

Toll-Like Receptors in Bacterial Meningitis

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Abstract Bacterial meningitis is still an important infectious disease with a high morbidity and mortality rate. Bacterial infection of the cerebrospinal fluid (CSF) space causes a powerful inflammatory reaction that is largely responsible for meningitis-induced tissue damage and adverse outcome of the disease. In a landmark series of experiments in the mid-1980s, cell wall components including lipooligosaccharides and lipoteichoic acid were indicated to be the key bacterial elements that can trigger the host inflammatory response in the CSF. Ten years ago, the discovery of Toll-like receptor proteins (TLRs) that allow the detection of microbial components and initiate the host immune response opened up new horizons in research on the pathophysiology of meningitis. Cell culture approaches provided the first evidence for a crucial role of TLRs in sensing meningeal pathogens including *Streptococcus pneumoniae*, *Neisseria meningitidis*, *Streptococcus agalactiae*, and *Listeria monocytogenes*. Subsequently, studies in mice with single or combined deficiencies in TLRs demonstrated that TLR activation is a key

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event in meningeal inflammation and, even more interestingly, a pivotal factor for meningitis-associated tissue damage. A detailed understanding of the mechanisms of host–pathogen interactions in the CSF space may generate new opportunities for specific treatment strategies for bacterial meningitis.

Abbreviations

BCG	Bacillus Calmette-Guérin
cfu	Colony-forming unit
CHO	Chinese hamster ovary
CNS	Central nervous system
CR	Complement receptor
CSF	Cerebrospinal fluid
DAI	DNA-dependent activator of interferon regulatory factors
GBS	Group B streptococci
HEK	Human embryonic epithelial kidney
HIB	<i>Haemophilus influenzae</i> type B
IL	Interleukin
ie-DAP	Meso-diaminopimelic acid
ISH	In situ hybridization
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MDP	Muramyl dipeptide
MR	Mannose receptor
MyD	Myeloid differentiation factor
NALP3	Nacht domain, leucine-rich repeat and PYD-containing protein 3
NOD	Nucleotide-binding oligomerization domain
NLR	NOD-like receptor
PAMP	Pathogen-associated molecular pattern
PCV	Pneumococcal conjugate vaccine
PGN	Peptidoglycan
PPR	Pattern recognition receptor
RIP	Protein kinase receptor-interacting protein
SR	Scavenger receptor
TLR	Toll-like receptor
TNF	Tumor necrosis factor

1 Introduction

From its first description by Vieusseux in 1806 until the early twentieth century, acute bacterial meningitis was quite uniformly considered a fatal disease. Although the introduction of antibiotics made it curable, mortality and morbidity from the

disease remain unacceptably high (Van de Beek et al. 2004). While endemic bacterial meningitis remains relatively rare, particularly in developed countries, the potential occurrence of epidemic bacterial meningitis in any part of the world heightens its profile as a major infectious disease. Large epidemic waves of meningococcal meningitis occur with a periodicity of 8–12 years in sub-Saharan territories of Africa, the so-called meningitis belt. In 1996, Africa experienced the largest recorded outbreak of epidemic meningitis in history, with over 250,000 cases and 25,000 deaths. Over 35,000 of the cured patients are expected to have sustained permanent neurological, otological, and psychological deficits (Robbins et al. 1997). In addition to these epidemics, at least 1.2 million cases of bacterial meningitis are estimated to occur each year; 135,000 of them are fatal. These numbers have made bacterial meningitis one of the top ten infectious causes of death worldwide. Clinical and neuropathological studies have clearly indicated that fatal disease outcome is predominantly caused by intracranial complications, including raised intracranial pressure, brain edema formation, and cerebrovascular insults (Kastenbauer and Pfister 2003; Van de Beek et al. 2004). During the past two decades, the frontier of investigation has therefore focused on the pathophysiology of meningitis-associated brain injury. It became evident that the host immune response to the pathogen, rather than the pathogen itself, is largely responsible for the damage that results from bacterial meningitis (Koedel et al. 2002a; Weber and Tuomanen 2007). As a result, the therapeutic approach to bacterial meningitis has to be widened from eradicating the pathogen with antibiotics to preventing the detrimental effects of the host immune response. The intention of this chapter is to provide an overview of the current state of our knowledge in this field by addressing the following questions: what do we know about the immune milieu at the site of infection? Which pathogens are the major causes of purulent meningitis? How are these bacteria recognized by immune cells? What is the impact of these “recognition systems” on the clinical course of the disease?

2 An Immunological Approach to the Cerebrospinal Fluid Compartment

Bacterial meningitis is mainly an acute purulent infection of the leptomeninges and subarachnoid space caused by bacteria. The leptomeninges are the two innermost layers of tissue (the arachnoid mater and pia mater) that envelop the brain. The subarachnoid space is the interval between the leptomeninges, which is filled with cerebrospinal fluid (CSF). The meninges and the CSF serve to cushion the brain, thereby protecting it from injury.

From an immunological point of view, the subarachnoid space is a unique site in the body (Fig. 1). Specialized blood–CSF barriers seclude the subarachnoid space from the circulating blood and prevent most blood components from entering the CSF (Pachter et al. 2003). Moreover, soluble pattern recognition receptors (PRR, e.g., complement factors) that recognize bacteria and enhance their uptake

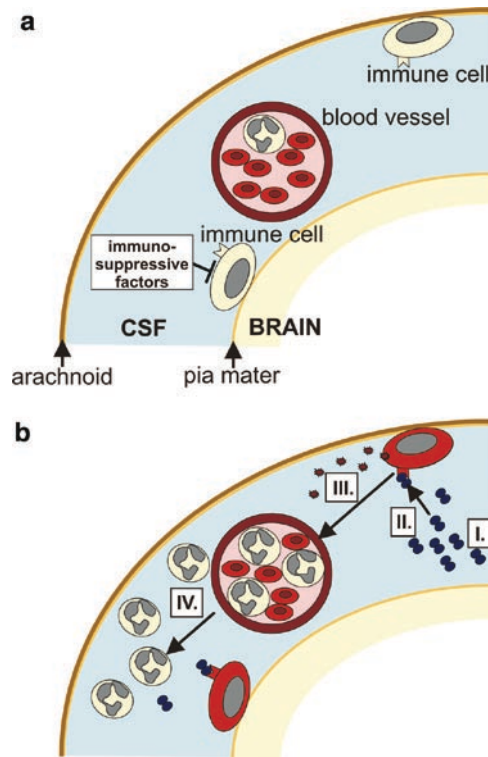


Fig. 1a–b Schematic illustration of the (a) normal and (b) inflamed subarachnoid space. **a** From an immunological point of view, the subarachnoid space is a unique anatomical compartment. It shares several features with immune-privileged organs. For example, soluble pattern recognition receptors that recognize bacteria and enhance their uptake by phagocytes are largely absent in the CSF. Moreover, the normal CSF contains an array of immunosuppressive factors that actively suppress immune reactivity. However, contrary to immune-privileged organs, functionally active immune cells are present in all non-neural structures that are in direct contact with the CSF. These cells can function as sentinel cells that are able to detect the presence of bacteria in the CSF. **b** Once pathogens have entered the subarachnoid space, they take advantage of the immunodeficiency within the CSF and multiply easily and efficiently (*I*). Phagocytes recognize pathogens by means of their cell surface receptors, so-called pattern recognition receptors (*II*). The activated phagocytes can destroy pathogens without any additional help, but in almost all cases they amplify immunity by releasing cytokines and chemokines (*III*). As a consequence, large numbers of neutrophils are recruited into the CSF (*IV*), a pathologic hallmark of acute bacterial meningitis

