



Review

Using Knockout Mice to Study Experimental Meningitis

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Abstract. Despite the use of antibiotics, the prognosis of bacterial meningitis is still poor due to central nervous system (CNS) complications, such as brain edema formation, cerebrovascular alterations, and intracranial hemorrhage. Experimental studies with animal models have given new insights into its pathophysiology during the acute phase of the disease. In recent years, genetically engineered mice have become a powerful tool in investigating the role of particular genes by targeted deletion and have also been applied in bacterial meningitis research. By using knockout mice, new knowledge of the roles of the different cytokines, proteases, and oxidants involved in the inflammatory cascade has emerged. In the future, temporal and cell type-specific control of gene expression will provide even more information on the impact of a particular gene on meningitis-induced brain damage.

Key words: bacterial meningitis; animal model; knockout mice; inflammation; CNS.

Introduction

Despite the use of antibiotics, mortality and morbidity from bacterial meningitis remain unacceptably high. In pneumococcal meningitis, the most common form of bacterial meningitis in adults, death occurs in 25–30% of all patients and neurological and neuropsychological sequelae are reported to affect up to 50% of survivors³⁵. Clinical and neuropathological studies have shown that the fatal outcome of the disease is mainly a result of complications secondary to bacterial meningitis. These complications include intracranial hypertension due to brain edema or hydrocephalus, arterial and venous cerebrovascular alterations (cerebral ischemia, venous thrombosis), intracranial hemorrhage, and systemic spread of bacteria leading to septic shock and multi-organ failure^{35, 47}.

Whereas the incidence of bacterial meningitis caused by *Haemophilus influenzae* type B, the predomi-

nant pathogen among infants in the 1980s, experienced a dramatic decline due to routine immunization^{84, 105}, clinicians are increasingly faced with the problem of antibiotic-resistant strains of *Streptococcus pneumoniae*. In the United States the proportion of *S. pneumoniae* strains resistant to penicillin increased from 21% in 1995 to 24% in 1998²³, and macrolide resistance almost doubled from 10.6% in 1991 to 20.4% in 1999²⁹ and it is predicted that more than 40% of all pneumococci will be double resistant by 2005⁶⁰. This imminent threat emphasises the need for adjunctive therapies to reduce the severity and frequency of complications due to bacterial meningitis.

The serious prognosis of bacterial meningitis strongly encouraged the development of animal models which mimic features of the human disease and which are suitable for the determination of the pathophysiology of bacterial meningitis and the evaluation of new treatment strategies⁴⁵. Many animal models meningitis

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have been applied using different species, pathogens, and sites of inoculation. Most of studies have been done with the adult rabbit model, as originally described by DACEY and SANDE⁹, and the adult rat model developed by QUAGLIARELLO et al.⁷⁵, where animals are infected by direct injection of the pathogen into the cerebrospinal fluid (CSF) via intracisternal puncture. These models can reproducibly cause lethal infections with a predictable outcome, bypassing the natural dissemination of bacteria from the intravascular compartment to the central nervous system (CNS). In other models, bacteria have been inoculated intranasally, subcutaneously, or intraperitoneally, mimicking the pathogenetic steps of bacterial meningitis in humans. Because adult animals will not reliably develop CNS inflammation by these kinds of challenge with live organisms, infant animals, mostly rats, must be used. Therefore, these models have been widely applied in studies investigating the pathogenic events in meningitis caused by *H. influenzae*^{62, 85} and *Escherichia coli*^{3, 37}, the most common pathogens of meningitis in neonatals and infants.

The use of relatively large animals like rats and rabbits has many advantages. Experimental and surgical procedures, e.g. puncture of the cisterna magna for pathogen inoculation, ventilation of the animals, and catheterization of vessels, are easily feasible. In addition, CSF and blood samples in amounts sufficient to perform multiple molecular tests, e.g. PCR, ELISA and Western blots, can be collected. Drawbacks of the use of rats and rabbits are the need for fairly large amounts of substances to be applied and the limited availability of biological reagents and genomic information for these species. Mice models of bacterial meningitis, previously used mainly for the study of the pathophysiology of *Listeria monocytogenes* meningoencephalitis^{57, 94}, overcome these restrictions and offer new possibilities for studying the role of particular proteins by targeted mutagenesis.

