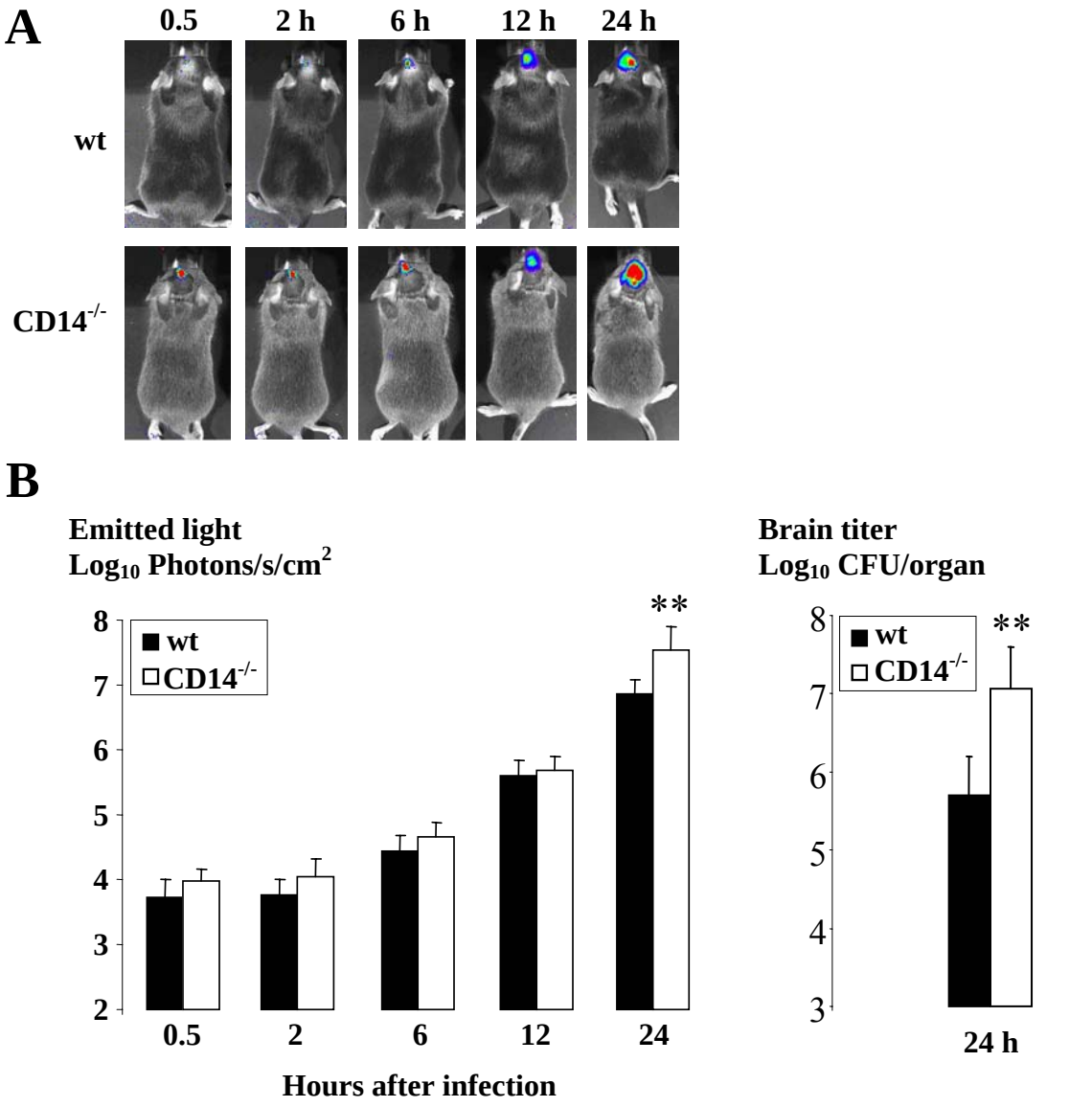

Figure 2. Time course of infection in CD14$^{-/-}$ and wt mice after infection with 3 x 10^3 CFU of luciferase-tagged *S. pneumoniae*. ***A***, Emitted photons in live animals were detected by a highly sensitive CCD camera (IVIS imaging system; Xenogen), and images from one animal out of 9 at 0.5, 2, 6, 12 and 24 h after infection are shown.

B, Emitted light was also quantified by LivingImage software and expressed as Photons/s/cm^2. CD14$^{-/-}$ brains showed more emitted photons [left] and more CFU [right] than wt brains 24 h after infection ($P < .01$).

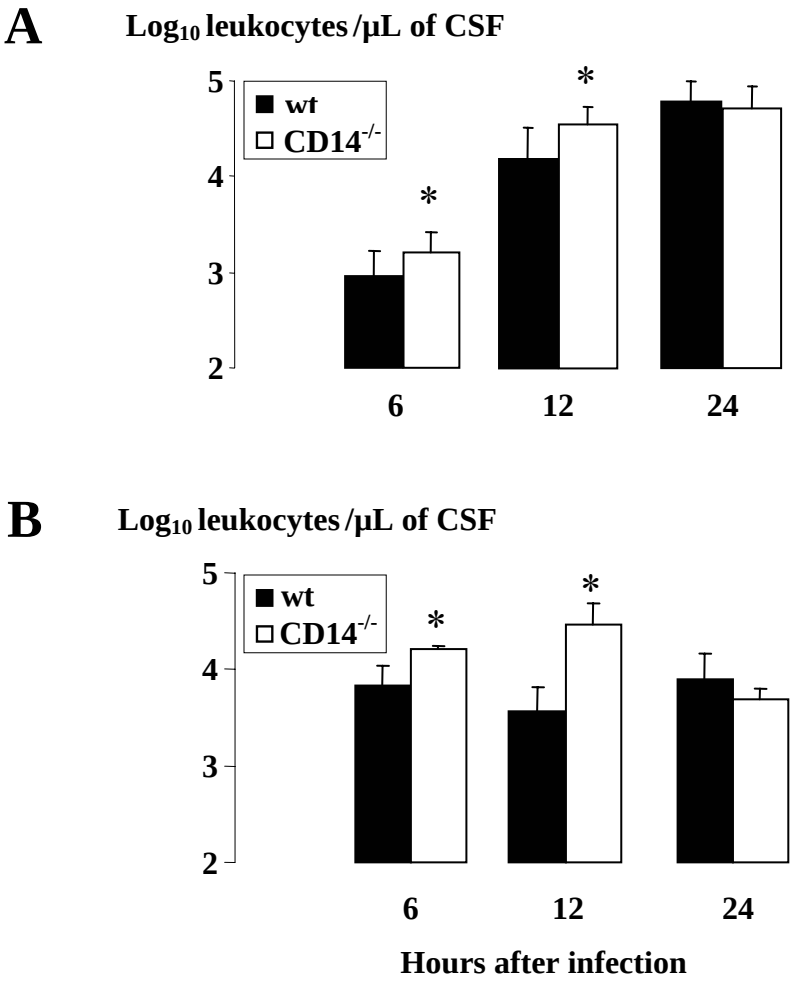

Figure 3. Time course of CSF pleocytosis in $CD14^{-/-}$ and wt mice after injection with either (***A***) live *S. pneumoniae* (3 x 10^3 CFU) or (***B***) PCW (300 µg/kg). CSF was collected at indicated times and infiltrating leukocytes were counted at 6 h (n = 15), 12 h (n = 11) and 24 h (n = 11). Results are represented as mean ± SD. *P < .05.

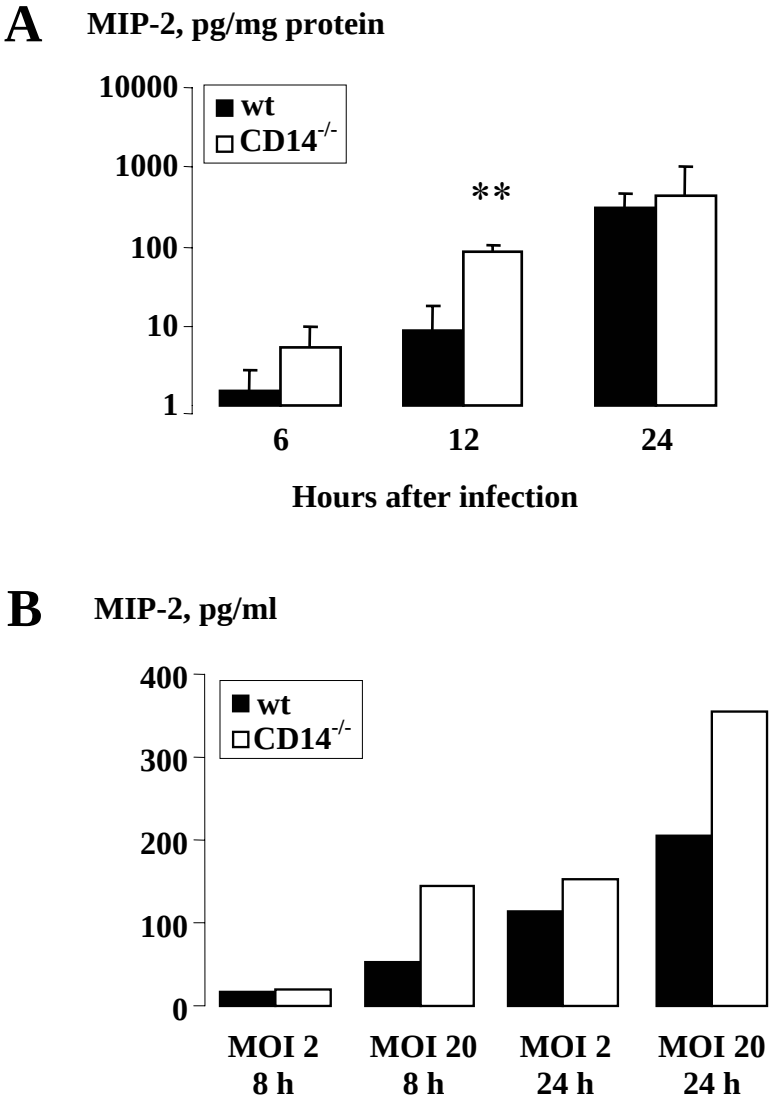

Figure 4. ***A***, Time course of MIP-2 levels in brain homogenates after infection with 3 x 10^3 CFU of *S. pneumoniae*. Mean ± SD of 3 experiments are shown. * $P < .05$.