by phagocytes are largely absent in the CSF (Dujardin et al. 1985; Stahel et al. 1997). Additionally, the subarachnoid cavity lacks fully organized drainage via lymphatic vessels (Johnston et al. 2004). Lymphatic vessels are of crucial importance for the migration of antigen-presenting dendritic cells from tissues to lymph nodes, where they interact with T and B lymphocytes to mount and shape an adaptive immune response (Steinman 2007). Finally, the normal CSF contains an

array of anti-inflammatory and immunosuppressive factors that actively suppress immune reactivity (Niederborn 2006).

However, in contrast to the brain, which lacks competent antigen-presenting cells (microglia, the resident cells for immune defenses in the brain, are in a resting state under physiological conditions, with weak expression of molecules associated with antigen presentation; Schwartz et al. 2006), functionally active macrophages and dendritic cells are present in all non-neural structures that are in direct contact with the CSF, namely the leptomeninges, the perivascular spaces and the choroid plexus (Pashenkov and Link 2002; Guillemin and Brew 2004). These cell types can function as sentinel cells to detect the presence of bacteria in the CSF through PRRs. Gene transcription and protein analyses have verified that diverse PRRs are expressed in tissues lining the CSF compartment. For instance, macrophage scavenger receptors (SR) that can bind and internalize a variety of microbial pathogens (Mukhopadhyay and Gordon 2004) are found on stromal and epiplexus macrophages of the choroid plexus, meningeal macrophages and on perivascular sites, but not on brain microglia (Naito et al. 1991). Likewise, the mannose receptor (MR) that recognizes a range of carbohydrates present on microbes and mediates microbial phagocytosis (McGreal et al. 2005) is exclusively expressed by perivascular, meningeal, and choroid plexus macrophages in the brain (Galea et al. 2005). The phagocytic complement receptors like CR1 (Roozendaal and Carroll 2006) exhibit a brain tissue expression pattern identical to that of SRs and MR (Singhrao et al. 1999). In addition, signaling PRRs, including Toll-like receptors (TLR) and nucleotide-binding oligomerization domain (NOD) proteins, have been implicated as being present on and in immunocompetent cells within the brain (Bsibsi et al. 2002; Letiembre et al. 2007; Rodriguez-Martinez et al. 2005). In situ hybridization (ISH) studies revealed a high constitutive expression of TLR2 and TLR4 mRNA in the choroid plexus. A strong hybridization signal for TLR4 was also observed along leptomeningeal vessels (Laflamme and Rivest 2001; Laflamme et al. 2001, 2003). The brain expression of other TLRs (e.g., TLR8 and TLR9) was below the detection limit of the ISH technique (De Luigi et al. 1998). Collectively, the normal milieu in the CSF seems to be much more “immune friendly” compared to that of the intact brain parenchyma, which constitutes a milieu that is unfavorable for immune responses. The difference in immune reactivity between both CNS compartments is exemplarily illustrated by experiments in which heat-killed bacillus Calmette–Guérin (BCG) was injected into either site (Matyszak and Perry 1996). Intra-CSF administration induced extensive leukocyte infiltration within the first few hours, comparable to that in immunocompetent tissues like the skin, whereas the same dose of BCG evoked only a minimal inflammatory response in the brain parenchyma (Matyszak and Perry 1996). However, in contrast to immunocompetent tissues, the host immune response within the CSF compartment is largely inefficient at controlling and overcoming microbial infections. For instance, *Streptococcus pneumoniae*, a major meningeal pathogen, can multiply within the CSF almost as efficiently as it can in vitro, reaching high titers of up to 10^9 colony-forming units (cfu)/ml. Moreover, intracisternal inoculation of 10^4 cfu of *S. pneumoniae* in mice resulted in the death of all infected animals, whereas only

one quarter of mice died after intratracheal application of an identical dose of this pneumococcal strain (Gerber et al. 2001; Rijnveld et al. 2001). Similar data have also been reported for humans. In the preantibiotic era, pneumococcal meningitis was a lethal disease, while 75% of patients survived pneumococcal pneumonia (Heffron 1979). This local host defense deficiency seems to be due to a local absence of soluble PRRs that flag the pathogens for phagocytosis, the local expression of immunosuppressive factors that interfere with the uptake and killing of pathogens, or both. The failure to eliminate the pathogens is the “driving force” for the immune response that is a key mediator of meningitis-associated tissue injury, and is thus largely responsible for the adverse disease outcome.

3 Bacteriologic Profile of Community-Acquired Bacterial Meningitis

During the past decades, we have witnessed significant changes in the epidemiology of bacterial meningitis. When all cases occurring after the neonatal period are taken into account, the leading cause of bacterial meningitis 20–30 years ago was *Haemophilus influenzae* type B (HiB), with a relative frequency of about 45%, followed by *S. pneumoniae* with 18% and *Neisseria meningitidis* with 14% (Swartz 2004). The introduction of the HiB conjugate vaccine in the early 1990s has virtually eliminated invasive HiB disease in countries that have included the HiB vaccine in their national immunization programs. By the end of 2005, HiB vaccines were part of the routine infant immunization program in 101 of the 192 WHO member states. Unfortunately, the proportion of infants in the world who had benefited from the HiB vaccination by the end of 2005 was still fairly low (21%), as eight countries with large birth cohorts had not introduced the vaccine (Rossi et al. 2007). In countries with HiB vaccination programs, *S. pneumoniae* became the leading species (with a total of 47% cases in the US), followed by *N. meningitidis* (25%), group B streptococci (12%) and *Listeria monocytogenes* (8%) (Schuchat et al. 1997). Further breakthroughs were the introduction of pneumococcal and meningococcal conjugate vaccines in the early 2000s (Makwana and Riordan 2007). For instance, the pneumococcal conjugate vaccine (PCV7) protects against the seven most common pneumococcal strains (out of over 90 serotypes) that cause invasive disease, including bloodstream infections and meningitis. After the implementation of routine childhood vaccination with PCV7 in the US, the average hospitalization rates for pneumococcal meningitis decreased by about 66% among children younger than 2 years and by 33% among adults aged 65 years and older (Tsai et al. 2008). The decline in invasive diseases in older adults probably occurs because of a decreased community transmission of vaccine-type pneumococci from young children to adults. Admittedly, the effectiveness of the vaccine against all invasive diseases, without regard to serotype, could be diminished in that nonvaccine pneumococcal serotypes account for larger portions of the disease (Whitney et al. 2006). All in all, the introduction of these bacterial vaccines has led to a decrease in the rate of