During the last two decades technology has been developed that allows the specific modification of the genes of many organisms, permitting the *in vivo* alteration of their expression or function. This technology provides a powerful method for investigating the physiological and pathophysiological relevance of proteins. Mice possess a distinct advantage over other species in that they are more closely related to humans from a physiological perspective. With some exceptions, where rats were used, most of the genetic studies have been done in mice, since it has not been possible to date to create pluripotent rat embryonic stem cell lines, which are needed to create specific mutants⁷.

Since the first publications addressing the correction of mutant genes by gene targeting in embryonic stem cells in the late 1980s^{50, 52, 93}, numerous genetically modified mice with distinct deletions have been created and studied. Mice deficient in a particular gene, so-called knockout (KO) mice, are generated by targeted insertion of DNA into embryonic stem cells by homologous recombination⁹⁹. Typically, a targeting vector is designed in a way that a positive selectable marker gene (e.g. neomycin resistance gene, *neo^r*) is flanked by large sequences of cloned genomic DNA homologous to the endogenous target gene. The homologous sequences enable insertion of the vector into the target gene by homologous recombination, while the *neo^r* gene disrupts the wild-type DNA sequence, replacing the original allele by a non-functional one ("null" allele, *gene^{-/-}*). Mating of heterozygous mice with one "null" allele will produce a strain of KO mice homozygous for the non-functional gene (*gene^{-/-}*). To date, hundreds of KO mice have been genetically engineered and numerous animals have been designed with specific mutations of genes related to the immune or CNS, offering new tools for research on bacterial meningitis^{6, 17, 73, 74}.

The pathophysiology of bacterial meningitis consists of a complex network of different interacting bioactive substances, such as cytokines, chemokines, oxidants, and proteases, that ultimately leads to cell injury, cerebrovascular alterations, and brain edema (Fig. 1). Genetic targeting of some of the pathways has offered new insights into the pathophysiological mechanisms underlying these detrimental complications (Table 1).

KO Mice in Experimental Meningitis

Immune activation in bacterial meningitis

Cell-wall components of bacteria are sufficient to induce inflammatory host response against bacteria. It was shown that heat-killed, unencapsulated pneumococci, their isolated cell walls, lipoteichoic acid, and peptidoglycan intracisternally injected activate the immune system in the CNS and cause an inflammation resembling the characteristics of naturally acquired meningitis⁹⁶. The first step in immune activation is mediated by the binding of bacterial cell membrane components to the pattern-recognition receptors of host cells³¹. Response to infections of both Gram-negative as well as Gram-positive bacteria is, at least in part, mediated by membrane CD14 (mCD14)¹⁵. Since mCD14 cannot transmit the activation signal into the

Table 1. Knockout mice investigated in models of bacterial meningitis

Target	Major results	References
Immune activation		
TLR2	disease severity [↑] , ICP [↑] , BBBP [↑] , bacterial titer ^{↑a} disease severity [↑] , survival rate [↓] , BBBP [↑] , bacterial clearance [↓]	39 16
Cytokines		
TNF- α	disease severity [↑] , survival rate [↓] , bacterial titers ^{↑b} , inflammation ^c	104
TNF receptors	inflammation [↓] , disease severity and neuronal damage ^c	104
IL-1R	survival rate [↓] , inflammation [↓] , bacterial titers [↑]	110
Caspase-1	disease severity [↓] , ICP [↓] , BBBP [↓] , IL-1 β [↓] , MIP-1 α [↓] , MIP-2 [↓] , iNOS [↓] , CSF-WBC [↓]	48
IL-6	ICP [↓] , BBBP [↓] , CSF-WBC [↑] , IL-1 β [↑] , TNF- α [↑] , MIP-2 [↑] , GM-CSF [↓]	69
M-CSF	disease severity, inflammation, and neuronal damage ^c	24
Adhesion molecules		
P-selectin	CSF-WBC [↓] , BBBP [↓]	92
P- and E-selectin	CSF-WBC ^{↓↓} BBBP ^{↓↓}	92
ICAM-1	disease severity, inflammation, and neuronal damage ^c survival rate ^{↓d}	24 91
Proteases		
MMP-9	bacterial titers ^b , inflammation and disease severity ^c	4
Oxidants related enzymes		
eNOS	ICP [↑] , BBBP [↑] , IL-1 β [↑] , KC [↑] , MIP-2 [↑] , P-selectin [↑] , CSF-WBC [↑]	43
iNOS	BBBP [↓] , IL-1 β [↓] , IL-6 [↓] , TNF- α [↓] , MIP-1 α [↓] , MIP-2 [↓] , CSF-WBC [↑]	106
PARP1	disease severity [↓] , ICP [↓] , BBBP [↓] , IL-1 β [↓] , TNF- α [↓] , IL-6 [↓] , CSF-WBC [↓]	49

