MyD88 is required for mounting a robust host immune response to Streptococcus pneumoniae in the CNS

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Summary
Myeloid differentiation factor 88 (MyD88) is an essential intracellular signal transducer in Toll-like receptor (TLR) and interleukin (IL)-1 receptor family member-mediated cell activation. In order to characterize the role of MyD88 in pneumococcal meningitis we used gene-targeted mice lacking functional MyD88 expression. At 24 h after intracisternal infection, MyD88-deficient mice displayed a markedly diminished inflammatory host response in the CNS, as evidenced by reduced CSF pleocytosis and expression of cytokines, chemokines and complement factors. The reduced CNS inflammation was paralleled by a marked reduction in the prognostic relevant CNS complications, such as brain oedema formation. Nevertheless, MyD88 deficiency was associated with a worsening of disease which seemed to be attributable to severe bacteraemia. This notion was supported by the unexpected observation that infected MyD88-deficient mice displayed enhanced mRNA expression of inflammatory mediators [such as the proinflammatory cytokine tumour necrosis factor α (TNF-α) and the CXC chemokine macrophage inflammatory protein (MIP-2)] in the lung and consequently increased cell influx in the bronchoalveolar lavage fluid, compared with infected wild-type mice. Thus, the present study demonstrated for the first time an important role of MyD88 in immune activation to bacterial pathogens within the CNS. The role played by MyD88 in mounting an immune response to Streptococcus pneumoniae, however, seems to be dependent on the anatomical compartment involved.

Keywords: brain; lung; complement; inflammation; innate immunity

Abbreviations: BAL = bronchoalveolar lavage; BBB = blood–brain barrier; Crry = complement receptor-related protein y; ELISA = enzyme-linked immunosorbent assay; ICP = intracranial pressure; Ig = immunoglobulin; IL = interleukin; KC = keratinocyte-derived cytokine; MIP = macrophage inflammatory protein; MyD88 = myeloid differentiation factor 88; PBS = phosphate-buffered saline; TLR = Toll-like receptor; TNF = tumour necrosis factor


Introduction
Streptococcus pneumoniae is a human pathogen of major importance, causing life-threatening, invasive diseases such as pneumonia, bacteraemia and meningitis (Obaro and Adegbola, 2002). Despite appropriate antimicrobial therapy, invasive pneumococcal diseases are still associated with considerable mortality (Parsons and Dockrell, 2002). In the USA, the case-fatality rate is about 10% for all invasive diseases, but it is significantly higher for pneumococcal meningitis than for all other syndromes combined (Robinson et al., 2001). The higher mortality of meningitis is assumed to be due to the inefficiency of the host immune response within the CSF in controlling and overcoming Streptococcus pneumoniae infection. In this body compartment, Streptococcus pneumoniae has been demonstrated to multiply almost as efficiently as it can in vitro, reaching high titres, of up to 108 colony-forming units/ml (Small et al., 1986). As a result
of the unrestrained proliferation and autolysis of Streptococcus pneumoniae, large quantities of proinflammatory subcapsular bacterial components are released into the CSF, resulting in an excessive inflammatory reaction (Tuomanen et al., 1985). This inflammatory host response has been indicated to cause important damage to the brain, thus largely accounting for the unfavourable outcome of meningitis (for reviews see Koedel et al., 2002a; Meli et al., 2002; Kim, 2003).