B, Time- and MOI-dependent MIP-2 production by CD14-deficient and wt brain-derived microvascular endothelial cells. Mean of triplicate cultures are shown.

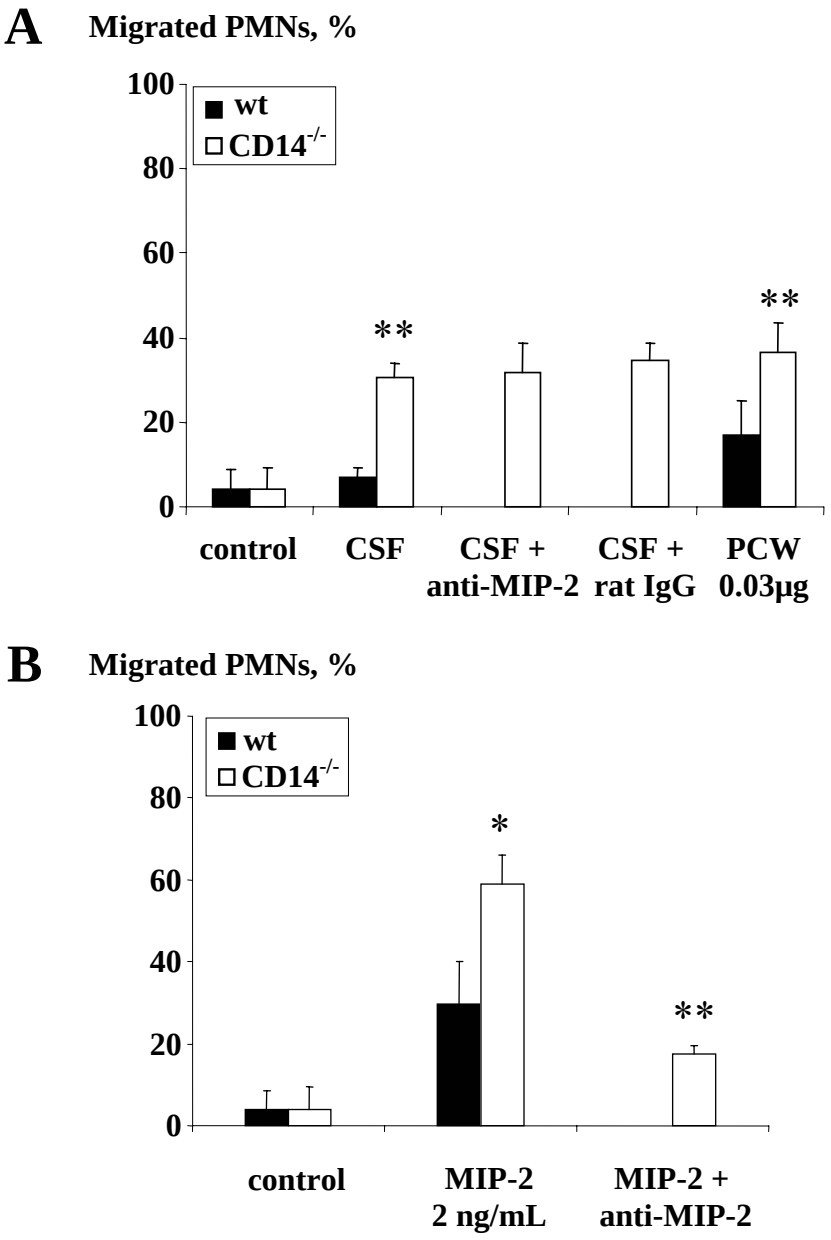

Figure 5. Chemotactic activity mediated by either CSF from CD14$^{-/-}$ and wt mice (subarachnoidally injected with 3 x 10^6 CFU of heat killed R6 *S. pneumoniae*) or by PCW. ***A***) CSF (final concentration of 50%), treated or not with neutralizing anti-MIP-2 (30 µg/mL) or PCW (3 µg or 0.03 µg), (***B***) rmMIP-2 (2 ng/mL) with or without neutralizing anti-MIP-2 (30 µg/mL) was placed into the lower wells of a 48-well microchemotaxis chamber and the chemotactic activity upon blood PMN was measured.

The percentage of migrated neutrophils was determined by counting 600 to 900 cells per well. Mean ± SD of three independent experiments. * $P < .05$; ** $P < .01$.

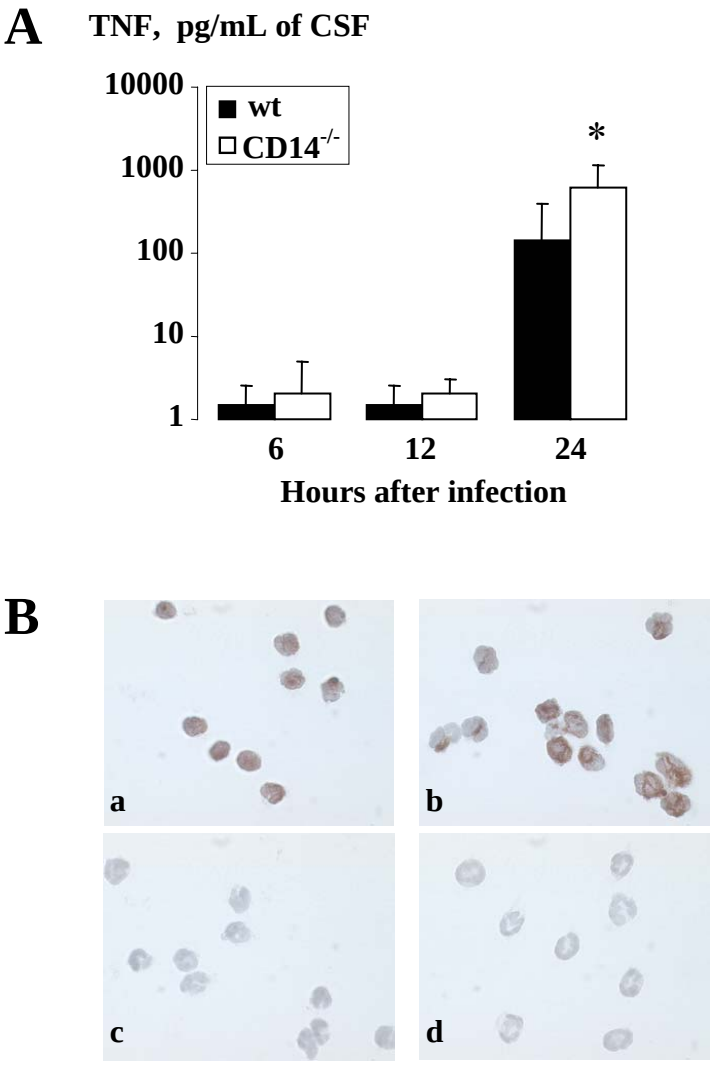

Figure 6. ***A***, Time course of TNF release into the CSF of $CD14^{-/-}$ and wt mice after infection with 3 x 10^3 CFU of *S. pneumoniae.*

CSF was collected at indicated times and TNF levels were determined by a TNF bioassay. Mean ± SD of three independent experiments. * $P < .05$.

B, Intracellular TNF staining of CSF infiltrating PMN harvested from $CD14^{-/-}$ and wt mice 24 h after infection. Cells from $CD14^{-/-}$ (**b** and **d**) and wt (**a** and **c**) mice were stained for intracellular with anti-TNF (40 µg/mL) (**a** and **b**) or control rat IgG1 (40 µg/mL) (**c** and **d**) mAbs by immunocytochemistry. Similar staining intensities were observed in $CD14^{-/-}$ and wt PMN.

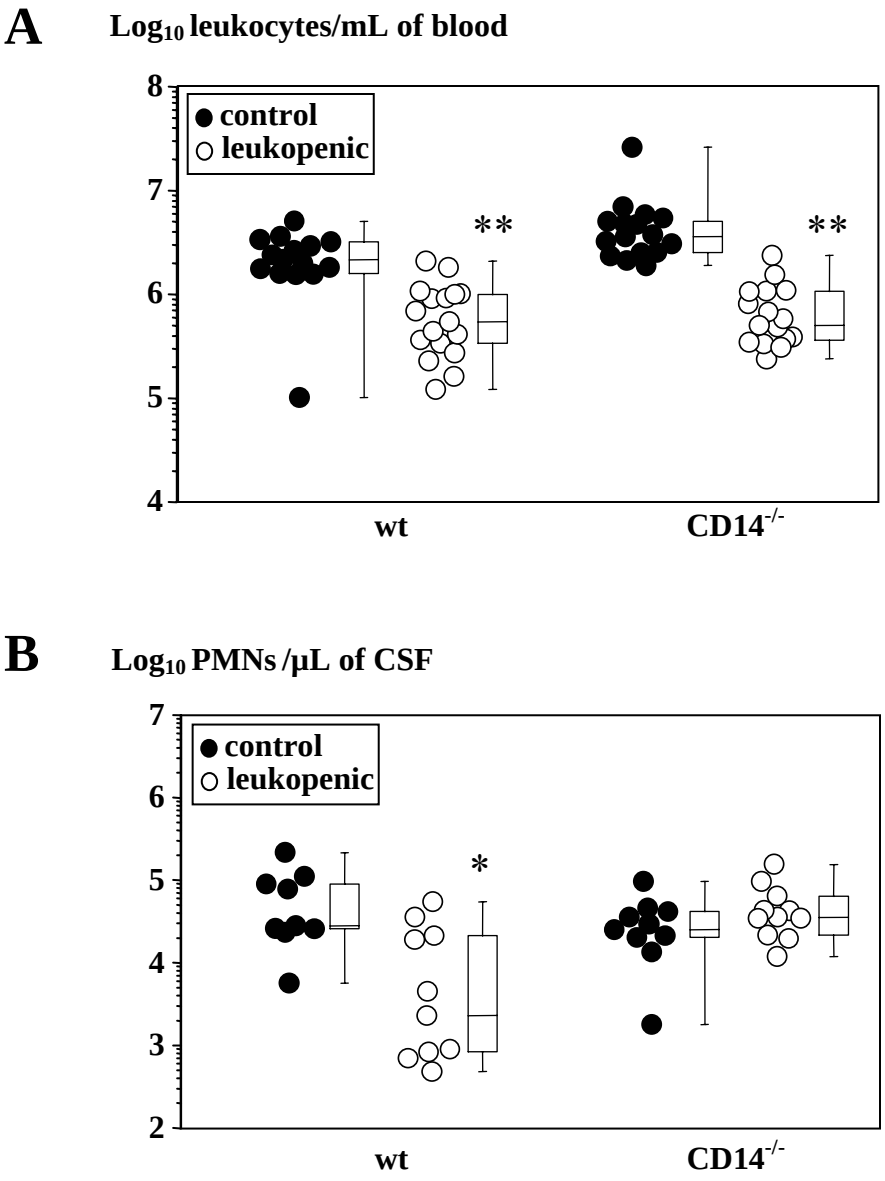

Figure7. Leukocyte numbers in (***A***) blood (n = 15 or 17) and (***B***) CSF (n = 9 or 11) in non treated and leukocyte-depleted animals 24 h after infection with 3 x 10^3 CFU of *S. pneumoniae.* 300 mg/kg of cyclophosphamide was injected i.p. 48 h before *S. pneumoniae* inoculation. Mean ± SD of three independent experiments. * $P < .05$.