ICP – intracranial pressure, BBBP – blood-brain barrier permeability, CSF-WBC – cerebrospinal fluid white blood cell count.

^a in blood and brain; ^b in blood and spleen not in brain; ^c no difference, from wild-type; ^d after infection with *S. pneumoniae*.

cell, a second receptor is needed to promote cellular response⁵⁵. Recently, it was shown that the Toll-like receptor 4 (TLR4) and TLR2 can recognize pathogen-associated molecular patterns such as peptidoglycans, lipoproteins, lipoteichoic acids and lipopolysaccharides in conjunction with CD14^{1, 61}. Accordingly, coexpression of CD14 and TLR2 in CHO fibroblasts resulted in an activation of the nuclear factor κ B (NF- κ B) signaling pathway in response to pneumococcal peptidoglycan and heat-killed *S. pneumoniae*, suggesting a role of TLR2 as a sensor and trigger of pneumococcal CNS infection¹⁰⁸. Here, the use of TLR2-deficient mice offered an elegant way to address this issue. Intracerebral as well as intracisternal injection of live *S. pneumoniae* induced an inflammatory response in TLR2^{-/-} mice, leading to an increased disease severity and aggravated intracranial complications (e.g. increased intracranial pressure) which were associated with higher brain and blood bacterial titers^{16, 39}. Expression of tumor necrosis factor α (TNF- α), interleukin 1 β (IL-1 β), macrophage inflammatory protein 2 (MIP-2), IL-6, inducible nitric oxide synthase (iNOS) and complement factor C3 showed no differences between infected wild-type and

TLR2-deficient mice at 24 h, indicating that mice can still sense CNS infection with *S. pneumoniae* in the absence of TLR2^{16, 39}. The message of these results is 2-fold: first, TLR2 is involved in the host immune response initiating bacterial killing after challenge with *S. pneumoniae* and second, other TLR2-independent, yet undefined cellular recognition pathways of *S. pneumoniae* must exist which induce host defense.

After the recognition of the pathogen and immune activation, the host reacts with an arsenal of bioactive molecules, such as cytokines, proteases, and oxidants, to attack and destroy the intruding organisms.

Cytokines in bacterial meningitis

Cytokines are important mediators of the inflammatory reaction to bacterial challenge¹⁴. TNF- α , IL-1 β , and IL-6 play a significant role in the early-response phase, triggering a cascade of inflammatory mediators, including other cytokines, chemokines, arachidonic acid metabolites, and reactive oxygen and nitrogen species^{47, 82}. All three cytokines can be produced by several cell types within the CNS, including endothelial