In mammalian species, the Toll-like receptors (TLRs) provide a repertoire of receptors for the recognition of conserved molecular patterns produced by infectious microbes. Once engaged, TLRs activate signalling pathways that culminate in the induction of key inflammatory and immune genes, thereby provoking the immune system into action (for reviews see Barton and Medzhitov, 2003; O’Neill, 2003). The first evidence for TLR involvement in the recognition of Streptococcus pneumoniae was provided by results from in vitro experiments in which transfection of TLR2 rendered Chinese hamster ovary fibroblasts responsive to pneumococcal peptidoglycan (Yoshimura et al., 1999). Subsequent studies revealed that exposure to either antibiotic- or ethanol-killed pneumococci resulted in NF-kB-driven reporter gene activation in human embryonic kidney 293 cells transfected either with TLR2 or TLR4 (Koedel et al., 2003; Malley et al., 2003). In more recent studies, the pneumococcal cell wall molecule lipo-teichoic acid was reported to act as an additional ligand for TLR2 (Schröder et al., 2003), whereas TLR4 was shown to mediate the innate immune responses to the pneumococcal cytotoxin pneumolysin (Malley et al., 2003). Since TLR2 and TLR4 are constitutively expressed in the choroid plexus lining the lateral ventricle (Laflamme et al., 2001; Laflamme and Rivest, 2001), where meningeval pathogens were reported to accumulate in large numbers (Daum et al., 1978), it seemed conceivable that TLR2 and/or TLR4 are key sensors of pneumococcal CSF infection. However, genetic depletion of TLR2 in mice was associated with only modest effects on the inflammatory reaction and the clinical course of experimental pneumococcal meningitis (Echchannaoui et al., 2002; Koedel et al., 2003). This finding was supported by experiments using macrophages from mice lacking TLR2 and/or functional TLR4 that revealed no differences in cytokine release upon exposure to antibiotic-killed pneumococci between the different genotypes (Koedel et al., 2003). It is also noteworthy that pneumolysin, which has been proved to be both a ligand for TLR2 or TLR4, further signalling pattern recognition receptors are involved in mounting the immune response to Streptococcus pneumoniae within the CSF compartment. The present work was undertaken to analyse the requirement of the adapter protein myeloid differentiation factor 88 (MyD88), which associates with TLRs and interleukin (IL)-1 receptors, in the host immune response in pneumococcal meningitis. MyD88 is essential for the induction of a full inflammatory response to the known TLR ligands, except for the TLR3 ligands (Wesche et al., 1997; Adachi et al., 1999; Medzhitov et al., 1998; O’Neill, 2003). In order to characterize the role of MyD88 in meningitis, we used gene-targeted mice lacking functional MyD88. Thereby, we characterized (i) pneumococcal outgrowth within the CNS, lung and blood; (ii) the inflammatory host response to Streptococcus pneumoniae within the CNS and lung; (iii) the development of the CNS complications that are assumed to occur as a consequence of an uncontrolled host immune response; and (iv) the (short-term) outcome of bacterial meningitis, which is dependent mostly on the extent of CNS alterations in the absence of functional MyD88 expression.

**Material and methods**

**Mouse model of pneumococcal meningitis**

A well-characterized mouse model of pneumococcal meningitis was used in this study (Koedel et al., 2002b, 2003; Paul et al., 2003). Briefly, meningitis was induced by transcutaneous injection of 15 μl of a bacterial suspension containing 10⁷ colony-forming units/ml of Streptococcus pneumoniae type 3 into the cisterna magna under short-term anaesthesia with halothane. Mice were weighed, put into cages, and allowed to wake up. Twenty-four hours after infection, the mice were evaluated clinically. The clinical score comprised the following criteria: (i) presence of tremor, piloerection, and seizures; (ii) spontaneous motor activity; (iii) vigilance; (iv) body proprio-reception; (v) a beam-balancing test; and (vi) a postural reflex test. Thereafter, the body temperature was measured with a rectal probe, and the mice were reweighed and anaesthetized with ketamine/xylazine. Subsequently, a catheter was inserted into the cisterna magna to measure intracranial pressure (ICP) and to determine CSF leucocyte counts. Then, blood samples were taken by transcardial puncture. After deep anaesthesia with ketamine, mice were perfused transcardially with 15 ml of ice-cold phosphate-buffered saline (PBS) containing 10 U/ml heparin. The brain was removed and rapidly frozen. In addition to the brain, the lungs were collected and immediately frozen.