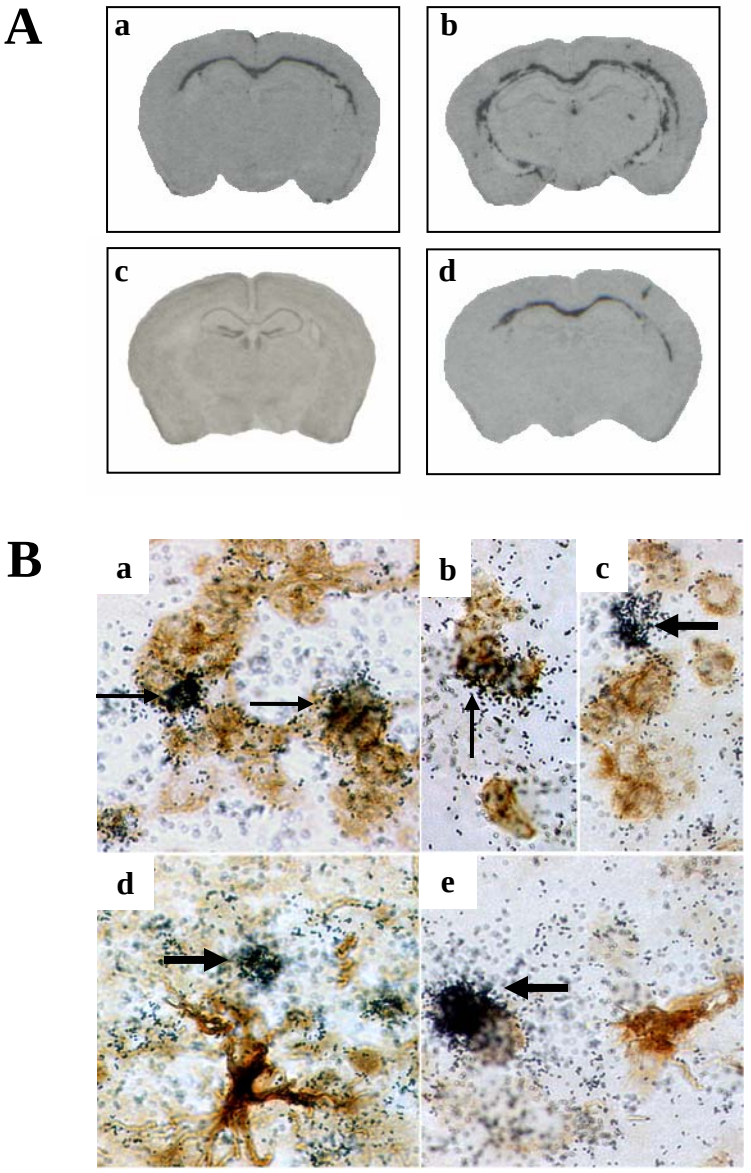

Figure 8. *In situ* hybridization of TNF mRNA in brains from CD14$^{-/-}$ and wt mice 24 h after infection with 3 x 10^3 CFU of *S. pneumoniae*. ***A***, Bright field photomicrographs of coronal brain cryosections (20 µm) hybridized as described in *Materials and Methods*. Expression of TNF mRNA transcripts in non treated (**a** and **b**) and leukocyte-depleted (**c** and **d**) infected animals. Results from one experiment representative of three independent experiments are shown (CD14$^{-/-}$, **b** and **d**; wt, **a** and **c**).

B, Colocalization of TNF mRNA in infiltrating and brain resident cells in mouse brains 24 h after infection with 3 x 10^3 CFU of *S. pneumoniae.* (**a**) CD45, (**b**) F4/80, (**c**) Gr1, (**d**) GFAP and (**e**) Iba1 immunostainings were performed on brain sections. Large arrows, TNF mRNA-positive cells; small arrows, double-labelled cells.

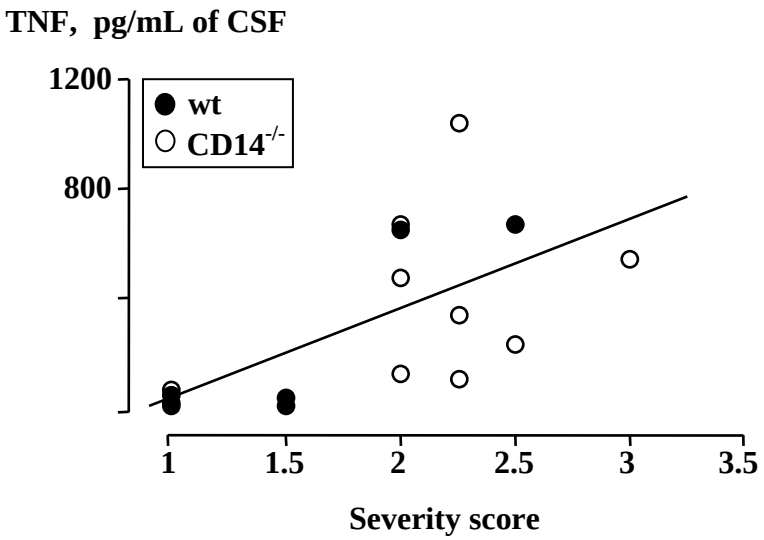

Figure 9. Association between disease severity and TNF release in CSF of mice after infection with 3 x 10^3 CFU of *S. pneumoniae.* The disease severity was assessed as described in *Materials and Methods*.

The correlation between TNF activity and severity of the disease was assessed in a linear regression model using a Spearman rank correlation test. ** $P < .01$. Filled circles = wt mice (n = 7), open circles = $CD14^{-/-}$ mice (n = 9).

Table I. Bacterial load in blood and CSF of $CD14^{-/-}$ and wt mice after infection with 3 x 10^3 CFU of *S. pneumoniae*.

	CFU/mL of blood	CFU/μL of CSF		
Hours after infection	24 h	6 h	12 h	24 h
wt	50 ± 300	89 ± 87	5217 ± 6268	31775 ± 37750
$CD14^{-/-}$	40 ± 300	52 ± 18	10150 ± 6784	28288 ± 20375

NOTE. CFU were determined in blood and CSF as described in *Materials and Methods*. Data are presented as median ± SD. Similar CFU numbers in $CD14^{-/-}$ and wt mice ($P > .05$).

Table II. MIP-2, KC and sICAM-1 levels in CSF of $CD14^{-/-}$ and wt mice 6 and12 h after i.c. infection with 3 x 10^3 CFU of *S. pneumoniae*.

Mouse strain	wt		$CD14^{-/-}$	
Hours after infection	6 h	12 h	6 h	12 h
MIP-2 (pg/mL)	395 ± 341	1747 ± 1708	623 ± 651	2740 ± 2711
KC (pg/mL)	1820 ± 1640	9830 ± 7262	4851 ± 4923	11662 ± 9720
sICAM-1 (ng/mL)	2435 ± 1667	2389 ± 893	2753 ± 1334	2495 ± 464

NOTE. MIP-2, KC and sICAM-1 measurements were determined in CSF from 5 to 6 mice using an ELISA kit. Data are presented as mean ± SD. Similar chemokines and sICAM-1 levels in $CD14^{-/-}$ and wt mice ($P > .05$).

Section III

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Antibiotics Rescue C57BL/6, CD14$^{-/-}$ and TLR2$^{-/-}$/CD14$^{-/-}$ Mice from Pneumococcal Meningitis; Adjuvant TACE Inhibitor Treatment is Required for TLR2$^{-/-}$ Mice

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Running title: TACE inhibitor, TLR2 and CD14 in pneumococcal meningitis

Abbreviations: **cfu**, colony forming units; **CSF**, cerebrospinal fluid; **TLR**, Toll-like receptor; **TNF**, tumor necrosis factor; **wild-type**, wt.

Abstract

Toll-like receptor (TLR)-2 and CD14 mediate host responses to Gram-positive bacterial wall components. $TLR2^{-/-}$, $CD14^{-/-}$ and $TLR2^{-/-}/CD14^{-/-}$ (double Knockout, KO) mice died earlier from murine *Streptococcus pneumoniae* meningitis than wt mice. Modulation of outcome by antibiotic and TNF-alpha converting enzyme (TACE) inhibitor treatment was evaluated in the four mouse strains. The three KO and the wt strain underwent ceftriaxone therapy. All wt, $CD14^{-/-}$ and $TLR2^{-/-}/CD14^{-/-}$ mice were rescued, 27% of the $TLR2^{-/-}$ mice died ($P < .04$). Treatment failure in $TLR2^{-/-}$ mice was related to high bacterial load and cerebrospinal fluid TNF levels in this strain. TACE inhibitor treatment significantly reduced TNF in CSF in $TLR2^{-/-}$ mice. It did not rescue, but prolonged survival in $TLR2^{-/-}$ and $CD14^{-/-}$ mice to the values observed in untreated wt mice. 90% of $TLR2^{-/-}$ mice were rescued by combined antibiotic and TACE inhibitor treatment. $TLR2^{-/-}/CD14^{-/-}$ mice behaved during meningitis like single $CD14^{-/-}$ mice. This illustrates that the two pattern recognition receptors TLR2 and CD14 have differing functions *in vivo*.