bacterial meningitis (in countries with the power to or the support of the international community necessary to implement these costly programs), and bacterial meningitis is now a disease predominantly found in adults rather than in infants and young children. Meningitis due to *N. meningitidis* is the most common form among young adults, whereas meningitis due to *S. pneumoniae* meningitis dominates in older adults (Dery and Hasbun 2007; Schut et al. 2008) (Table 1). These two pathogens account for over 80% of all cases of bacterial meningitis. Other etiologic agents include *Streptococcus agalactiae*, *Escherichia coli*, *L. monocytogenes*, and *Mycobacterium tuberculosis* (Schuchat et al. 1997; Van de Beek et al. 2004). *S. agalactiae* and *E. coli* are the leading causes of meningitis among newborns (Heath et al. 2003). *L. monocytogenes* is an uncommon cause of meningitis in the general population, but an important pathogen in neonates, the elderly, transplant recipients and other patients with impaired cell-mediated immunity (Doganay 2003). *M. tuberculosis* very rarely causes meningitis in Western countries but is feared in countries where tuberculosis is endemic (Thwaites et al. 2000).

Bacterial meningitis typically begins with host acquisition of the abovementioned pathogens by nasopharyngeal colonization, followed by systemic invasion, development of high-grade bacteremia, and invasion of the subarachnoid space via the blood–brain/CSF barrier. Pathogens can also enter the subarachnoid space by direct migration from nearby infections (e.g., sinusitis, mastoiditis) or through exterior openings in normally closed CSF pathways (e.g., due to meningocele or neurosurgical procedures). Once the pathogens have entered the subarachnoid space, they take advantage of the immunodeficient CSF compartment and multiply easily and efficiently. The host immune response does not appear to be activated until the pathogens reach a relatively high density (i.e., $>10^5$ cfu/ml for *S. pneumoniae*) (Tuomanen et al. 1985b). During recent decades, the mechanisms underlying host–pathogen recognition and immune activation have been intensively investigated using *in vitro* and *in vivo* approaches. The following sections will address how TLRs contribute to the recognition of the most common pathogens that cause acute bacterial meningitis in developed countries, namely *S. pneumoniae*, *N. meningitidis*, *S. agalactiae*, and *L. monocytogenes*.

4 Host Cellular Sensors of Meningeal Pathogens

In a landmark series of studies on immune activation in pneumococcal meningitis (Tuomanen et al. 1985a,b, 1986), the cell wall was found to be the key bacterial element that can trigger the host inflammatory response. Both of the major pneumococcal cell wall components, lipoteichoic acid (LTA) and peptidoglycan (PGN), can contribute to the induction of meningeal inflammation. Each has specific activity that is high enough to produce an immune response if given at doses of $\geq 10^5$ bacterial equivalents (Tuomanen et al. 1985a). Intracisternal inoculation of the cell wall induces clinical symptoms of meningitis (Tuomanen et al. 1989). The greater the amount of cell wall components in the CSF, the worse the clinical outcome of

Table 1 Epidemiologic data for acute bacterial meningitis

Pathogen	Age group			Frequency			Case fatality rate		
				Netherlands	France	Central African Republic	Netherlands	France	Central African Republic
<i>S. pneumoniae</i>	Neonates	Children	Adults	(n = 696) 51%	Children (n = 1,084) 33%	Children (n = 167) 37%	Adults (n = 696) 30%	Children (n = 1,084) 11%	Children (n = 167) 47%
	Adults								
<i>N. meningitidis</i>	Children	Adults		37%	55%	5%	7%	8%	13%
<i>S. agalactiae</i>	Neonates			0.7%	5%	— ^a	— ^b	19%	
<i>L. monocytogenes</i>	Neonates	Adults		4%	0.5%	— ^a	— ^b	17%	
Others:	Children			2%	2.5%	28%	— ^b	4%	33%
<i>H. influenzae</i>									

The occurrence and outcome of acute bacterial meningitis varies according to socioeconomic aspects (developed vs. developing countries), age, and the pathogen causing the acute bacterial meningitis. Data are taken from van de Beek et al. (2004); Bingen et al. (2005) Bercion et al. (2008)

^aNot detected

^bData not reported

the disease (Schneider et al. 1999). When challenged with pneumococcal cell wall components, isolated immunocompetent cells produce a wide range of cytokines, like interleukin (IL)-1 β , IL-6, tumor necrosis factor (TNF)- α , CXCL1, or CXCL2 (Riesenfeld-Orn et al. 1989; Heumann et al. 1994; Hanisch et al. 2001). Similar to this Gram-positive pathogen, the inflammatory activity of the Gram-negative bacteria *N. meningitidis* and HiB was mainly attributed to the release of bacterial cell wall material. Thus, in rabbits, intra-CSF injection of HiB lipooligosaccharide (LOS, but not capsular polysaccharide) induces an inflammatory reaction in the subarachnoid space comparable to that of live HiB (Syrogiannopoulos et al. 1988). Likewise, injection of meningococcal lipopolysaccharide (LPS) into the CSF compartment results in meningitis (Waage et al. 1989). In patients with meningococcal disease, the compartmentalization of LPS production correlates with the clinical presentation, meaning that high LPS levels in CSF and low LPS in plasma are associated with meningitis, whereas reciprocal findings are obtained from patients with septicemia (Brandtzaeg et al. 1992). Collectively, it is virtually certain that components of the bacterial cell walls “can do it all” when it comes to initiating the host immune response in meningitis. The essential question, however, concerns how bacterial cell wall components can trigger host responses.