**Determination of the blood–brain barrier (BBB) integrity**

To assess BBB integrity, mouse brain homogenates were examined for infiltration by both albumin and immunoglobulin (Ig) G, abundant serum proteins that are normally excluded from the brain by the intact BBB, using enzyme-linked immunosorbent assay (ELISA) or Western immunoblotting.
Detection of brain IgG by immunoblotting analysis

Mouse brain protein extracts (20 μg of protein per lane) were separated on a 4–12% gradient NuPage Tris-Bis gel (NoveX, Frankfurt, Germany), transferred to a polyvinylidene fluoride membrane, and probed with an anti-mouse IgG peroxidase-conjugated rabbit polyclonal antibody (1:2500; Sigma Chemicals, Deisenhofen, Germany). Immunoreactive protein bands were visualized by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech, Freiburg, Germany). X-ray films were digitized and optical densities were determined using a computer imaging analysis system (VisiTron Systems, Puchheim, Germany).

Measurement of brain albumin by ELISA

Maxisorb plates (Nunc, Wiesbaden, Germany) were coated and incubated for 60 min at room temperature with a mouse albumin-specific rabbit polyclonal antibody (Acris, Bad Nauheim, Germany), diluted in coating buffer (0.05 M sodium carbonate, pH 9.6) to a concentration of 0.5 μg/ml. Plates were washed with washing buffer (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0) and blocked using blocking buffer (50 mM Tris, 0.14 M NaCl, 1% bovine serum albumin, pH = 8.0). Mouse brain protein extracts diluted in lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl2 and a mixture of protease inhibitors including phenyl-methylsulphonyl fluoride, aprotinin, leupeptin and pepstatin A; 0.5 μg protein per well) were transferred to assigned wells and plates were incubated for 60 min at room temperature. Bound albumin was detected using a goat polyclonal peroxidase-conjugated anti-mouse albumin antibody, diluted in sample conjugate buffer (50 mM Tris, 0.14 M NaCl, 1% BSA, 0.05% Tween 20, pH 8.0) to a concentration of 0.1 μg/ml. Plates were incubated for 60 min at room temperature. Enzyme substrate reagent (R&D Systems, Wiesbaden, Germany) was added to the wells and the wells were incubated for 10 min at room temperature. The colorimetric reaction was stopped by adding 2 M sulphuric acid and absorbance was read at 450 nm.

mRNA isolation and reverse transcription–PCR analysis

Total RNA was extracted from frozen sections (brain and lung) with Trizol-LS reagent (Gibco, Gaithersburg, MD, USA) and reverse-transcribed using Superscript II (Gibco). The cDNA was amplified by PCR with gene-specific primers of the following sequences: IL-1β sense, 5'-TCATGGGATGATGATAACCTGCT-3'; IL-1β antisense, 5'-CCCATCCTTTAGGAAGACACGGATT-3'; IL-6 sense, 5'-CCAAAGACCTGTCTATACCAC-3'; IL-6 antisense, 5'-CCCTCTGTGACTCAGCTTATC-3'; tumour necrosis factor (TNF-α) sense, 5'-AGCAGAAGAAGCATGATCGG-3'; TNF-α antisense, 5'-CAGAGCAATGACTCAGCTTATC-3'; macrophage inflammatory protein (MIP)-2 sense, 5'-AGTTGGGATGATATCTTGGTCT-3'; MIP-2 antisense, 5'-TTGGTTGGGATGATATCTTGGTCT-3'; KC sense, 5'-AACGGAGAAAGACGCAGACTTG-3'; KC antisense, 5'-GACGAGGACGCAGGAAACAGGG-3'; complement C3 sense, 5'-CACCGCGAAAGATAGCTGCT-3'; C3 antisense, 5'-GACGAGGACGCAGGAAACAGGG-3'; complement C1r sense, 5'-CTTCCGTACATCACACCAC-3'; complement C1rA antisense, 5'-GCTAATCTTCTCTGTGTTA-3'; complement C1q A sense, 5'-TCTCAGCCATTCGCCAGAAC-3'; complement C1qA antisense, 5'-TAACACCGGAGAGCCCTTT-3'; complement receptor-related protein y (Crry) sense, 5'-CATCACAGCTTCC-TTGTGACCTT-3'; Crry antisense, 5'-ATCGTGCGGTGATCAGATATA-3'; MyD88 sense, 5'-ATGGTGCTACACTGCTTGG-3'; MyD88 antisense, 5'-ACTGCTTTCCACTCTGGC-3'. Mouse β-actin was co-amplified as an internal control using the following primer sequences: sense 5'-GGACTCCTATATGGGTCGAGGAG-3'; antisense 5'-GGGAGAGCAGCTCCTCTGATAG-3'. Linearity of DNA amplification was determined for each primer set in experiments establishing the PCR procedures in terms of cDNA amounts and cycle number. PCR products were separated on a 1.5% agarose gel, stained with ethidium bromide, visualized by UV illumination, and photographed. Densitometry was performed on the negative image, and the relative absorbances of MyD88, IL-1β, IL-6, TNF-α, MIP-2, KC, C3, C1qA, C1rA and Crry were normalized in relation to the absorbance of β-actin reverse transcription-PCR products.