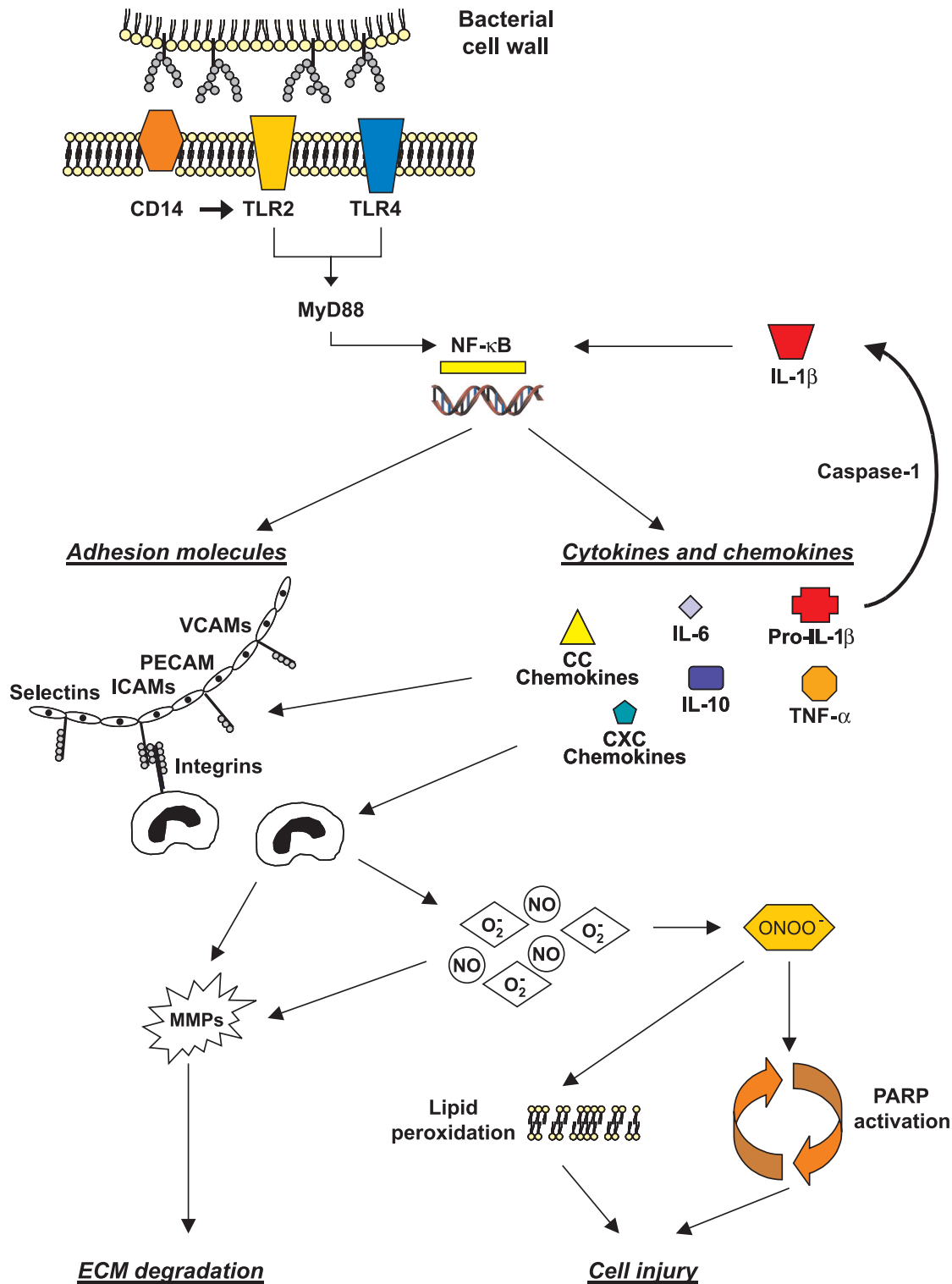


Fig. 1. Mechanisms of brain damage in experimental meningitis. Host immune response to pathogens is induced by interaction of bacterial cell-wall components with host Toll-like receptors (TLR), especially TLR2 and TLR4, together with CD14. Subsequently, the transcriptional activator NF-κB drives the expression of cytokines, chemokines, and adhesion molecules via the MyD88-regulated pathway. Leukocytes are attracted and activated by chemokines and cytokines, and migrate over the endothelium using adhesion molecules. Activated leukocytes release a complex assortment of potentially cytotoxic agent, such as oxidants (O_2^-), nitric oxide (NO), proteases, and cytokines. Pro-IL-1β is converted to its active form by caspase-1, which leads to an exaggerated activation of NF-κB, initiating an uncontrolled expression of proinflammatory mediators. The toxic reagent product of NO and O_2^- , peroxynitrite ($ONOO^-$), initiates lipid peroxidation and activates PARP, which ultimately results in cell injury and death. Proteases, especially matrix metalloproteinases (MMPs), degrade extracellular matrix (ECM), causing a disruption of the blood-brain barrier