Two studies in the mid-1990s solved the mystery of pathogen recognition by the immune system. One study showed that signaling through the mammalian receptor TLR4 activates the immune system (Medzhitov et al. 1997). The other study found that TLR4 plays a critical role in mediating the inflammatory activity of the Gram-negative cell wall component LPS (Poltorak et al. 1998). These discoveries were foreshadowed by the observation that fruit flies require the protein Toll, the namesake of the TLR family, to sense fungal infection (Lemaitre et al. 1996). The fact that a single receptor family serves as a sentinel of microbial infection in such widely different species suggested a fundamental role of this recognition mechanism. It soon became clear that the recognition of microbial invaders is based on the detection of conserved microbial molecules (pathogen-associated molecular patterns or PAMPs) (Medzhitov and Janeway 2002; Beutler and Rietschel 2003; Akira et al. 2006) that are (1) produced only by microbes, (2) invariant between microorganisms of a given class, and (3) essential for microbial survival. These microbial molecules are recognized by specific receptors of the innate immune system, the PRRs. A recently published database of PRR (called PRRDB) contains around 500 PRRs from 77 distinct organisms ranging from insects to humans. This includes 177 TLRs, 124 SRs, and 67 NOD-like receptors (NLRs) (Lata and Raghava 2008). A total of 13 TLRs have been identified in mammals; humans express TLR1–10, while mice express TLR1–7, TLR9 and TLR11–13 (Beutler et al. 2006). To date, TLRs are the best-characterized class of PRRs. These transmembrane receptors are expressed on the cell surface or intracellularly on endosomal membranes (TLR3, 7, 8, and 9), and binding of the respective ligands elicits inflammatory and antimicrobial responses of the host. The first insights into the role of TLRs as sensors of major meningeal pathogens were provided by cell culture studies (Fig. 2).

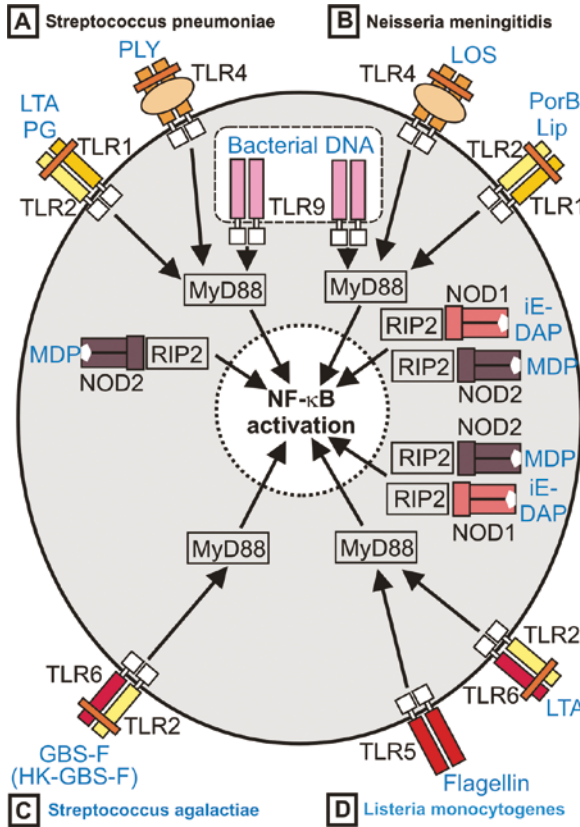


Fig. 2a–d Model for engagement of TLRs and NODs by major meningeal pathogens, as deduced from cell culture experiments. *S. pneumoniae* (a) generally has the potential to activate cells through TLR1/2, TLR4, TLR9, NOD2, and presumably as-yet unidentified pattern recognition receptors. Pneumococcal peptidoglycan (PGN) and lipoteichoic acid (LTA) are recognized through TLR1/2, whereas TLR4 senses the presence of pneumolysin (PLY). Moreover, pneumococcal DNA and internalized muramyl dipeptide (MDP) are detected through TLR9 and NOD2, respectively. A similar subset of pattern recognition receptors has been implicated in the host recognition of *Neisseria meningitidis* (b). While meningococcal lipooligosaccharide (LOS) and DNA interact with TLR4 and TLR9, respectively, the meningococcal outer membrane proteins porin B (PorB) and Lip are recognized through TLR2 with the help of TLR1 and CD14. In addition to MDP, the meningococcal cell wall contains dipeptide meso-diaminopimelic acid (iE-DPP), which induces activation of the transcription factor NF-κB through NOD1-RIP2-dependent signaling. In contrast to these pathogens, the mechanisms involved in the sensing of *S. agalactiae* (c) are still largely unclear. Recently, a heat-labile factor named GBS-F has been found to engage the TLR2/TLR6 heterodimer as an essential signaling molecule. Similar to *N. meningitidis*, *L. monocytogenes* (d) has been found to express NOD1- and NOD2-dependent immunostimulatory activity in vitro. Extracellular listerial cell wall components such as LTA are recognized by TLR2 (with the help of CD14 and TLR6), whereas extracellular listerial flagellin signals through TLR5. Other TLRs including TLR4 and TLR9 have been reported as being dispensable for *Listeria*-induced immune cell activation

4.1 Immune Recognition of *S. pneumoniae* In Vitro

In 1999, Yoshimura and colleagues (Yoshimura et al. 1999) reported that heterologous expression of human TLR2, but not TLR4, in Chinese hamster ovary (CHO) fibroblasts confers responsiveness to heat-killed *S. pneumoniae* (D39, a serotype 2 strain), as evidenced by inducible translocation of the transcription factor NF- κ B. The role of TLR2 in the sensing of *S. pneumoniae* was further strengthened by the following findings: first, the human embryonic epithelial kidney cell line HEK293 becomes responsive to heat-killed (D39 or TIGR4, a serotype 4 strain), antibiotic-lysed encapsulated (serotype 3 strain), or live unencapsulated pathogen (strain R6x) when transfected with TLR2 (Malley et al. 2003; Koedel et al. 2003; Schmeck et al. 2006). Second, the uptake and killing of pneumococci (serotype 1 and 3 strains) by isolated murine polymorphonuclear leukocytes is impaired in the absence of TLR2 (Letiembre et al. 2005). The cell wall components, PGN and LTA were identified as the main pneumococcal ligands for TLR2 (Yoshimura et al. 1999; Schroder et al. 2003). TLR2 was reported to recognize pneumococcal LTA in cooperation with TLR1 (Han et al. 2003) and with the help of CD14 (Schroder et al. 2003; Han et al. 2003). In addition to TLR2, other TLRs may also contribute to sensing the presence of *S. pneumoniae*. For instance, Malley and colleagues (Malley et al. 2003) demonstrated that ethanol killed *S. pneumoniae* (D39 strain) stimulated HEK293 cells transfected either with TLR2 or TLR4. Similarly, antibiotic-lysed pneumococci (serotype 3 strain) were found to trigger activation of transfected HEK293 cells in a TLR2- and TLR4-dependent manner (Koedel et al. 2003). The observations that ethanol-killed pneumolysin-deficient pneumococci (isogenic mutant of D39) failed to stimulate TLR4-expressing HEK293 cells, and that recombinant pneumolysin failed to activate TLR4-deficient macrophages but was effective on macrophages from TLR4-positive mice, suggested that TLR4 mediates host immune responses to pneumococci through its interaction with one of the most important virulence factors of the organism, the cholesterol-dependent cytolysin pneumolysin (Malley et al. 2003; Schmeck et al. 2006). Interestingly, TLR4 seems to be dispensable for the cellular recognition of viable *S. pneumoniae* (Mogensen et al. 2006; Schmeck et al. 2006). Instead, TLR9 was implicated as another sensor for the live bacterium. Mogensen and colleagues (Mogensen et al. 2006) reported that viable *S. pneumoniae* (TIGR4 strain) induced cell activation in HEK293 cells that were stably transfected with TLR9. TLR9-dependent cell activation was also observed when isolated genomic DNA from *S. pneumoniae* was used as a stimulus (Mogensen et al. 2006). In contrast to viable pneumococci and their genomic DNA, heat-killed (serotype 4 and 14 strain) and antibiotic-lysed pneumococci (serotype 3 strain) were almost completely unable to activate TLR9 (Koedel et al. 2003; Mogensen et al. 2006; Lee et al. 2007). The reported differences in the role of single TLRs in pneumococcal sensing are most likely attributable to the fact that distinct pneumococcal strains and preparations express and/or release distinct PAMPs. For example, heat inactivation of bacteria can lead to unwanted inactivation of PAMPs, as demonstrated for