Determination of bacterial titres in blood and organs

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

Determination of the cell influx in bronchoalveolar lavage (BAL) fluid

In a subset of experiments (see Experimental groups in the mouse model), the trachea was exposed through a midline incision and cannulated with a 22-gauge catheter prior to transcardiac perfusion. BAL was performed by instilling two 0.5 ml aliquots of sterile isotonic saline. A total of 0.85–0.95 ml of BAL fluid was retrieved per mouse, contaminating erythrocytes were lysed with ammonium chloride, and total cell numbers were counted using a haemocytometer.

Experimental groups in the mouse model

The following experimental groups were investigated: (i) wild-type mice injected intracisternally with 15 μl PBS (n = 8); (ii) wild-type mice injected intracisternally with Streptococcus pneumoniae (n = 11); and (iii) MyD88-deficient mice injected intracisternally with Streptococcus pneumoniae (n = 11). In an additional experimental series, bacterial titres in the lung, total cell numbers in BAL, cerebellar titres, and CSF leucocyte counts were determined in infected wild-type mice and MyD88-deficient mice (five mice per group). MyD88-deficient mice backcrossed eight times to the C57BL/6 background were kindly provided by Professor S. Akira (Osaka, Japan). Wild-type C57BL/6 mice were purchased from Charles River Germany (Sulzfeld, Germany). Age- and sex-matched groups of wild-type and MyD88-deficient mice were used for the experiments. All the experiments were approved by the Government of Upper Bavaria.

Statistical analysis

The principal statistical test was the unpaired Student’s t test. Differences were considered significant at P < 0.05. Data are expressed as mean ± SD.
Results

Upregulation of MyD88 mRNA expression in the brain during pneumococcal meningitis

In brain homogenates from PBS-injected control mice, a modest basal expression of MyD88 mRNA was detected by reverse transcription–PCR. Pneumococcal infection caused a substantial increase in the mRNA expression of MyD88 in brains obtained from wild-type mice 24 h after challenge. The constitutive MyD88 expression in the lung was not affected by intracisternal pneumococcal inoculation (Fig. 1).

Effect of MyD88 deficiency on the clinical course of pneumococcal meningitis

Within 24 h after inoculation, all infected wild-type mice exhibited a similar degree of disease, as evidenced by loss of weight, hypothermia, piloerection, lethargy, and impaired motor activity and function (Fig. 2). None of these mice died during the 24 h observation period. In MyD88-deficient mice, however, meningitis was associated with substantial mortality; 5 of 11 (45%) infected MyD88-deficient mice died within 24 h after infection. MyD88-deficient mice also showed a more pronounced reduction in body temperature. In addition, abnormal breathing (notably gasping and audible crackles) were observed more frequently in infected MyD88-deficient mice (9 of 11; 82%) than in wild-type mice (2 of 11; 18%).

None of the control mice injected intracisternally with PBS exhibited signs of infection within the observation period. Body temperatures were within the normal range. No breathing abnormalities could be detected.