Introduction

Streptococcus pneumoniae is the cause of the most severe and most frequent form of adult bacterial meningitis (1). The case fatality rate remained unchanged at 25% since 20 years and intracranial complications with neurological sequelae are frequent (2). In view of increasing resistance of pneumococcus against beta-lactam antibiotics (3), new treatment strategies are required. Adjuvant therapy with dexamethasone is used in children and adults. Pathophysiological parameters and outcome have been found improved (4). Experimental studies have however shown a neurotoxic potential of steroids (5). Therefore other adjunctive therapies (6) have been evaluated which all aimed at reducing inflammatory components or toxic products released by inflammatory cells. TNF and matrix metalloproteases are major targets, both molecules are upregulated early during meningitis (7) and TNF contributes to brain injury in this disease (8, 9). TNF and its receptors are shed from the membrane by TNF alpha converting enzyme (10). A recently developped combined inhibitor of matrix metalloproteases and TNF alpha converting enzyme has proven non toxic and efficient in experimental meningitis and has initiated a new therapeutic area in bacterial meningitis (8).

TLR2 recognizes microbial components and mediates inflammatory responses to cell wall peptidoglycan, cell wall and teichoic acid, and membrane lipoproteins of Gram-positive bacteria (11, 12). Direct TLR2 binding was shown only for peptidoglycan (13); binding sites for the other ligands have not been identified. Live Gram-positive bacteria, - although they express TLR2 ligands on their surface - do not depend on TLR2 for phagocytosis and killing, nor for induction of inflammation, but TLR2 modulates

phagocytosis and inflammation. The mechanism and direction of TLR2 action was found variable depending of the disease model. In pneumococcal meningitis and in *Borrelia* arthritis, bacterial clearance was accelerated by TLR2 (9, 14, 15). In contrast, in pneumococcal pneumoniae bacterial clearance was unaltered in $TLR2^{-/-}$ mice (16). Finally, modulation of inflammation by TLR2 was not restricted to Gram-positive infections. Indeed, a virulence factor released from Gram-negative *Yersinia* colitis induced IL-10 production and thereby immunosupression in a TLR2-dependent way, therefore in this model TLR2-deficient mice were less susceptible than wt mice (17).

The glycosylphosphatidylinositol-anchored CD14 binds lipopolysaccharide, peptidoglycan and lipoteichoic acids (18). It does not transmit a signal on its own, but most likely is the major ligand binding element and associates with TLR2 for signaling (11, 19). This conclusion comes from TLR2 and CD14-transfected cells, where CD14 alone did not cause but enhanced TLR2-mediated NFkB activation (11). Furthermore, in human monocytes anti-CD14 antibodies were able to completely prevent cytokine release induced by Gram-positive components, indicating that in these cells which coexpress CD14, and TLR2, CD14 is upstream TLR2 (18). Anti-CD14 pre-treatment led to a late delay in bacterial clearance in a rabbit *E. coli* pneumonia model (20), and to a more severe bacterial invasion and tissue destruction in shigellosis in rabbits (21). Bactericidal activity upon E. coli but not upon *Shigella* was found altered by anti-CD14 (21, 22), thus other defense mechanism may also be affected by CD14 including PMN migration and inflammatory product release. In murine Gram-positive infection, CD14 deletion was not associated with an altered disease outcome but with increased TNF levels in serum (23).

The deleterious outcome of TLR2$^{-/-}$ mice in meningitis was published by our group (9), the outcome of CD14$^{-/-}$ mice with meningitis has also recently been described by us and has been found similarly unfavorable (24). However, we observed that the two receptors conferred protection by different mechanisms. While the lack of TLR2 delayed bacterial clearance, leukocyte infiltration and enhanced inflammation by an unresolved pathway (9), the lack of CD14 had no effect on bacterial clearance, but led to enhanced inflammation by accelerating leukocyte infiltration into the CSF (24). In view of our observations, that different host effector functions were modulated by TLR2 and CD14 during meningitis, we asked the question whether response to therapy was also dependent on the presence of these two molecules. Therefore we compared the outcome in wt, TLR2$^{-/-}$ and CD14$^{-/-}$ mice with meningitis under antibiotic treatment and/or anti-inflammatory treatment with TACE inhibitor TNF484. In addition we investigated the course of meningitis and the treatment response of TLR2$^{-/-}$/CD14$^{-/-}$ mice.

Materials and Methods

Preparation of bacterial inocula

Streptococcus pneumoniae (clinical isolate of serotype 3, H14) was grown for 7 h in double Mueller Hinton broth (MHB) (DIFCO Laboratories Detroit, USA), then subcultured overnight in new MHB, and washed in 0.9% sterile saline (12,000 x g for 6 min) immediately before use. The inoculum size was calculated from optical density (OD) determinations (OD 0.4 = 1 x 10^8 cfu/mL) and was retrospectively assessed by CFU counting on blood agar plates.

Mouse meningitis model

Six to eight week old C57BL/6 (wild-type, wt), $TLR2^{-/-}$ mice were kindly provided by William J. Rieflin from Tularik, South San Francisco, CA) and $CD14^{-/-}$ mice were kindly provided by Mason Freeman, Massachusetts General Hospital, Boston. The two strains with targeted gene deletions were crossed to obtain $TLR2^{-/-}/CD14^{-/-}$ double knockout mice. All animals were kept under specific pathogen free conditions in the Animal House of the Department of Research, University Hospitals Basel according to the regulations of the Swiss veterinary law. Both transgene mouse strains had been backcrossed for 6 generations on a C57BL/6 background). Mice were anesthetized via intraperitoneal (i.p.) injection of 100 mg/kg Ketamine (Ketalar©; Warner-Lambert AG, Baar, Switzerland) and 20 mg/kg Xylazinum (Xylapan©; Graeub AG, Bern, Switzerland),

and subsequently subarachnoidally inoculated into the left forebrain with either 25 µL 0.9% NaCl or live *Streptococcus pneumoniae* (2×10^2 or 3×10^3 cfu).

The health status of the mice was assessed by the following scores as described previously (8) (1) normal motor activity and turned upright in < 5s when put on their back; (2) decreased spontaneous activity, but still turned up in < 5s; (3) turned up in > 5s; (4) did not turn up; (5) did not move. After 6, 12, 24, 48 and 72 h or if they presented score 5, mice were sacrificed by i.p. injection of 100 mg/kg pentobarbital (Abbott Laboratories, North Chicago, IL). Animals were perfused with Ringer's solution (Braun Medical AG, Emmenbrücke, Switzerland) into the left cardiac ventricle until the effluent became clear. Cerebrospinal fluid (CSF) was obtained by puncture of the cisterna magna as described previously (25) Due to the small volumes (3-6 µL) obtained from each animal, CSF from 3 mice were pooled.

Treatment

Mice were treated with either 80 mg/kg ceftriaxone (Rocephin, Hoffmann-La Roche, Switzerland, dissolved in 0.1 mL saline) via i.p. injection twice daily during 5 days and/or with subcutaneous (s.c.) injection of 1 mg/kg water soluble inhibitor of matrix metalloproteases and TACE TNF484 twice daily during 4 days. Treatment with antibiotics was started 18 h and treatment with TACE inhibitor was started 12 h after infection.

Determination of bacterial counts and inflammatory parameters

Pooled CSF samples were serially diluted in 0.9% saline to assess the bacterial load after plating and incubation at 37°C for 24 h. Thereafter it was centrifuged at 800 x g for 7 min (room temperature) to obtain cell free CSF. Thereafter, samples were stored at -20°C until cytokine determination. Brains were removed 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% saline on blood agar plates. The concentration of the pro-inflammatory cytokine TNF in CSF was determined with a bioassay, measuring the degree of cytotoxicity on WEHI cells in the presence of 1 μg/mL actinomycin D, using mouse rTNF as a standard.

Statistical analysis

Differences in survival between different mouse strains and treatments were analyzed using the log rank test and Kaplan-Meier analysis. Data of disease severity scores were assessed by ANOVA corrected for repeated measurements, followed by post hoc analysis (Fisher's PLSD, Scheffe and Bonferroni/Dunn tests). Differences in CSF parameters between wt, $TLR2^{-/-}$ and $CD14^{-/-}$ mice were analyzed with the non-parametric Kruskall Wallis test. In all statistical tests, $P < .05$ was accepted statistically significant.

Results

Effect of TLR2- and CD14-deficiency on survival in pneumococcal meningitis

We and others have previously shown that TLR2 is protective in pneumococcal meningitis, since TLR2-deficient mice presented a higher disease severity both in an intracisternal and a subarachnoidal meningitis model and died earlier than wt mice (9, 14). Similarly, we could show, that $CD14^{-/-}$ mice died earlier during pneumococcal meningitis than wt mice (24).

Antibiotic treatment of meningitis in $TLR2^{-/-}$- and $CD14^{-/-}$ mice

Antibiotic treatment is efficient in mouse (26) and human meningitis (27), if treatment starts early. Accordingly, ceftriaxone was administered starting as early as 18 h after infection. All wt and $CD14^{-/-}$ mice were rescued (figure 1*A* and *B*), whereas 27% of $TLR2^{-/-}$ mice still died, which was significantly different from the outcome in wt mice ($P < .05$). The higher bacterial load in the former strain explained the difference between $TLR2^{-/-}$ and $CD14^{-/-}$ mice in response to antibiotics. Indeed, we previously showed that $TLR2^{-/-}$ mice had a significantly higher bacterial brain load than wt mice, starting two hours after infection (9). In contrast, $CD14^{-/-}$ mice had similar numbers of cfu as wt mice in brain up to 24 h after infection ($3.2 \pm 2.4 \times 10^3$ and $4.9 \pm 3.8 \times 10^3$ CFU/brain, respectively 3 h after infection). Therefore it can be concluded that the high bacterial number in $TLR2^{-/-}$ mice rapidly precluded rescue by antibiotics alone but required anti-inflammatory treatment.