cells, astrocytes, and microglia⁹⁰. Elevated CSF levels of these cytokines were found in patients with bacterial meningitis^{57, 102}, and the concentration of TNF- α was significantly correlated with the clinical outcome of the disease⁶⁶. In rodent models of bacterial meningitis, brain mRNA as well as protein expressions of TNF- α , IL-1 β , and IL-6 were upregulated in the acute stage of the inflammation^{43, 106}. Moreover, intracisternal injection of recombinant IL-1 β , alone or in combination with TNF- α , can induce meningitis in rats with influx of neutrophils into the CSF space and disruption of the blood-brain barrier resembling the features of bacterial-induced CNS inflammation⁷⁶. Studies with mice deficient in one of these cytokines or their receptors provided a deeper insight into their role during bacterial meningitis.

In a murine model of pneumococcal CNS inflammation, the lack of TNF- α was associated with worse clinical outcome and shorter survival time compared with wild-type mice, but meningeal inflammation was not distinguishable between the groups¹⁰⁴. However, bacterial titers were significantly higher in the blood and spleen of TNF- α ^{-/-} mice, indicating that the clearance of *S. pneumoniae* from the bloodstream was severely affected. By contrast, mice with targeted disruption of the two TNF receptors p55 and p75 showed decreased meningeal inflammation with reduced leukocyte infiltration¹⁰⁴. These results indicate that other TNF receptor ligands than TNF- α (e.g. lymphotoxin a) may contribute to the inflammatory reaction in pneumococcal meningitis.

IL-6 is a multifunctional cytokine with diverse actions, e.g. regulation of inflammation including the induction of the acute-phase reaction, immune response, and cellular differentiation^{20, 21}. In IL-6-deficient mice, intracisternal injection of pneumococci caused a 3-fold increase in CSF leukocyte counts compared with wild-type mice⁶⁹. This was associated with a higher expression of IL-1 β , TNF- α and MIP-2 in IL-6^{-/-} mice, indicating that in wild-type animals these proinflammatory substances are downregulated by IL-6. Surprisingly, the meningitis-induced increase in cerebral vascular permeability was attenuated in IL-6^{-/-} mice, leading to decreased intracranial pressure by a yet unknown mechanism. These results indicate that migration of leukocytes across cerebral vessels is not necessarily accompanied by the disruption of the blood-brain barrier.

The role of IL-1 in bacterial meningitis was studied in two KO models. First, IL-1 signaling was investigated using IL-1 receptor type I (IL-1R)-deficient mice in a model of pneumococcal meningitis induced by intranasal inoculation¹¹⁰. In this study, enhanced bacterial

growth in the CSF of IL-1R^{-/-} mice was found in the early phase of the disease, which was associated with a higher mortality rate in these mice compared with wild-type controls. Moreover, the infected KO mice showed decreased infiltration of leukocytes in the meninges compared with wild-type controls, though cerebrospinal fluid white blood cell counts did not differ between the groups. Therefore, the author concluded that IL-1 is essential for host defense during meningitis. In a second approach, IL-1 signaling after intracisternal inoculation of pneumococci was investigated in mice deficient in caspase-1. Caspase-1, also known as IL-1 β converting enzyme (ICE), is essential in generating biologically active IL-1 β . Expression of caspase-1 mRNA and protein is upregulated in the brain during experimental pneumococcal meningitis and is associated with increased levels of IL-1 β ^{48, 101}. In caspase-1^{-/-} mice, the increase in IL-1 β during meningitis expression was significantly attenuated⁴⁸. This was paralleled by a reduced inflammatory host response to pneumococcal challenge: NF- κ B activity as well as TNF- α , MIP-1 α , MIP-2 expression in the brain, and CSF leukocyte count were reduced. Pharmacological inhibition of caspase-1 affirmed these findings suggesting that caspase-1 blockage may provide an efficient adjuvant therapeutic strategy in this disease^{5, 48}. These results demonstrate a crucial role of caspase-1 in the IL-1 β -mediated signaling pathway by inducing and amplifying host inflammatory response during pneumococcal meningitis.