the TLR4 ligand pneumolysin (Malley et al. 2003). The pneumococcal capsule might also interfere with the release of single PAMPs upon the heat killing of pneumococci. This was demonstrated by the intracisternal challenge of animals with heat-killed pneumococci. Nonencapsulated, heat-killed pneumococci elicited an inflammatory response in intracisternally challenged animals, whereas encapsulated heat-killed bacteria did not. In contrast, such a difference was not observed when viable encapsulated and unencapsulated pneumococci were compared (Tuomanen et al. 1985b). Moreover, large disparities have been reported for the production of the TLR4 ligand pneumolysin by different strains. Benton and coworkers described two principal types of pneumolysin production during in vitro growth (Benton et al. 1997). For the D39 (serotype 2) strain, they found a rise of cytoplasmic pneumolysin but no pneumolysin release until the late log phase. In contrast, for the Wu (serotype 3) strain, a measurable extracellular pneumolysin titer became detectable prior to cytoplasmic titers, possibly due to small amounts of autolysis that can begin in the early- to mid-log phase (Benton et al. 1997). Furthermore, sequence variations in the pneumolysin gene were reported to occur at a frequency higher than 3% (Jefferies et al. 2007). Allelic variations can result in the expression of noncytolytic pneumolysin in certain clonal groups of pneumococci, namely lineages of the serotypes 1 and 8 (Jefferies et al. 2007). In a mouse model of bronchopneumonia, mice infected with an isogenic mutant strain that expresses noncytolytic pneumolysin showed a pattern of neutrophil infiltration distinct from that observed in mice infected with the wild-type strain. In the early phase after infection (12 h), the numbers were the same with both strains, but neutrophil numbers were subsequently significantly lower in mice infected with the mutant strain than in animals inoculated with the wild-type strain (Jounblat et al. 2003). The existence of pneumolysin variants among pneumococcal strains suggests that, although the reduced inflammatory activity of noncytolytic pneumolysin is not due to a loss of binding to TLR4 (Malley et al. 2003), differences in PAMP structure between pneumococcal strains may also contribute to the reported differences in the role of single TLRs in pneumococcal sensing. Collectively, in vitro studies have demonstrated that *S. pneumoniae* generally has the potential to activate cells through TLR2, TLR4, and TLR9 (Fig. 2). Utilizing primary immune cells isolated from mice with single, double or triple deficiencies, studies by Snapper's (Lee et al. 2007) and our group (Klein et al. 2008) recently provided evidence that a single deficiency of TLR2, TLR4 or TLR9 caused only selective and relatively modest reductions in cytokine production by pneumococci-stimulated immune cells, whereas the combined loss of TLR2, TLR4 and TLR9 recapitulated the phenotype of cells lacking MyD88, an essential adaptor molecule in the signaling cascade of all TLRs with the exception of TLR3 (Takeuchi and Akira 2002; Barton and Medzhitov 2003). These in vitro data strongly suggest that distinct TLRs must synergize to fully activate an immune cell response to *S. pneumoniae*.

While there is no doubt that TLRs are important for sensing pneumococcal infection, there is also substantial evidence for the involvement of other PRRs in pneumococcal recognition. The NLRs are one example of such a receptor (Kanneganti et al. 2007). Whereas TLRs are able to sense PAMPs at the cell surface

and within endosomes, NLRs detect PAMPs in the cytosol. The discovery of these cytosolic PRRs suggests that pathogens evading extracellular surveillance encounter another line of recognition in the cytosol (Kanneganti et al. 2007). Two NLRs, NOD1 and NOD2, sense the cytosolic presence of peptidoglycan fragments and lead to protein kinase receptor interacting protein (RIP)-2-dependent activation of NF- κ B. A different set of NLRs (like NALP1, NALP3 or IPAF) are qualified to recognize a plethora of molecules ranging from divergent PAMPs to endogenous danger molecules and promote the assembly of inflammasome complexes (Ogura et al. 2006) that are required for the activation of caspase-1 (and the generation of biologically active IL-1 family cytokines). Recently, Opitz et al. (2004) demonstrated that pneumococci can invade HEK293 cells and induce NF- κ B activation through NOD2- and RIP2-dependent signaling. Further studies revealed that NOD2 is activated by muramyl dipeptide (MDP), a component of virtually all types of PGN (Girardin et al. 2003b), whereas NOD1 recognizes the dipeptide meso-diaminopimelic acid (iE-DAP) which is found in many Gram-negative and certain Gram-positive bacteria (Girardin et al. 2003a). Accordingly, many bacteria, including the meningeal pathogens *N. meningitidis* (Girardin et al. 2003a) and *L. monocytogenes* (Hasegawa et al. 2006; Park et al. 2007), have been shown to express NOD1- and/or NOD2-stimulatory activity in vitro. Thus, NOD1 and NOD2 may act as sensors of microbial invaders of the CSF compartment—presumably in cooperation with TLRs (and additional PRRs).