Effect of TACE inhibitor on meningitis in TLR2$^{-/-}$- and CD14$^{-/-}$ mice

Since TLR2$^{-/-}$ mice could not be rescued with antibiotics, it was likely that the higher bacterial load in this strain led to excess inflammation, which contributed to their bad prognosis. Indeed TLR2$^{-/-}$ mice presented higher TNF concentrations in CSF 24 h and 48 h after infection than wt mice (9). This TNF was significantly related to blood brain barrier disruption and to disease severity (9). In the present study we confirmed that TNF in CSF was higher in TLR2$^{-/-}$ than in CD14$^{-/-}$ or wt mice (figure 2). Therefore we aimed at a TNF-reducing therapy with the TACE inhibitor TNF484. To confirm, that this substance blocked TNF in our model, TNF was measured in CSF of mice, which had or had not received TNF484 treatment 12 h after infection with *S. pneumoniae*. TNF was reduced from 1424 ± 824 pg/mL in TLR2$^{-/-}$ and from 191 ± 271 pg/mL in wt CSF to non-measurable values in both mouse strains during treatment with TACE inhibitor. Outcome was followed in infected TLR2$^{-/-}$, CD14$^{-/-}$ and wt mice, which were treated for 5 days with TNF484 (figure 3). No mice were rescued to 100% survival, but mortality was significantly delayed by TNF484 treatment in the KO strains. In TLR2$^{-/-}$ and CD14$^{-/-}$ animals, this therapy significantly slowed death ($P < .05$) to the rate observed in untreated wt mice (figure 3*A* and 3*B*). Survival of wt mice was non-significantly prolonged by the TACE inhibitor treatment (figure 3*A* and 3*B*, $P > .05$). These results indicate that TLR2 deficient mice with a high degree of inflammation profited best from TNF484.

Effect of antibiotics and TACE inhibitor combined in TLR2 $^{-/-}$ mice

All wt and CD14$^{-/-}$ mice, but not TLR2$^{-/-}$ mice were rescued by antibiotics According to the individual effects of ceftriaxone and TNF484 in TLR2$^{-/-}$ mice, an

additive result was expected by combining both treatments. Therefore this combination therapy of TNF484 and antibiotics was started 12 and 18 h after infection respectively. Wild-type mice were rescued as expected and TLR2$^{-/-}$ mice were significantly improved (P <.01), they had 89% survival rate, which was not significantly different from wt mice.

Survival and treatment effects in TLR2$^{-/-}$/CD14$^{-/-}$ mice

According to *in vitro* cell activation studies, both CD14 and TLR2 have a receptor function for cell wall components of Gram-positive bacteria including peptidoglycan and lipoteichoic acid (11, 19, 28). During experimental meningitis, each molecule was found to have a protective effect on its own, which was exerted by different mechanisms. The lack of TLR2 delayed bacterial clearance. Table 1 illustrates these findings by comparison of the fold increase in brain bacterial numbers between 6 h and 12 h after infection. The increase was 220 and 134 fold in wt and CD14$^{-/-}$ mice respectively, but twice as high, namely 442 fold in TLR2$^{-/-}$ mice. In addition, the lack of TLR2 delayed leukocyte infiltration, while the lack of CD14 accelerated infiltration (9, 24). Table 1 again demonstrates these findings; between 6 and 12 h after infection, leukocyte numbers increased 10 fold in wt, 2 fold in TLR2$^{-/-}$ and 22 fold in CD14$^{-/-}$ mice. Twenty four hours after infection TLR2$^{-/-}$ mice showed the strongest i.e. a 408 fold TNF increase in CSF, it was less in CD14 (333 fold) and wt mice (115 fold, table 1). We were curious to know the phenotype of mice deficient in both molecules. Therefore meningitis was induced in TLR2$^{-/-}$/CD14$^{-/-}$ mice. Interestingly, survival was similar to that observed in either TLR2$^{-/-}$ or CD14$^{-/-}$ mice and the double KO animals were rescued with antibiotics alone (figure 5). To understand the observations we measured increase of bacterial load in brain and

leukocyte infiltration in CSF. In these mice, unlike in TLR2$^{-/-}$ mice, early bacterial clearance was unimpaired (figure 6*A*) and cfu increased only 84 fold after 12 hours (table 1). Leukocyte immigration was neither delayed as in TLR2$^{-/-}$ nor accelerated as in CD14$^{-/-}$ mice (figure 6*B*, table 1). Following the modest increase in cfu and leukocytes, TNF rose only 27 fold in the double KO mice (table 1). This indicates that the lack of TLR2 and CD14 *in vivo* was compensated by other receptors or that the opposite effects of TLR2 and CD14 were neutralized.

Discussion

We compared treatment effects of antibiotics and TACE inhibitor TNF484 in mice during pneumococcal meningitis; in particular we studied differential modulation of the treatment response by the pattern recognition receptors TLR2 and CD14, either singly or combined. By simultaneous study of 4 mouse strains, we confirmed our earlier data that wt mice with untreated *S. pneumoniae* meningitis lived longer than infected mice with a genetic deletion of TLR2, CD14 or both receptors. We found, first, that antibiotics, which rescued wt and $CD14^{-/-}$ mice, were insufficient in the $TLR2^{-/-}$ strain; combination with TACE inhibitor was required for rescue in that group. Second, TACE inhibitor TNF484 therapy alone delayed death in $TLR2^{-/-}$ and $CD14^{-/-}$ but did not significantly improve in wt mice. Third, untreated and antibiotic-treated $TLR2^{-/-}/CD14^{-/-}$ mice behaved similarly as single $CD14^{-/-}$ mice.

In meningitis, the timing of antibiotic treatment is an essential variable determining outcome, early therapy was associated with a lower mortality than late therapy (27). This is most likely due to the fact, that antibiotics are less efficient, as soon as the deleterious inflammation, which follows accumulation of bacteria, has developed. In our study the bacterial load and consequently the extent of inflammation may also have decided on the antibiotic response of the different mouse strains. Indeed in wt mice, we found a 100% response in lethal meningitis with 10^3 cfu of *S. pneumoniae,* when cephalosporin was applied parenterally 18 h after infection. At that time, bacterial numbers in CSF and brains were similar in wt and $CD14^{-/-}$ mice, but significantly higher in brains of $TLR2^{-/-}$ mice. The different status of the disease in $TLR2^{-/-}$ as compared to the

other mice was further documented by the clinical picture; 24 h after infection, 50% of TLR2$^{-/-}$ mice showed clinical symptoms of meningitis, whereas in wt and CD14$^{-/-}$ mice, only a small proportion of mice had symptoms (20 and 10%, respectively). Therefore it is likely, that the bacteria could be cleared by antibiotics in wt and CD14$^{-/-}$, but not in TLR2$^{-/-}$ mice.

The inability of TLR2$^{-/-}$ mice to clear *S. pneumoniae* as fast as wt and CD14$^{-/-}$ mice, was followed by a stronger inflammation, which was recorded as higher TNF level in CSF from infected TLR2$^{-/-}$ as compared to CD14$^{-/-}$ and to wt mice. Accordingly the treatment of choice in TLR2$^{-/-}$ mice was an anti-inflammatory adjunctive drug. Based on our earlier studies with TACE inhibitor TNF484 in neonatal rat pneumococcal meningitis, we first confirmed that TACE inhibitor TNF484 used at 1 mg/kg twice daily blocked TNF in CSF of infected mice. The therapeutic effect was weak in wt, which at the time, when treatment was started, had a low TNF in CSF; it was significant in receptor-deficient mice, in which TNF release was higher after 24 h. So the profit from treatment in TLR2$^{-/-}$ and CD14$^{-/-}$ mice could be predicted from the TNF levels, which indicated a higher degree of inflammation than in wt mice.

Since TLR2$^{-/-}$ mice were rescued by neither antibiotics nor TACE inhibitors alone, combined treatment was applied and resulted in 80% survival in this mouse strain. It can be hypothetized, that TACE treatment was also effective, because it reduced the inflammation induced by cell wall active antibiotics, such as ceftriaxone (29). However, *in vitro* penicillin treatment of *S. pneumoniae* was found to induce host inflammation in a TLR2-dependent manner, since it led to IL-8 promoter activation in TLR2-transfected

cells (30). This finding suggests that antibiotic treatment did not augment inflammation in TLR2$^{-/-}$ mice.

So far, the *in vivo* consequences of a double TLR2/CD14 deletion in mice are unknown. The association of CD14 with TLR2 in a heteromeric complex was shown to be required for cell activation by TLR2 ligands (31). The evidence comes from the fact that cotransfection of CD14 with TLR2 increased peptidoglycan-induced reporter gene activation (11). Furthermore the association of CD14 and TLR2 was demonstrated by coimmunoprecipitation of TLR2 and CD14 in double transfected cells (32). It is however unknown, whether all TLR2-positive cells coexpress CD14 and whether for all signals CD14 is upstream TLR2. It is also unclear whether TLR2 is activated via ligand-loaden sCD14 in cells, which lack membrane CD14. Schwandner et al. showed that peptidoglycan-induced activation of TLR2 in transfected cells was stronger in the absence than in the presence of serum, which indicates that sCD14 may not act like membrane CD14 in the activation of TLR2 (11). Based on the *in vitro* findings the phenotype of infected double KO mice was not predictable.

Also, we had observed different phenotypes in TLR2$^{-/-}$ and CD14$^{-/-}$ mice during meningitis, which suggested that certain effects were dependent on both receptor and others not. We knew that TLR2 deficiency alone caused a very early delay of bacterial clearance, while CD14 did not affect early bacterial clearance (9, 24) (and table 1 this paper). Interestingly, double KO mice behaved like CD14$^{-/-}$ mice, they had a similar or better bacterial clearance and no change in leukocyte infiltration (table 1). Double KO died earlier than wt mice, but could be rescued with antibiotics like wt and CD14$^{-/-}$ mice.

This means that the lack of CD14 with its ensuing early infiltration compensated for the defect in bacterial killing of the TLR2-deficiency.