Macrophage colony-stimulating factor (M-CSF) is one of the most important activators of microglia. M-CSF is essential for the final step of maturation of microglia and not replaceable by other factors³⁰. Increased levels of M-CSF in the CSF of patients with bacterial meningitis could be found, suggesting a role of M-CSF in the pathophysiology of the disease²². However, in an animal model of *S. pneumoniae* meningitis, mice deficient in M-CSF did not show any differences in clinical score, meningeal inflammation or neuronal damage compared with their wild-type littermates. The lack of M-CSF totally blocks the activation of brain microglia, whereas most of the peripheral macrophages appear to be unaffected²⁴. Therefore, the authors of this study question whether recruited monocytes or other hematogenous cells, rather than resident microglia, might be involved in the disease progression of bacterial meningitis.

Many cytokines, especially IL-1 β and TNF- α , stimulate the expression of chemokines and adhesion molecules which facilitate the attraction and passage of leukocytes from the blood stream into the CSF space.

Adhesion molecules in bacterial meningitis

Leukocyte migration across the cerebral endothelium is a multistep process consisting of the sequential activation of adhesion receptors and their ligand on both leukocytes and endothelial cells^{51,63}. The adhesion cascade can be divided into the four steps of tethering, triggering, firm adhesion, and emigration. Tethering is mediated by the selectin family of adhesion molecules (P-selectin, E-selectin, L-selectin), each of which promotes leukocyte rolling under flow conditions^{26, 68}. P-selection was originally discovered on platelets, but is also found on endothelial cells. P-selectin binds to CD24 and the P-selectin glycoprotein ligand 1 on leukocytes, which is also a ligand for E-selectin, the second selectin expressed on endothelial cells. Firm adhesion of leukocytes on the endothelium is mediated by β_2 integrins, predominantly by CD11b/CD18 ($\alpha_M\beta_2$, Mac-1) and its endothelial counterpart, the intercellular adhesion molecule 1 (ICAM-1, CD54), a member of the IgG-superfamily⁵¹.

In an acute cytokine-induced meningitis model, partial inhibition of CSF leukocyte influx and blood-brain barrier leakage was noted in P-selectin-deficient mice. In P- and E-selectin double-KO mice, a nearly complete inhibition of these parameters was achieved, suggesting that both selectins cooperatively contribute to meningitis-induced intracranial complications⁹².

In contrast, mice deficient in ICAM-1 were not protected against the detrimental effects of CNS inflammation. In an infant mouse model of hematogenous bacterial meningitis, infection of ICAM-1^{-/-} mice with *S. pneumoniae* caused an increase in CSF leukocyte counts comparable with wild-type mice and mortality was even higher in the KO group⁹¹. Similar results were reported in a study using an adult mouse model of pneumococcal meningitis. Here, the intensity of inflammation, neuronal damage, and clinical course of bacterial meningitis in ICAM-1^{-/-} mice were not different from those seen in their wild-type littermates²⁴. This is in contrast to other studies which showed a beneficial effect of blocking leukocyte adhesion using antibodies directed against ICAM-1 or its counterpart, CD18^{97, 103}. There are several possible reasons for this discrepancy. First, different endpoints were chosen: in the study using the ICAM-1-deficient mice the duration of the experiments was between 24 and 32 h, whereas the blocking experiments with the anti-ICAM-1 antibody lasted only 6 h. Secondly, the positive results using antibodies directed against CD18 might be due to the fact that β_2 recognizes not only ICAM-1, but also other

members of the ICAM family⁸⁶. Thirdly, alternative adhesion mechanisms could compensate for the lack of ICAM-1 in deficient mice, supporting leukocyte migration and disease progression.

After migration across the blood-brain barrier, leukocytes release a complex assortment of potentially tissue-destructive agents, including reactive oxygen and nitrogen intermediates, such as nitric oxide (NO) and peroxynitrite, and proteolytic enzymes, e.g. matrix metalloproteinases (MMPs).