Effect of MyD88 deficiency on meningitis-induced CNS complications

Since CNS complications are major determinants of an unfavourable clinical outcome in bacterial meningitis (Kastenbauer and Pfister, 2003), we investigated the impact of MyD88 deficiency on BBB permeability and ICP in our meningitis models.

Pneumococcal infection induced a significant increase in ICP in wild-type mice. Surprisingly, at 24 h after pneumococcal challenge, infected MyD88-deficient mice showed a significantly attenuated rise in ICP and in brain albumin levels compared with infected wild-type mice. These findings are surprising with regard to recent observations indicating that intracranial alterations such as a rise in ICP are major determinants of an unfavourable clinical course of meningitis. Data are mean ± SD. *P < 0.05 compared with infected wild-type mice.
BBB by measuring the brain concentrations of IgG and albumin by immunoblotting and ELISA, respectively. In brain homogenates from PBS-injected control mice, only faint bands for IgG were present (integrated density 6300 ± 10 900 arbitrary units). Intracisternal injection of pneumococci caused extensive extravasation of IgG into the brain. Infected MyD88-deficient mice exhibited significantly less immunoreactivity for IgG than infected wild-type mice (11 400 ± 13 000 compared with 293 300 ± 225 700 arbitrary units in wild-type mice). Similar data were obtained when the brain concentrations of albumin were analysed by ELISA (Fig. 3). These findings suggest that the worsening of disease in infected MyD88-deficient mice is not due to aggravation of CNS complications.

**Effect of MyD88 deficiency on bacterial titres in blood and organs**
To test whether the worsening of disease is the result of altered bacterial growth in vivo, the numbers of bacteria in the cerebellum, blood and lung were determined 24 h after intracisternal pneumococcal infection. In cerebellar homogenates, the number of *Streptococcus pneumoniae* was about 50-fold higher in MyD88-deficient mice than in wild-type mice (Fig. 3). Similarly, the clearance of *Streptococcus pneumoniae* from the bloodstream was severely affected by MyD88-deficiency, resulting in bacterial blood titres which were >4000-fold higher than in infected wild-type mice. The more severe bacteraemia in MyD88-deficient mice was paralleled by increased bacterial titres (about 80-fold) in their lungs (Table 1). This dramatically impaired host defence in both the brain and the periphery may contribute to the worsening of the disease.

**Effect of MyD88 deficiency on the inflammatory host reaction**
To investigate whether the higher susceptibility to pneumococcal infection was due to a defective immune response, we first assessed the impact of MyD88 deficiency on the inflammatory host response in the brain in our meningitis model.

Pneumococcal infection led to massive leucocyte infiltration into the subarachnoid space in wild-type mice. Infected MyD88-deficient mice had significantly lower CSF leucocyte counts than infected wild-type mice (2303 ± 1168 compared with 11 849 ± 4563 cells/μl in infected wild-type mice). The reduction in leucocyte infiltration was associated with markedly reduced brain mRNA levels of the neutrophil chemoattractants MIP-2 and KC. In addition, the mRNA accumulation of either the proinflammatory cytokines IL-1β, IL-6 and TNF-α or the complement factors C1r and C3 was significantly lower in brain homogenates from infected MyD88-deficient mice than in brain homogenates of wild-type mice (Fig. 4). Of the mRNA levels analysed 24 h after infection, only the brain mRNA levels of the complement

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**Table 1** Effect of MyD88 deficiency on bacterial outgrowth and cellular influx in the lung and brain

<table>
<thead>
<tr>
<th></th>
<th>Wild-type mice (n = 5)</th>
<th>MyD88-deficient mice (n = 5)</th>
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</thead>
<tbody>
<tr>
<td>Lung titre (log 10 c.f.u./organ)</td>
<td>7.1 ± 0.7c</td>
<td>9.3 ± 0.4c</td>
</tr>
<tr>
<td>Total BAL cells (× 10⁵/ml BAL)</td>
<td>5.9 ± 3.7c</td>
<td>70.8 ± 60.5c</td>
</tr>
<tr>
<td>Cerebellar titre (log 10 c.f.u./organ)</td>
<td>8.9 ± 0.1c</td>
<td>10.8 ± 0.3c</td>
</tr>
<tr>
<td>CSF leucocytes (× 10⁵/μl)</td>
<td>10.1 ± 3.0c</td>
<td>1.9 ± 0.5c</td>
</tr>
</tbody>
</table>