Our treatment study reveals the different effects mediated by TLR2 and CD14 respectively in meningitis and illustrates the requirement and success of adjuvant therapy, when bacterial load is high and inflammation is strong in $TLR2^{-/-}$ mice.

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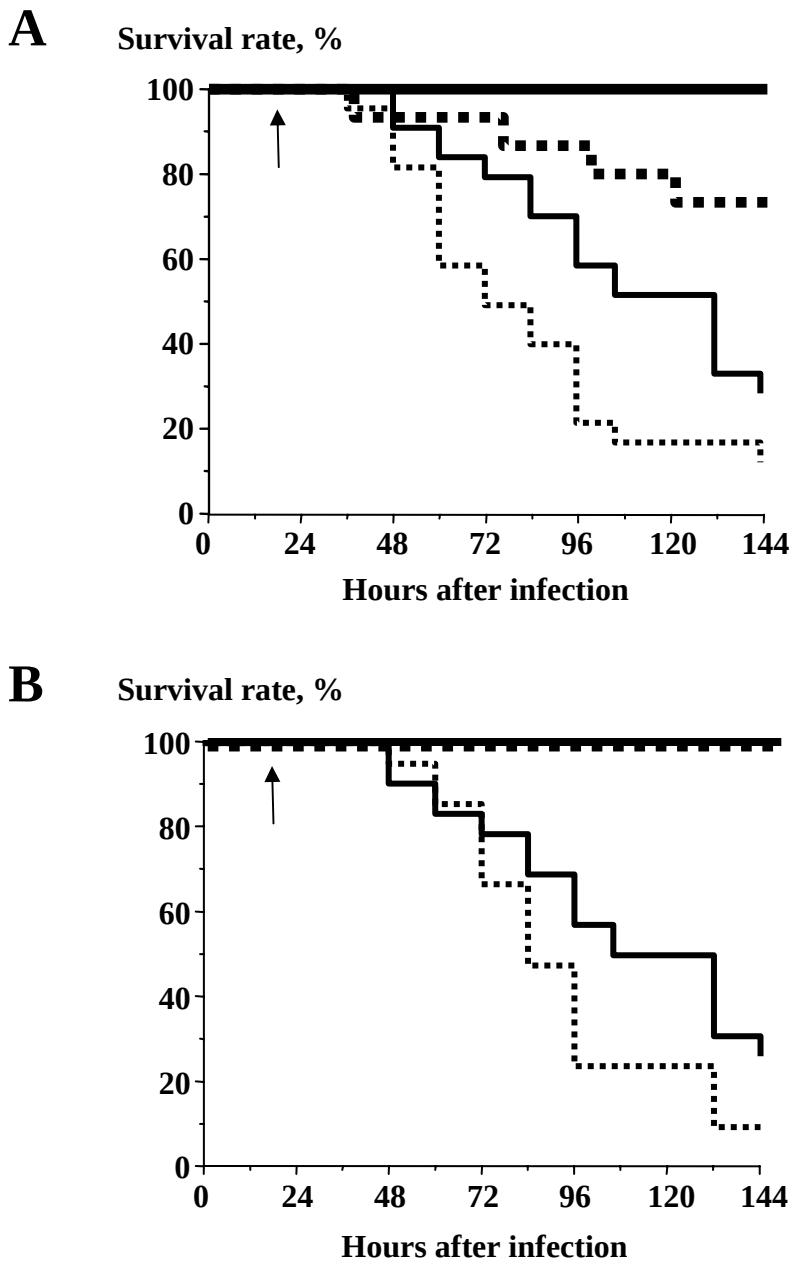

Figure 1. ***A***, Effect of antibiotic treatment on mortality of wt (without ceftriaxone, solid line (n = 33); with ceftriaxone, thick solid line (n = 15); P < .001) and TLR2$^{-/-}$ mice (without ceftriaxone, dashed line (n = 21); with ceftriaxone, thick dashed line (n = 15); P < .001).

B, Effect of antibiotic treatment on mortality of wt (without ceftriaxone, solid line (n = 33); with ceftriaxone, thick solid line (n = 15); P < .001) and CD14$^{-/-}$ mice (without ceftriaxone, dashed line (n = 21); with ceftriaxone, thick dashed line (n = 9); P < .001) in pneumococcal meningitis. Arrows indicate start of twice daily therapy with 80 mg/kg ceftriaxone i.p.

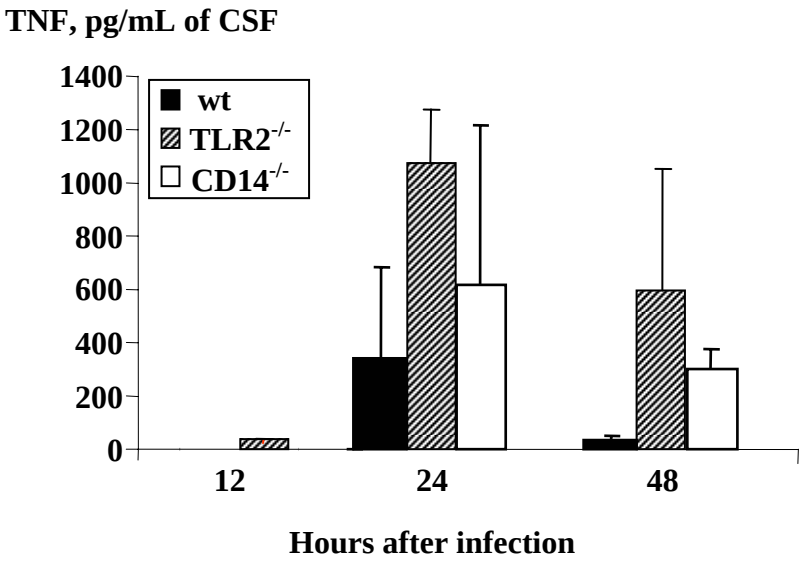

Figure 2. TNF release in CSF during pneumococcal meningitis in wt, $TLR2^{-/-}$ and $CD14^{-/-}$ mice. Values at 24 h (mean values; 329.4 pg/mL, 1077 pg/mL and 613.4 pg/mL in wt, $TLR2^{-/-}$ and $CD14^{-/-}$ mice, respectively) and 48 h (mean values; 48.4 pg/mL, 600.9 pg/mL and 300 pg/mL in wt, $TLR2^{-/-}$ and $CD14^{-/-}$ mice, respectively) after infection were compared by ANOVA repeated measures. Values at both time points were significantly ($P < .05$) different between wt, $TLR2^{-/-}$ and $CD14^{-/-}$ mice.

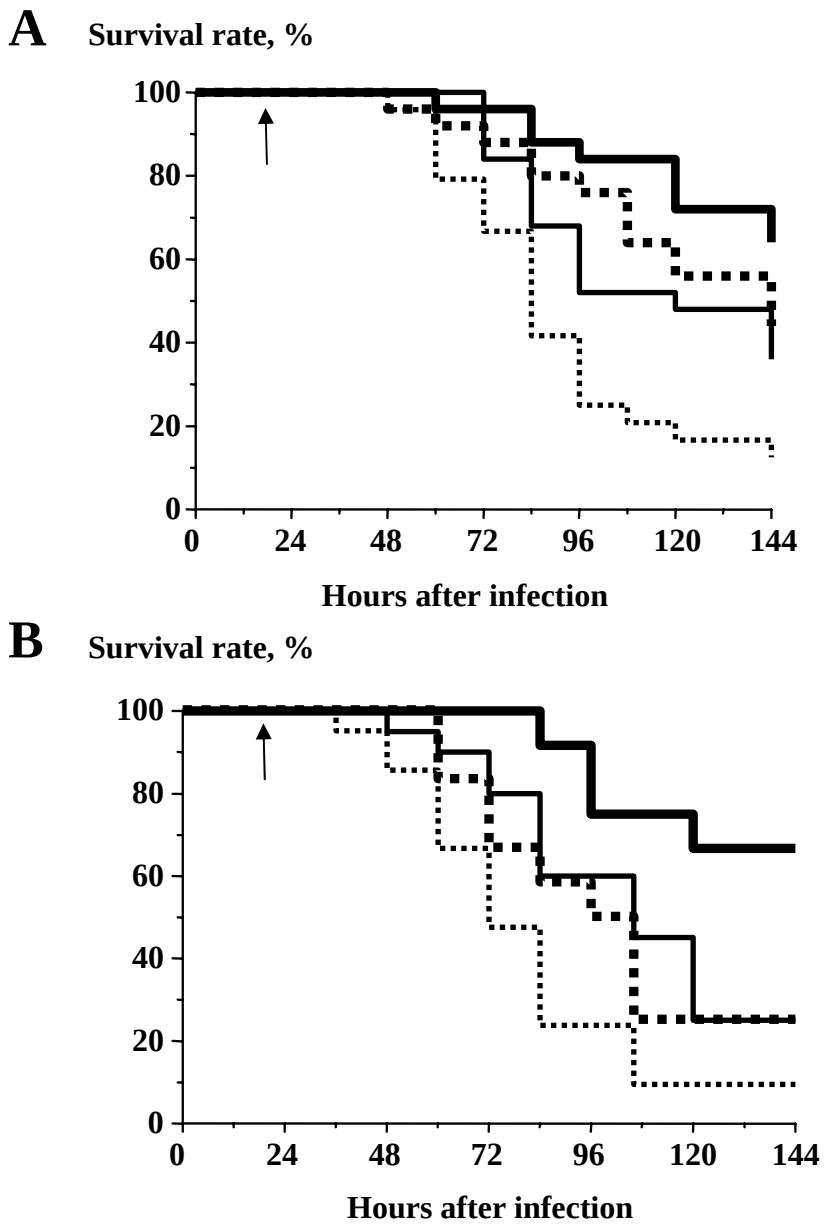

Figure 3. Effect of TACE inhibitor treatment on outcome of pneumococcal meningitis in wt, TLR2$^{-/-}$ and CD14$^{-/-}$ mice. ***A***, Survival rate in wt (without TNF484, solid line (n = 25); with TNF484, thick solid line (n = 25), P > .05) and TLR2$^{-/-}$ mice (without TNF484, dashed line (n = 25); with TNF484, thick dashed line (n = 25), P < .05; wt vs TLR2$^{-/-}$ treated, P > .05). ***B***, Survival rate in wt (without TNF484, solid line (n = 20); with TNF484, thick solid line (n = 12), P > .05) and CD14$^{-/-}$ mice (without TNF484, dashed line (n = 12); with TNF484, thick dashed line (n = 12), P < .05; wt vs CD14$^{-/-}$ treated, P > .05). Arrows indicate start of twice daily therapy with 1 mg/kg TNF484 s.c.