MMPs in bacterial meningitis

MMPs are a family of zinc-dependent endopeptidases which degrade components of extracellular matrix (ECM), including proteoglycans, glycoproteins, and various types of collagen¹⁰⁷. MMPs can be induced and activated by several inflammatory mediators, such as TNF- α , IL-1 β , IL-6, NO, peroxynitrite, and metabolites of the arachidonic acid pathway^{58,65,77}. Experimental studies showed that intracerebral injection of MMP-2 and MMP-9 can cause blood-brain barrier disruption^{64,78}. It has also been speculated that neutrophils use MMPs, in particular MMP-9, to digest ECM during transmigration from the bloodstream^{11,13}. Correspondingly, in a rat model of pneumococcal meningitis, application of the broad-spectrum MMP inhibitor BB-94 (batimastat) significantly reduced blood-brain barrier disruption and, to a lesser extent, CSF pleocytosis⁷⁰. Similarly, in an infant rat model of meningococcal meningitis, treatment with different MMP inhibitors significantly attenuated blood-brain barrier permeability and neuronal injury⁵⁶. In patients, MMP-9 and MMP-8 were upregulated in the CSF during bacterial meningitis^{36,56,70}. High MMP-9 concentrations were correlated with poor clinical outcome, suggesting a pivotal role of MMP-9 in bacterial meningitis. However, mice deficient in MMP-9 showed no reduction in disease severity, leukocyte recruitment, and brain bacterial titers after intracerebral injection of *S. pneumoniae*. Only spleen bacterial titers were significantly increased in MMP-9^{-/-} mice compared with wild-type mice, suggesting that MMP-9 deficiency impairs host defense⁴. These unexpected results may have several reasons. First, pharmacological inhibition targets not only MMP-9, but also other MMPs, e.g. MMP-3, MMP-8, and MMP-13, which might be involved in blood-brain barrier disruption. Secondly, the lack of MMP-9 could be compensated by other proteolytic enzymes which degrade components of the ECM.

Oxidants in bacterial meningitis

Besides proteolytic enzymes, reactive oxygen species (ROS, e.g. superoxide) and reactive nitrogen intermediates (RNI, e.g. NO) are important effector molecules, and animal studies have provided substantial evidence for a central role of ROS and RNI in the pathogenesis of bacterial meningitis^{33, 34, 40, 46}. ROS generation has been detected in models of bacterial meningitis and treatment with antioxidants showed beneficial effects on the course of the disease, attenuating (or even preventing) meningitis-induced intracranial complications^{44, 72}. Likewise, RNI have been demonstrated to be important mediators in the pathophysiology of bacterial meningitis^{34, 40}. In patients with bacterial meningitis, increased levels of nitrite/nitrate, as indicators for NO production, were detected in the CSF during the acute stage of the disease^{95, 100}. Similar results were obtained from animal studies of bacterial meningitis^{12, 32}. In contrast to ROS, where KO mice have not yet been applied to study the deficiency of particular genes during experimental meningitis, the lack of NO-producing enzymes has been extensively investigated.

NO is produced by three different NO synthases (NOS): two constitutive isoforms, which are present in endothelial cells (eNOS) and neuronal cells (nNOS), and one inducible isoform iNOS, which is expressed in many cells, e.g. neutrophils, macrophages, astrocytes and microglia, in response to stimulation with cytokines or bacterial cell-wall components^{28, 88}. The significance of pharmacological studies using NOS inhibitors is limited by three major problems: 1) NOS inhibitors are not completely selective for one isoform, 2) their bioavailability is questionable, and 3) they may exert additional pharmacological effects unrelated to the NOS system. Therefore, mice deficient in one distinct isoform offered a powerful tool to elucidate the role of eNOS and iNOS in experimental bacterial meningitis. In pneumococcal meningitis, iNOS^{-/-} mice showed significantly lower concentrations of inflammatory mediators (e.g., IL-1 β , TNF- α , and IL-6) compared with wild-type mice. This effect was paralleled by a reduction in blood-brain barrier disruption. However, iNOS deficiency increased CSF leukocyte count in response to pneumococcal challenge¹⁰⁶. This effect could not be elucidated yet, but there is evidence that NO is able to modulate the adhesiveness of leukocytes to the endothelium, possibly by downregulating surface expression of β_2 -integrins⁷⁹.

In contrast, lack of eNOS was associated with an aggravation of meningitis-induced pathological alter-

ations, including an increase in blood-brain barrier permeability and CSF leukocyte count. This effect seems to be due to an elevated expression of proinflammatory host factors such as IL-1 β , keratinocyte-derived cytokine, MIP-2, and P-selectin in the brains of eNOS^{-/-} mice. Furthermore, the increased inflammatory response was associated with a worsening of the disease and higher mortality rate⁴³.