4.2 Immune Recognition of *N. meningitidis* In Vitro

TLRs have also been ascribed a particularly important role in the initiation of the host immune response against *N. meningitidis* (Fig. 2). By genetic complementation in HEK293 cells, human TLR2, TLR4, and TLR9 were found to confer responsiveness to live *N. meningitidis* strains (Mogensen et al. 2006). The ability of *N. meningitidis* to activate TLR2 involves recognition of the neisserial porin, the major outer membrane protein of the pathogenic *Neisseria* (Massari et al. 2002). Thereby, TLR2 recognizes the porin PorB through direct binding, and engagement of the TLR2/TLR1 heterodimer is required for initiating signaling in transfected HEK293 cells and in murine B cells (Massari et al. 2006). Apart from neisserial porin, purified Lip lipoprotein, which contains a conserved epitope known as H.8 that is common to all pathogenic *Neisseria* species, is capable of stimulating the production of proinflammatory mediators from HEK293 cells in a TLR2-dependent manner. The Lip-induced activation of TLR2-expressing HEK293 cells is further enhanced by co-transfection of TLR1 (but not TLR6) (Fisette et al. 2003). With respect to TLR4, meningococcal LOS is necessary for receptor activation, as a LOS-deficient *N. meningitidis* mutant was unable to induce cell activation via TLR4/MD2 (Pridmore et al. 2001). TLR4-mediated activation requires engagement of both CD14 and MD-2 (Zughaier et al. 2004), which is typical of the pattern of TLR4 binding by Gram-negative bacteria. By using an isogenic mutant system,

truncation of the oligosaccharide and removal of the capsule were found to exert no effect on the ability of meningococcal LOS to signal via the TLR4/MD2 complex (Pridmore et al. 2003). More recent studies indicated that meningococcal KDO₂-lipid (KDO = 3-deoxy-D-manno-octulosonic acid) is the minimal structure required for immunostimulatory activity of meningococcal LOS (Zughaier et al. 2006, 2007). Moreover, natural DNA sequences present in Gram-negative bacteria including *N. meningitidis* were shown to produce innate immune cell stimulation via TLR9 (Magnusson et al. 2007). The involvement of TLR9 in meningococcal detection was further strengthened by the study of Mogensen et al. (2006) that demonstrated the ability of both purified meningococcal DNA and live *Neisseria* to stimulate cells through TLR9. This study also showed that *Neisseria* can activate parental HEK293 cells, devoid of any TLRs, suggesting the contribution of TLR-independent signaling pathways in *Neisseria*-induced cell activation.

4.3 Immune Recognition of *S. agalactiae* In Vitro

The first study that investigated the role of TLRs in the recognition of group B streptococci (GBS or *S. agalactiae*) was published in 2000 (Flo et al. 2000) (Fig. 2). This study reported that expression of neither TLR2 nor CD14 makes CHO cells responsive to heat-killed GBS, and that a blocking antibody directed against TLR2 does not inhibit monocyte activation upon exposure to heat-killed GBS. More recent experiments using peritoneal macrophages from MyD88- and TLR2-deficient mice indicated that TLR2 is involved in TNF- α production to heat-labile, extracellular GBS products but not the whole bacterium, whereas the induction of TNF- α by both types of stimuli depends entirely upon MyD88 (Henneke et al. 2001). The heat-labile factor (named “GBS-F”) engages the TLR2/TLR6 heterodimer as the essential signaling molecule (Henneke et al. 2001). The requirement of MyD88 to sense the whole heat-inactivated bacterium suggested that GBS is recognized by TLRs (or alternatively by receptors of the IL-1R family) other than TLR2. By using macrophages from mutant mice carrying spontaneous mutations or targeted deletions of individual TLRs, all of the TLRs that have been implicated as sensors of microbial products from Gram-positive bacteria (TLR1, TLR2, TLR4, TLR6, TLR9) could be excluded as being solely responsible for the induction of TNF- α release by heat-killed GBS (Henneke et al. 2001, 2002). However, a recent analysis of gene expression profiles in wild-type and TLR2-deficient macrophages demonstrated that, while the upregulation of 76% of genes (including TNF- α) induced by GBS in macrophages does not depend on TLR2, the induction of a small fraction of genes is impaired in the absence of TLR2 (Draper et al. 2006). Among these genes is the cytokine IL-1 β that signals through MyD88. This observation raises the possibility that the abrogation of cytokine production in MyD88-deficient cells in response to heat-killed GBS is, at least partly, due to an interrupted feedback loop via IL-1 family receptors. In addition, the engagement of TLR7 and/or TLR8 by microbial RNA may possibly contribute to MyD88-dependent macrophage activation by GBS.

4.4 Immune Recognition of *L. monocytogenes* In Vitro

While the story of GBS-induced immune activation is far from clear, *L. monocytogenes* infection is evidently sensed by diverse cell surface and cytosolic PRRs. Listerial cell wall components such as LTA are recognized by TLR2 (with the help of CD14 and TLR6, Fig. 2) (Flo et al. 2000; Seki et al. 2002; Janot et al. 2008), whereas extracellular listerial flagellin signals through TLR5 (Hayashi et al. 2001). Other TLRs including TLR3, TLR4 and TLR9 have been reported as being dispensable for *Listeria*-induced immune activation (Janot et al. 2008). Once in the cell, live bacteria replicating in the cytosol activate a macrophage transcriptional response distinct from that of *Listeria* trapped in phagosomal vacuoles (Leber et al. 2008). The vacuolar response was found to be entirely MyD88-dependent and to control the induction of many proinflammatory cytokines, including IL-1 β and TNF- α (Leber et al. 2008). The cytosolic response was reported to induce a distinct and significantly nonoverlapping set of host response genes including Type 1 interferons, and to be under the coordinated control of at least two pathways. The first pathway consists of recognition of bacterial DNA by cytosolic PRR(s), possibly DNA-dependent activator of IFN regulatory factors (DAI) (Takaoka et al. 2007; Wang et al. 2008). The second pathway involves the detection of listerial PGN components, presumably by NOD1 and NOD2 (Park et al. 2007; Hasegawa et al. 2006). Further studies showed that cellular activation upon *L. monocytogenes* infection critically depends on NOD1 and NOD2 in macrophages pretreated with TLR ligands, but not in naïve macrophages (Kim et al. 2008), and that other receptors of the NLR family including NALP3 and IPAF detect cytosolic *Listeria* and subsequently activate caspase-1 (Mariathasan et al. 2006; Franchi et al. 2007; Warren et al. 2008). These data suggest that multiple NLRs are crucial for microbial recognition and host defense against *L. monocytogenes*, particularly when proinflammatory responses are compromised by tolerization induced by TLR stimulation.

Collectively, in vitro studies indicated that distinct yet overlapping sets of TLRs and other PRRs (namely the NLRs NOD1 and NOD2) are used to sense major meningeal pathogens including *S. pneumoniae*, *S. agalactiae*, *N. meningitidis*, and *L. monocytogenes*. However, in vivo studies using mice with targeted deletions of individual TLRs (and other PRRs) are needed to assess whether these results can be extrapolated to in vivo models of bacterial meningitis. So far, the in vivo relevance of TLRs for mounting an inflammatory response to meningeal infection has been demonstrated only for *S. pneumoniae*, as outlined in the next section.