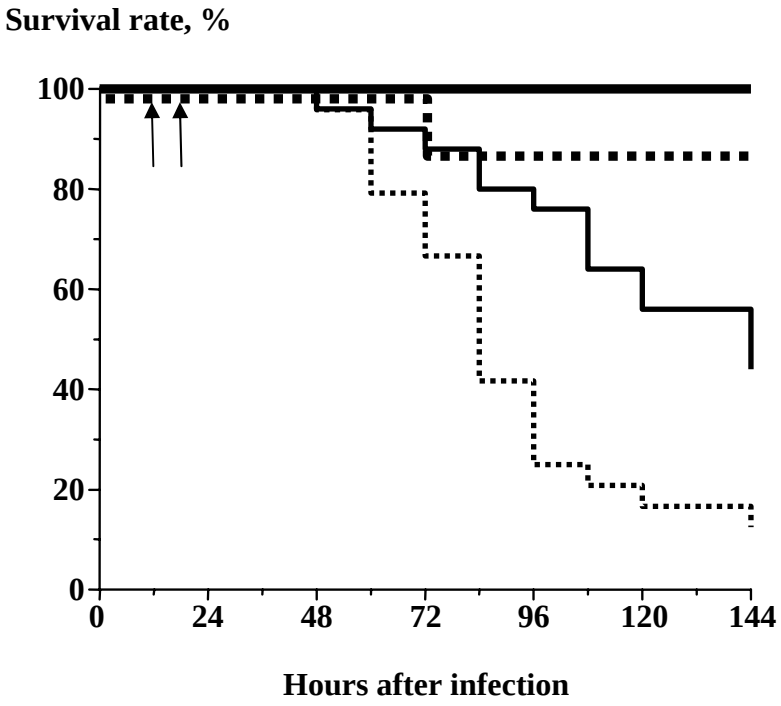

Figure 4. Effect of antibiotic and TACE inhibitor treatment on outcome of pneumococcal meningitis in wt and TLR2$^{-/-}$ mice.

Survival rate in wt (without treatment, solid line (n = 10); with treatment, thick solid line (n = 10), P < .001) and TLR2$^{-/-}$ mice (without treatment, dashed line (n = 10); with treatment, thick dashed line (n = 10), P < .001); wt vs TLR2$^{-/-}$ treated, P > .05).

Arrows indicate start of therapy with 1 mg/kg TNF484 s.c. and 80 mg/kg ceftriaxone i.p. twice daily, starting 12 h and 18 h after infection, respectively.

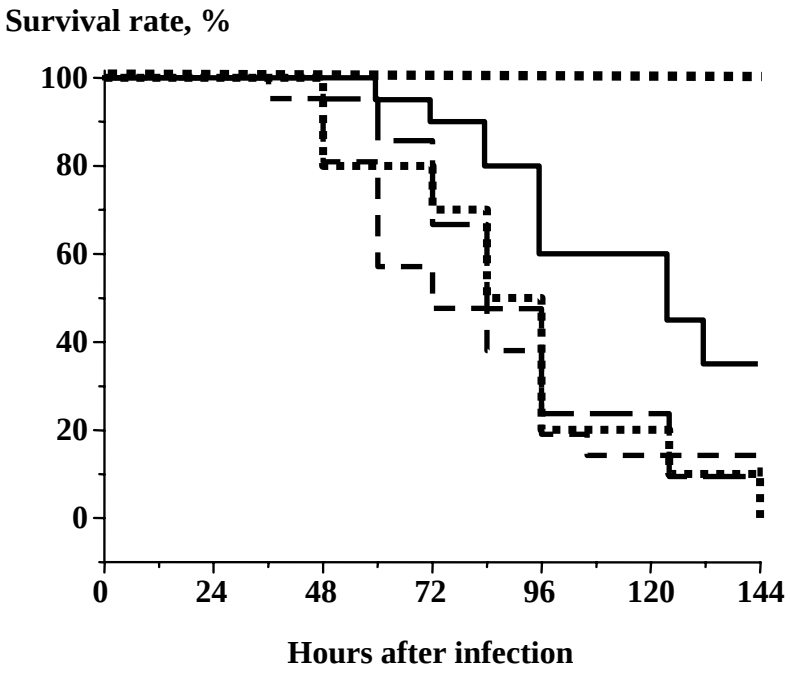

Figure 5. Survival of wt (—) mice was significantly longer than survival of TLR2$^{-/-}$ (- - -), CD14$^{-/-}$ (– – –), and TLR2$^{-/-}$/CD14$^{-/-}$ (···) mice during pneumococcal meningitis. Survival in the three deficient strains did not differ. Antibiotic treatment rescued all TLR2$^{-/-}$/CD14$^{-/-}$ (•••) mice.

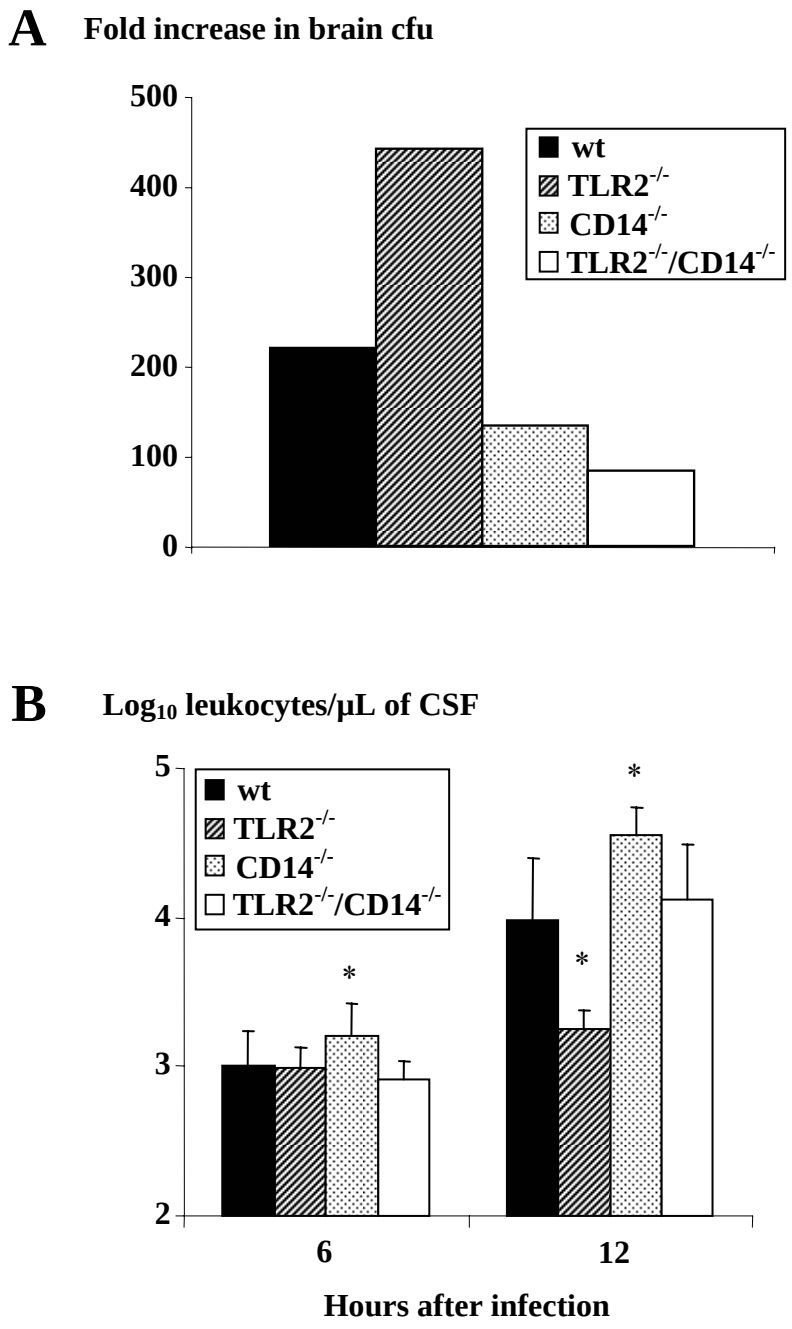

Figure 6. Colony-forming units (cfu) in brain and leukocyte numbers in CSF in 4 mouse strains early during meningitis. ***A***, Fold increase of brain cfu between 6 and 12 h after infection in wt, TLR2$^{-/-}$, CD14$^{-/-}$ and TLR2$^{-/-}$/CD14$^{-/-}$ mice; values are average of 4 mice. ***B***, Leukocyte numbers in CSF 6 and 12 h after infection (values are average of 4 mice, * $P < .05$).

Table. Fold increase in brain bacterial numbers, leukocyte counts and TNF in CSF during pneumococcal meningitis.

Mouse strain	Fold increase cfu [a]	Fold increase leukocytes in CSF [a]	Fold increase TNF in CSF [b]
$CD14^{+}/TLR2^{+}$	220	10	115
$CD14^{+}/TLR2^{-/-}$	442	2	408
$CD14^{-/-}/TLR2^{+}$	134	22	333
$CD14^{-/-}/TLR2^{-/-}$	84	15	27

NOTE. [a] Fold increase between 6 and 12 h after *S. pneumoniae* infection.
[b] Fold increase between 12 and 24 h after *S. pneumoniae* infection.