*Two of the MyD88-deficient mice died within the observation period, one of these 24 h after bacterial inoculation, thus allowing tissue and fluid sampling. Right lungs were used for the determination of bacterial titre. *P* < 0.05 compared with wild-type mice. Data are mean ± SD. c.f.u. = colony-forming unit.
inhibitor Crry and the complement factor C1q were similar in the two mouse strains. Taken together, the results of this expression analysis suggest that the marked reduction in meningitis-associated CNS complications in MyD88-deficient mice is linked to substantial attenuation of the host immune response within the brain.

**Fig. 4** Brain and lung mRNA expression of meningitis-relevant inflammatory mediators. (A–F) In brain homogenates from PBS-injected control mice, no or only low basal expression of (A) IL-1β, (B) TNF-α, (C) MIP-2, (D) KC, (E) C3 and (F) C1rA mRNA was detected by reverse transcription–PCR. Pneumococcal infection caused a significant increase in the mRNA expression of all these host factors in brains obtained from wild-type mice 24 h after challenge. In brains obtained from infected MyD88-deficient mice, the brain expression levels of all these inflammatory mediators were markedly reduced. (G–M) In contrast to the brain, in the lung basal expression of IL-1β, TNF-α, MIP-2, KC, C3, and C1rA mRNA was detectable in homogenates from PBS-injected control mice. Meningitis-associated bacteraemia was accompanied by upregulation of lung mRNA expression of IL-1β, TNF-α, MIP-2 and C1rA. Moreover, after 24 h of pneumococcal infection, differences in the lung expression of (G) IL-1β, (K) KC, (L) C3 and (M) C1rA were not detectable except for the lung expression levels of (H) TNF-α and (I) MIP-2, which were significantly higher in infected MyD88-deficient mice than in infected wild-type mice. Data are mean ± SD. *P < 0.05 compared with infected wild-type mice.
**Effect of MyD88 deficiency on lung involvement associated with pneumococcal infection**

Since *Streptococcus pneumoniae* can spread from the CNS to peripheral organs via the blood (Scheld et al., 1979), internal complications, notably septic shock and pneumonia, can also contribute to an adverse outcome of pneumococcal meningitis (Kastenbauer and Pfister, 2003). Thus, we investigated the impact of MyD88 deficiency on the inflammatory host response in the lung in our mouse model.

Twenty-four hours after intracisternal pneumococcal inoculation, substantial upregulation of the mRNA expression of IL-1β, TNF-α, C1r and MIP-2 (but not IL-6, KC, C3, C1q and Crry) was detectable in lung homogenates from infected wild-type mice compared with PBS-injected control mice. Targeted disruption of the MyD88 gene was associated with increased expression of TNF-α and MIP-2 mRNA, while IL-1β and C1r mRNA expression did not differ between the two genotypes. In addition, MyD88-deficient mice had significantly more cells in their BAL fluid when compared with wild-type mice (Table 1). These findings suggest that the worsening of disease is, at least in part, due to aggravation of the host immune response outside the brain in MyD88-deficient mice.