5 Role of TLRs in Pneumococcal Meningitis

The first evidence for TLR involvement in pneumococcal meningitis was provided by Echchannaoui et al. (2002), who demonstrated that while intracerebral infection (into the left forebrain) with *S. pneumoniae* was fatal in both wild-type and

TLR2-deficient mice, the clinical course of meningitis was aggravated in mice lacking TLR2. In this pneumococcal infection model, the worsening of disease was accompanied by a higher bacterial load in the brain and a more pronounced increase in both cerebral TNF- α activity and blood–brain barrier disruption (Echchannaoui et al. 2002). The observations that TLR2 deficiency led to a moderate increase in disease severity, cerebral bacterial titers and blood–brain barrier compromise were confirmed by our experiments in which pneumococcal meningitis was induced by direct inoculation of the pathogen into the CSF compartment (Koedel et al. 2003). Supplemental analyses of the inflammatory host response in our meningitis model revealed that, in early meningitis (4 h after pneumococcal infection), brain expression of TNF- α and the CXC chemokine CXCL-2 (but not of IL-1 β , inducible nitric oxide synthase, and complement C3) was significantly lower in TLR2-deficient mice than in wild-type mice. However, in later stages of the disease, the brain cytokine and chemokine expression levels did not differ between the two mouse strains. Accordingly, CSF leukocyte counts were similar in wild-type and TLR2-deficient mice (Koedel et al. 2003). These observations hinted at higher pneumococcal toxin concentrations (e.g., pneumolysin and hydrogen peroxide) (Braun et al. 2002) as a major factor in deterioration to more clinical symptoms in TLR2-deficient mice. Moreover, the increased release of pneumococcal toxins due to the increased bacterial burden in TLR2-deficient mice was suggested to compensate for the anti-inflammatory properties exerted by TLR2 deficiency. Finally, since the immune milieu in the CSF compartment is different to that of the brain (as depicted above), the site of pneumococcal inoculation may account for the differences observed in brain TNF- α expression between our experiments and those by Echchannaoui et al. (2002). Altogether, these initial studies argued for an important role of TLR2 in host defense against pneumococcal infection in the CSF, but also for the involvement of further PRRs in mounting the host immune response in pneumococcal meningitis. In order to get a better insight into the role of TLR signaling in this disease, we then used gene-targeted mice lacking functional MyD88, which is used by all TLRs apart from TLR3. Compared to TLR2-deficient mice, the phenotype of MyD88-deficient mice in our meningitis model was much more impressive (Koedel et al. 2004). For example, while none of the wild-type mice died during the first 24 h after pneumococcal infection, the mortality rate of infected MyD88-deficient mice was approximately 45%. The more adverse outcome observed in MyD88-deficient mice was paralleled by a dramatically impaired host defense in both the CSF and the periphery, as evidenced by substantially higher bacterial loads in the brain, blood and lung. The higher susceptibility to intracisternal pneumococcal infection was due to a defective host immune response inside the CSF. Compared to infected wild-type mice, MyD88-deficient mice showed an 80% reduction in CSF leukocyte counts, which was associated with a near-complete abrogation of the brain expression of proinflammatory cytokines and chemokines (Fig. 3). Since the substantial attenuation of meningeal inflammation resulted in a marked reduction of meningitis-induced intracranial complication (such as blood–brain barrier compromise), it is most likely that the worsening of pneumococcal disease in MyD88-deficient mice is due to the severe bacteremia and

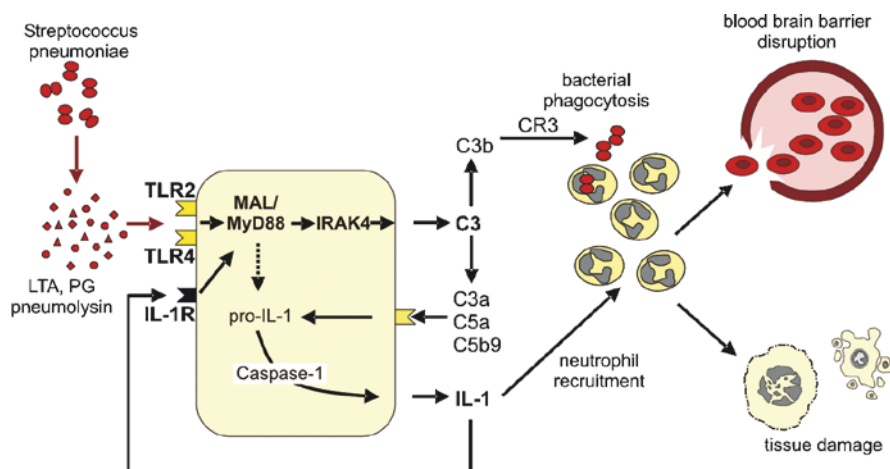


Fig. 3 Schematic diagram of the main pathogenic steps of pneumococcal meningitis. Inside the CSF compartment, the presence of *S. pneumoniae* seems to be recognized initially by TLR2 and TLR4 (as well as by as-yet unidentified pattern recognition receptors). TLR signaling via MyD88/Mal results in the induction of complement factor 3 (C3) expression, which is absent from normal CSF. After its activation/cleavage, C3 contributes to the killing of pneumococci, but also promotes the immune response, presumably via upregulation of the expression of IL-1 family cytokines, which are essential for the recruitment of neutrophils to the site of infection. An autocrine (and possibly paracrine) positive feedback loop via IL-1 and MyD88 contributes to the exaggeration of the host inflammatory reaction, which is a key mediator of meningitis-associated blood-brain barrier disruption and tissue injury

the aggravation of internal complications including sepsis and pneumonia (Koedel et al. 2004). These data strongly indicated a fundamental role of MyD88 in the immune reaction to pneumococcal infection of the CSF compartment. The fact that MyD88 is an adapter molecule common to signaling pathways of IL-1Rs and all TLRs activated *in vitro* by *S. pneumoniae* suggested that the phenotype of MyD88-deficient mice can be linked to defects in either the IL-1 pathway or TLR-mediated signaling, or in both. By utilizing mice with single or combined deficiencies of TLR2, TLR4, and TLR9, all three of which were implicated in the sensing of *S. pneumoniae* *in vitro*, we were able to demonstrate that the concerted action of both TLR2 and TLR4 plays a crucial role in host defense and immune activation in pneumococcal meningitis (Klein et al. 2008). TLR2-TLR4 double-deficient mice had a 50% reduction in CSF pleocytosis and a selective inhibition of cytokine production. Moreover, similar to the TLR2-deficient state, the single deficiency in TLR4 was found to have no significant impact on either bacterial outgrowth in the CSF or on meningeal inflammation. However, contrary to our expectations deduced from the *in vitro* findings, our *in vivo* analyses did not suggest TLR9 as an additional sensor for pneumococcal infection of the CSF, since additional TLR9 deficiency did not result in further attenuation of the inflammatory reaction observed in TLR2-TLR4 double-deficient mice (Klein et al. 2008). The observation that TLR2-TLR4

double-deficient mice were less severely impaired in their immune response than MyD88-deficient mice suggests that the MyD88 phenotype may be—at least partly—due to the blocking of secondary autocrine effects of IL-1 family cytokines. This concept is supported by recent studies in mice with deficiencies of individual genes of the IL-1/IL-18 pathway. Recently, we 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 in the CSF compartment (Koedel et al. 2002b).