Future perspectives

Despite highly active antibiotics and the development of capsular polysaccharide vaccines, bacterial meningitis is still associated with high morbidity and mortality in both children and adults. *Streptococcus pneumoniae* remains the major cause of acute bacterial meningitis in adults in developed countries [1]. Standard anti-microbial drugs, which are administered orally, may not achieve therapeutic concentrations in the subarachnoid space, since the penetration of an antibiotic into cerebrospinal fluid is primarily influenced by characteristics of the antibiotic and the integrity of the blood-brain barrier [2]. Vancomycin, which represents an effective treatment for *S. pneumoniae* meningitis in the era of ß-lactam resistance, has limited penetration into the CSF [3]. In addition, antibiotic therapy of suspected bacterial meningitis is usually given empirically, which may not be efficient in the presence of multi-drug-resistant pneumococci. Moreover, effective antimicrobial drugs, such as ß-lactam antibiotics, can induce the release of lipoteichoic acid (LTA) and teichoic acid (TA) from *S. pneumoniae* [4], which may contribute to an unfavorable outcome [5]. Indeed LTA and TA have been shown to induce severe meningeal inflammation when injected into the subarachnoid space of rabbits [6] and their concentrations in CSF were significantly associated with neurological sequelae and mortality in patients with pneumococcal meningitis [7]. However, the release of proinflammatory components, such as LTA and TA, occurs also during spontaneous bacterial lysis.

Our increasing understanding of the pathogenesis and the host response in bacterial meningitis has suggested new approaches to modulate host immune activation. Indeed, the inflammation within the subarachnoid space and neuronal damage, which

occur during bacterial meningitis, are not mediated simply by the presence of live bacteria but occur as a consequence of the host's inflammatory response to bacterial components [8]. Indeed, experimental studies have clearly demonstrated that pneumococcal cell wall components induce CSF inflammation [5] and cellular damage in primary astrocyte and microglial cell cultures from newborn rats [9]. It was further shown that LTA, peptidoglycans and pneumococcal DNA were capable of damaging neurons and glia in organotypic hippocampal cultures [10]. Therefore, in order to prevent or attenuate harmful subarachnoid space inflammation and neurological damage, additional therapies, which can modulate the host immune system response to pneumococci have been considered.

Corticosteroids were then used as anti-inflammatory adjuvant in the treatment of bacterial meningitis [11]. In 1997, a meta-analysis of all randomized clinical trials of dexamethasone as adjunctive therapy in bacterial meningitis from 1988 to 1996 concluded that dexamethasone treatment has beneficial effects on patients with *Haemophilus influenza* type b meningitis and children with pneumococcal meningitis when the treatment was started with or before parenteral antibiotics [12]. Recently, a prospective, randomized, double-blind European trial of adjuvant treatment with dexamethasone in 301 adults with acute bacterial meningitis showed that early steroid use (before or with the first dose of antibiotic) is associated with improved survival and outcome [13]. In this study, among the patients with pneumococcal meningitis, there were unfavorable outcomes in 26% of the dexamethasone group, as compared with 52% of the group which received placebo. The dexamethasone treatment did not increase the incidence of severe neurological disability or the risk of gastrointestinal bleeding [13]. A

recent meta-analysis of adjunctive steroid therapy in five trials, involving 623 adults with acute bacterial meningitis (pneumococcal meningitis = 234, meningococcal meningitis = 232, others = 127, unknown = 30) showed that treatment with dexamethasone was associated with a significant reduction in mortality and in neurological sequelae in pneumococcal meningitis [14]. In this study, subgroup analysis for patients with meningococcal and other bacterial meningitis also showed a favorable trend in mortality and neurological sequelae in the absence of any excess adverse events. However, there is one caveat to these beneficial effects of dexamethasone in experimental model of pneumococcal meningitis [15]. The authors showed that dexamethasone treatment increased hippocampal apoptosis and reduced learning capacity in pneumococcal meningitis in infant rats.

Bacterial meningitis is characterized by neutrophil accumulation into the CSF. Therefore, another approach was used to attenuate PMN recruitment in the subarachnoid space and thus prevent meningeal inflammation. It was shown that inhibition of leukocyte recruitment into the CSF by blocking $ß_2$-integrins with anti-CD18 antibody markedly reduced meningeal inflammation in a rabbit model of pneumococcal meningitis [16]. Other studies have used similar approach with the polysaccharide fucoidin, which is a selectin blocker that inhibits leukocyte rolling and subsequent leukocyte transendothelial migration [17]. Treatment with fucoidin intravenously has been found to attenuate pleocytosis and reduce inflammatory parameters, such as intracranial pressure in experimental pneumococcal meningitis in rats [18]. However, in this study, rats were intracisternally injected with pneumococcal cell wall components instead of live bacteria, which does not represent a real experimental meningitis model. In a recent study,

fucoidin was used in rabbits intracisternally injected with live serotype 3 *S. pneumoniae* strain [19]. In this study, fucoidin treatment prevented CSF pleocytosis and thus inhibited the release of proinflammatory cytokines into the CSF such as IL-1ß.

Cytokines modulate chemokine expression, i.e MIP-2 is found higher in IL-$6^{-/-}$ mice than in wt mice after intracisternal pneumococcal infection [20] and chemokines themselves are causative of the pleocytosis, since it has been shown that anti-MIP-2 antibodies reduced leukocyte infiltration.

In addition to cytokines and chemokines, matrix metalloproteins, which contribute to the integrity of the BBB are found increased in patients with meningitis [20]. Accordingly, treatment with matrix metalloproteinases inhibitors reduced CSF TNF, BBB permeability and the extent of neuronal injury in infant rat model of pneumococcal meningitis [21] [22].

The approach in our study was to understand the mechanisms of cellular activation triggered by the bacteria-host immune cells interactions in an experimental model of pneumococcal meningitis. We therefore investigated the *in vivo* role of two specific pattern recognition receptors, TLR2 and CD14, which mediate cellular activation towards Gram-positive cell wall components. The function of these two receptors has been intensively studied *in vitro* in response to heat killed pneumococci or pneumococcal cell wall components but no *in vivo* study using experiemental model of bacterial meningitis was reported. We were the first to investigate the *in vivo* function of TLR2 in experimental pneumococcal meningitis model using $TLR2^{-/-}$ mice [23]. We could show that TLR2-deficiency was associated with reduced pneumococcal clearance from the

central nervous system (CNS), increased meningeal inflammation and BBB permeability, and earlier death due to pneumococcal meningitis. These results demonstrated the importance of TLR2 in the induction of protective innate immunity to pneumococcal infection. TLR2 most likely modulates inflammatory response induced by *S. pneumoniae* or its cell wall components, thus preventing harmful meningeal inflammation, which leads to brain damage. This observation was further confirmed by the fact that antibiotic treatment could not rescued all $TLR2^{-/-}$ mice (27% died), since these mice showed a strong meningeal inflammation at the time of treatment. TLR2 is not only involved in the modulation of TNF response in the subarachnoid space but plays a major role in mediating phagocytosis and/or killing of *S. pneumoniae*, as shown by a greater bacterial load in the CNS of $TLR2^{-/-}$ mice as compared to wt mice [23]. Indeed, it was recently observed that in $TLR2^{-/-}$ macrophages infected with *Staphylococcus aureus*, phagosomes showed deficient fusion with lysosomes, which are crucial for bacterial killing [24].

In the second paper, which is submitted, we investigated the *in vivo* role of CD14 in the pathogenesis of pneumococcal meningitis. CD14 binds Gram-positive cell wall components [25] [26] [27] but requires interaction with TLR2 to initiate signal transduction in response to Gram-positive bacteria [28]. However, the pathophysiologic role of CD14 in Gram-positive bacterial infection is still unknown. In our study, CD14 deficiency was associated with early strong PMN recruitment into the subarachnoid space, which leads to increased meningeal inflammation. This strong CSF pleocytosis was due to more MIP-2 release in brain. Moreover, brain endothelial cells from $CD14^{-/-}$ mice, *in vitro* infected with live *S. pneumoniae* released more MIP-2 than those from wt mice. In contrast to TLR2, CD14 does not play any role in bacterial clearance, but is

rather involved in controlling PMN recruitment into the CSF, and thus prevents harmful and deleterious meningeal inflammation. This is a new function of a pattern recognition receptor to modulate PMN migration in pneumococcal infection. It will be interesting to understand the mechanisms by which CD14 interferes with MIP-2 release.

In the third paper, which is also submitted, we were mainly interested in the modulation of TLR2 and CD14-mediated cellular functions by antibiotic and TACE inhibitor treatment in pneumococcal meningitis. We could show that both wt and $CD14^{-/-}$ mice were rescued with ceftriaxone therapy, whereas $TLR2^{-/-}$ mice required additional anti-inflammatory drug (TACE inhibitor) to be cured. These results could be explained by an excessive CSF inflammation observed in $TLR2^{-/-}$ mice as compared to wt or $CD14^{-/-}$ mice. Therefore we investigated the course of meningitis and the treatment response of $TLR2^{-/-}/CD14^{-/-}$ mice. It appears that $TLR2^{-/-}/CD14^{-/-}$ mice behaved like $CD14^{-/-}$ mice. Indeed all $TLR2^{-/-}/CD14^{-/-}$ mice could be rescued by ceftriaxone therapy. This suggests that early lack in bacterial clearance, which was characteristic for $TLR2^{-/-}$ mice was somehow compensated by early leukocyte infiltration (characteristic for $CD14^{-/-}$ mice).

Taken all together, our data showed that although both TLR2 and CD14 recognize Gram-positive cell wall components *in vitro*, their *in vivo* functions towards live Gram-positive bacteria are completely different. Our findings show a direct implication of TLR2 and CD14 in the host response towards *S. pneumoniae* and contribute to a better understanding of the host innate immunity in the experimental pneumococcal meningitis model.

Besides vaccination, which remains a priority in populations who are at risk, such as elderly and patients who are immunocompromised, we believe that our studies help understanding modulation of the innate immune response to pneumococci and thus to design adjunctive therapies which are promising in reducing CSF inflammation and neurological sequelae and thus improve outcome in pneumococcal meningitis.

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Publications

2004 **Hakim Echchannaoui**, Karl Frei, Robert M. Strieter, Yoshiyuki Adachi, Christian Schnell, and Regine Landmann (2004). CD14 deficiency leads to early death in pneumococcal meningitis due to a high MIP-2, CXCR2, neutrophil transmigration and inflammation. Submitted in *J immunol*.

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