**Discussion**

Recent studies have indicated a dual role for the IL-1R/TLR adapter molecule MyD88 in host defence against bacterial infections *in vivo*. MyD88-deficient mice have been reported to be highly susceptible to intravenous *Staphylococcus aureus* infection (Takeuchi et al., 2000). In contrast, MyD88-deficient mice have been demonstrated to be resistant either to lethal shock induced by administration of a high dose of *Escherichia coli* lipopolysaccharide (Kawai et al., 1999) and less susceptible to peritonitis induced by bacterial polymicrobial infection (Weighardt et al., 2002). These results suggest that the role played by the MyD88 protein is strongly dependent on the type of pathogen. Furthermore, the requirement for MyD88 in order to mount a robust immune response in the host is assumed to be dependent also on the anatomical compartment involved. Thus, the polymicrobial sepsis-induced production of cytokines and chemokines was normal in the spleen of MyD88-deficient mice but almost completely abolished in the lung and liver (Weighardt et al., 2002). It has been speculated that tissue-specific expression of a splice variant of MyD88 (MyD88 short) that acts as a dominant negative inhibitor of IL1R/TLR-mediated signalling may account for the differences in immune reactivity between the spleen and the lung (or liver) (Janssens et al., 2002; Weighardt et al., 2002; Burns et al., 2003). In the present study we demonstrated that MyD88-deficient mice showed a strongly compromised inflammatory reaction to *Streptococcus pneumoniae* infection within the CNS, including diminished leucocyte infiltration and diminished production of cytokines (IL-1β, IL-6, TNF-α), chemokines (MIP-2, KC) and complement components (C1r, C3). However, in the lung, which is affected following pneumococcal spread from the CNS to the bloodstream (Scheld et al., 1979; Koedel et al., 2002b), the production of inflammatory mediators was similar in MyD88-deficient and wild-type mice. This observation contrasts with the results obtained in a murine model of polymicrobial sepsis that demonstrated that the production of immune mediators in the lung is mainly MyD88-dependent (Weighardt et al., 2002). It also brought into question an overall role of the expression of MyD88 short in the differences in immune reactivity between organs, since the immune response to *Streptococcus pneumoniae* in the brain, where MyD88 short was expressed, was mainly MyD88-dependent, whereas MyD88-independent pathways seem essential in the pneumococcus-induced immune response in the lung, where MyD88 short was not expressed (Janssens et al., 2002). Thus, yet undefined factors may determine the immune reactivity to *Streptococcus pneumoniae* in different organs.

MyD88 is an adapter protein common to signalling pathways of IL-1R, IL-18R and all TLRs (except TLR3) (Janssens and Beyaert, 2002; Takeuchi and Akira, 2002). Thus, the effects of MyD88 deficiency observed in our model of experimental pneumococcal meningitis can be linked to defects either in IL-1/IL-18 pathways or in TLR-mediated signalling or in both. We have previously demonstrated that targeted disruption of the gene for the cysteine protease caspase-1, which is crucial for the generation of both active IL-1 and IL-18, significantly diminished the inflammatory host response to pneumococci within the CNS (Koedel et al., 2002b). The similarity in the inflammatory reaction within the CNS between caspase-1-deficient and MyD88-deficient mice suggests that, in the regulation of the immune response to pneumococcal infection of the CNS, MyD88 acts mainly as an adapter for IL-1R/IL-18R signalling. Recent results obtained in a mouse model of haematogenous meningitis indicate that both IL-1 and IL-18 pathways contribute to the exaggerated inflammation in the CNS during pneumococcal meningitis (Zwijnenburg et al., 2003a, b). In IL-1R-deficient mice, pneumococcal meningitis was associated with less severe leucocyte infiltration (histopathological observation) and with lower brain levels of cytokines and chemokines (not significant, due to the large interindividual variation) (Zwijnenburg et al., 2003a). IL-18-deficient mice were also reported to show a suppressed inflammatory response, as evidenced by a less profound inflammatory infiltrate and lower brain levels of TNF-α, IL-6, IL-12, IL-10, MIP-2 and KC (differences in IL-6 and MIP-2 brain levels were not significant due to the great interindividual variation) (Zwijnenburg et al., 2003b). The great interindividual variation limits the validity of models of haematogenous meningitis using adult animals and is related to the facts that (i) only a portion of animals inoculated peripherally develop meningitis, and (ii) the time interval for the development of meningitis is variable. A second weakness which must be borne in mind is that, in these models, animals die...
predominantly from septic shock but not from CNS complications (Koedel et al., 2002a), making this model system not well suited for studies on meningitis-related brain damage and death. However, the existing data obtained from two model systems of pneumococcal meningitis indicate a crucial role of the IL-1/IL-18–MyD88 pathway in the host immune response to *Streptococcus pneumoniae* within the CNS.