Using a mouse model of hematogeneous meningitis, Zwijnenburg et al. (2003b,a) provided further evidence for the crucial role of both IL-1 and IL-18 pathways in the immune regulation in pneumococcal meningitis. In IL-1R-deficient mice, pneumococcal meningitis was associated with less severe leukocyte infiltration and with lower brain levels of cytokines and chemokines (Zwijnenburg et al. 2003b). IL-18-deficient mice were also reported to show a suppressed inflammatory response, as evidenced by a less profound inflammatory infiltrate as well as lower brain cytokine and chemokine levels (Zwijnenburg et al. 2003a). Thus, an interrupted autocrine feedback loop via IL-1 receptors is likely to contribute to the phenotype of MyD88-deficient mice. The fact that MyD88 deficiency resulted in strong but not complete inhibition of the host immune response furthermore indicated the presence of additional PRRs in the recognition of *S. pneumoniae*. Cell culture experiments indicated that internalized *S. pneumoniae* can be sensed by NOD2 (Opitz et al. 2004). However, mice lacking RIP2, which is crucial for NOD1- and NOD2-dependent immune activation (Park et al. 2007), did not show any alterations in the host immune response to *S. pneumoniae* in our meningitis model (unpublished data). This finding is strengthened by a recent study which monitored the trafficking of pneumococcal cell walls (PCW) in the host as well as its consequences on the host after intravascular injection (Fillon et al. 2006). Intravascular PCW was found to bind to endothelial cells and cause rapid lethality in both wild-type and NOD2-deficient mice to a similar degree. Since PCW was also observed to be internalized into host cells, these data argue against an important role of NOD2 in the sensing of pneumococcal fragments in vivo.

Collectively, experiments using mouse meningitis models supported the idea that TLRs are crucial for the detection of pathogens in the CSF compartment. Contrary to the in vitro observations, only TLR2 and TLR4, not TLR9, act as sensors of *S. pneumoniae* infection of the CSF. Engagement of these TLRs by pneumococcal ligands leads to MyD88-dependent production of proinflammatory cytokines of the IL-1 family. Secretion of IL-1 family cytokines forms a positive feedback loop that boosts MyD88-dependent production of inflammatory mediators. The impressive phenotype of MyD88-deficient mice illustrates the extraordinary importance of TLR-IL-1R-MyD88 signaling in regulating the host immune response to pneumococcal infection of the CSF compartment.

Based upon the experimental evidence described in this article, molecular genetic studies have been performed in humans to assess whether genes of the TLR pathway influence immunity to pneumococcal infection. A listing of all TLR-related gene polymorphisms and studies investigating associations with susceptibility to

pneumococcal diseases is beyond the scope of this chapter, but two examples are mentioned here. First, children with an inherited deficiency of the IL-1 receptor kinase (IRAK)-4, which is essential for NF- κ B activation in TLR- and IL-1R signaling pathways, are highly susceptible to severe (and often recurrent) invasive pneumococcal disease (Ku et al. 2007). Secondly, heterozygous carriage of a leucine substitution at serine 180 of Mal, another key molecule in the TLR/IL-1R signal transduction cascade, was reported to roughly halve the risk for invasive pneumococcal disease (Khor et al. 2007). The authors speculated that heterozygosity of Mal may confer protective immunity, characterized by an intermediate TLR/IL-1R activation state and a well-balanced inflammatory response, whereas homozygosity of Mal (or IRAK-4) may result in impaired host-defense mechanisms and overwhelming infection. Altogether, these studies suggest that TLR/IL-1R pathways are vital for immunity to *S. pneumoniae*.

6 Conclusions

Over the past 20 years it has become clear that intracranial complications (including brain edema formation and alterations in cerebral blood flow) are major determinants of an unfavorable outcome in bacterial meningitis, and are largely caused by the host immune response (Koedel et al. 2002a; Weber and Tuomanen 2007). Recent studies showed that direct injection of synthetic TLR2 or TLR9 agonists can induce inflammatory and associated neuropathological changes typically associated with bacterial meningitis (Deng et al. 2001; Hoffmann et al. 2007). Moreover, targeted disruption of both TLR2 and TLR4 led to a substantial reduction of meningitis-induced intracranial complications and tissue damage in a murine model of pneumococcal meningitis (Klein et al. 2008). The neuroprotective effects of TLR deficiency, together with the meningitis-inducing potency of TLR agonists, strongly suggest that targeted interference with TLR signaling is a promising strategy for dampening meningeal inflammation and thus improving the outcome of the disease. However, the road toward the ultimate goal of helping patients with meningitis by using TLR antagonists is still long. First of all, in vivo studies are absolutely warranted to clarify the mechanisms of immune activation in pathogen recognition in bacterial meningitis due to other pathogens besides *S. pneumoniae*. While in vitro data indicate a crucial role of TLRs in the detection of *N. meningitidis* infection, their contribution to immune activation upon infection with either *S. agalactiae* or *L. monocytogenes* in vivo is largely unclear and needs to be addressed in future studies using mutant mice with single or combined deficiencies in genes of the TLR signaling cascade. Moreover, based on studies performed using pneumococcal meningitis models, TLRs are likely to be key triggers of the immune response to the pathogen, but TLR-independent, IL-1R-dependent MyD88-signaling pathways seem to be crucial for the aggravation and, quite possibly, the perpetuation of meningeal inflammation. Therefore, it must be evaluated whether pharmacological interference with TLR signaling is still effective at

reducing inflammation and tissue injury once the inflammatory reaction has been initiated. In this context, it is of special interest to assess the efficacy of TLR antagonists (e.g., neutralizing antibodies) as adjuvant therapy (given either simultaneously with or at defined time points after the start of antibiotic therapy) in experimental bacterial meningitis. Finally, targeted disruptions of either MyD88 or TLR2/4 were found to result in the worsening of disease in a mouse model of pneumococcal meningitis. Disease deterioration was associated with severe bacteremia, presumably leading to an aggravation of internal complications such as septic shock and pneumonia. These data make it conceivable that the benefit to the brain of TLR antagonism may be overshadowed by the risk of impaired bacterial eradication and uncontrolled infection of the host. Therefore, studies evaluating whether adjuvant therapy resulting from TLR antagonists impairs the penetration and action of antibiotics in the CSF are strongly warranted. Deciphering the exact role of TLRs (and other PRRs) in bacterial eradication, immune activation and immune resolution in the CSF compartment is a challenge for future meningitis research.

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