Another major finding of the present study was that the bacterial numbers in the CNS were ~50-fold higher in MyD88-deficient mice than in wild-type mice. In a previous study, we demonstrated that TLR2-deficient mice displayed ~10-fold higher cerebellar bacterial titers than infected wild-type mice. In contrast, genetic depletion of caspase-1, IL-1R or IL-18 had no or only minimal impact on pneumococcal outgrowth within the CNS (von Mering et al., 2001; Zwijnenburg et al., 2003a, b). These findings imply a critical role of MyD88 in the control of pneumococcal infection by mediating not only IL-1 and IL-18 signalling but also TLR signalling. The dominant host defence mechanism against systemic infection with *Streptococcus pneumoniae* is complement-mediated opsonophagocytosis (AlonsoDeVelasco et al., 1995). The classical pathway appears to be decisive for activation of the complement system during innate immunity to *Streptococcus pneumoniae* (Brown et al., 2002). Tuomanen and colleagues (Tuomanen et al., 1986) reported that complement depletion (by treatment with Egyptian cobra venom factor) resulted in diminished opsonophagocytosis of encapsulated *Streptococcus pneumoniae* and consequently to increased bacterial titers in the CSF of rabbits with pneumococcal meningitis, which is analogous to what occurs in experimental models of systemic pneumococcal infection. Thus, complement factors appear to mediate partial killing of *Streptococcus pneumoniae* from the CSF, though not the clearance of this organism. In the present study we observed meningitis-induced up-regulation of the brain mRNA expression of two components of the C1 complex (C1r) and complement component C3, but not of the complement inhibitor Crry. Infected MyD88-deficient mice had lower brain mRNA levels of both C1r and C3 than infected wild-type mice. Taking these data together, it seems conceivable that the higher cerebellar bacterial titers in MyD88-deficient mice correlate with diminished brain expression of the complement factors that are relevant for successful phagocytosis of *Streptococcus pneumoniae*.

Apart from higher bacterial titers in the CNS, MyD88-deficient mice displayed ~4000-fold higher bacterial numbers in the bloodstream than infected wild-type mice. Since previous studies have clearly demonstrated that secondary bacteraemia is a common occurrence in meningitis and is directly related to the concentration of the microorganisms within the CSF (Scheld et al., 1979), it is conceivable that the higher concentrations of *Streptococcus pneumoniae* in the blood occur as a consequence of the higher bacterial titre in the CNS. This severe bacteraemia may contribute substantively to the more adverse outcome observed in MyD88-deficient mice. This suggestion is supported by the following observations: (i) hypothermia, an indicator of severe septicaemia in mice, was more pronounced in MyD88-deficient mice than in wild-type mice; (ii) MyD88-deficient mice displayed breathing problems more frequently than wild-type mice; (iii) the inflammatory reaction in the lung (expression of the inflammatory mediators TNF-α and MIP-2 and cell influx in BAL fluid) was more pronounced in MyD88-deficient mice than in wild-type mice; and (iv) CNS complications, the most important determinants of an unfavourable clinical outcome, were substantially reduced in MyD88-deficient mice compared with infected wild-type mice.

In conclusion, the present study demonstrates a crucial role of MyD88 in mounting a robust host immune response to *Streptococcus pneumoniae* within the CNS. Although diminished CNS inflammation was paralleled by an attenuation of meningitis-associated CNS complication, MyD88 deficiency resulted in worsening of this disease. The worsening of disease was associated with severe bacteraemia, which presumably led to aggravation of systemic complications such as septic shock and pneumonia. This might be supported by the substantial contribution of MyD88-independent pathways to the immune activation in response to pneumococcal infection outside the CNS.

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