Thus, NO generated by separate NOS isoforms may have different roles and opposing effects in pneumococcal meningitis. While NO formed by iNOS seems to contribute to some of the detrimental pathophysiological alterations, eNOS-derived NO apparently plays a protective role in bacterial meningitis.

Simultaneous production of oxygen-centred and nitrogen-centred free radicals can result in the formation of a toxic reaction product, the oxidant peroxynitrite². Besides the initiation of lipid peroxidation⁶⁷, which can ultimately lead to loss of cellular membrane function and integrity, peroxynitrite can also cause DNA strand breakage and subsequent activation of poly(ADP ribose) polymerase 1 (PARP1)^{2, 89}. PARP1 is a nuclear enzyme involved in the detection and signaling of DNA strand breaks and is proposed to be critical for cellular processes such as DNA repair and transcription. Extensive PARP1 activation can initiate an energy-depleting intracellular cycle leading to cell dysfunction and death. Poly(ADP ribosyl)ated protein, as a marker for PARP catalytic activity was increased in brains of mice and rats challenged intracisternally with *S. pneumoniae* after 24 h⁴⁹. Mice with targeted disruption of the *PARP1* gene were protected against the development of meningitis-induced intracranial complications, such as blood-brain barrier leakage and CSF pleocytosis. Moreover, infected PARP1^{-/-} mice had significantly reduced concentrations of IL-1 β , TNF- α , and IL-6 in the brain compared with infected wild-type mice⁴⁹. Interestingly, PARP activation during bacterial meningitis was not associated with DNA strand breaks in the brain parenchyma, as assessed by TUNEL staining. Therefore, mechanisms other than DNA damage may be involved in PARP activation in bacterial meningitis resulting in the disruption of the blood-brain barrier.

Limits of Genetically Engineered Mouse Models

As shown in this review, targeted deletion of specific genes offers a powerful tool in investigating their role in the pathophysiology of bacterial meningitis. However, this method is also associated with several limits and drawbacks. First, some gene deletions result

in a lethal phenotype, either already during embryogenesis or shortly after birth. For example, endothelins (ET) and their receptors, which are involved in the pathophysiology of bacterial meningitis^{41, 42}, cannot be investigated, since ET-1- and ET_A receptor-deficient mice die shortly after birth due to respiratory failure and ET_B receptor KO mice become progressively ill with megacolon within several days after weaning^{8, 27, 54}. Likewise, the role of transforming growth factor β 1 (TGF- β 1) and TGF- β 2 in bacterial meningitis can only be studied incompletely. It was shown that TGF- β acts as an anti-inflammatory cytokine, since treatment with TGF- β 1 or TGF- β 2 attenuated cerebrovascular changes and brain edema formation in experimental pneumococcal meningitis⁷¹. However, TGF- β 1 or TGF- β 2 deficiency cannot be studied, since TGF- β 1^{-/-} mice succumb to a wasting syndrome leading to organ failure and death, and TGF- β 2^{-/-} mice exhibit perinatal mortality and a wide range of developmental defects^{80, 83}.

Furthermore, results obtained from experiments using mutant mice should be treated with caution, since the lack of one gene is often compensated by altered expression of other genes which might hamper the significance of the results. For example, it was shown that IL-6^{-/-} mice have profound differences in the basal mRNA content of GM-CSF and MIP-2 compared with uninfected wild-type mice⁶⁹. In this study the phenotype of the KO mice was affirmed by the systemic treatment of rats with anti-IL-6 antibodies, which yielded similar results. However, in other studies (e.g. investigating adhesion molecules) contradictory findings were obtained using KO mice and antibody inhibition. Among other things (see above), this might be due to compensatory gene expression in ICAM-1-deficient mice replacing the disrupted adhesion mechanism.

Moreover, the expression of a phenotype in mice carrying a mutation may depend on a number of factors not directly associated with the disrupted gene. One factor is the genetic background, defined as the sum of all genes present in an organism that influences a trait. The genetic background, depends on so-called genetic modifiers (allelic variants at loci other than the one being genetically modified) in the inbred strain genome, which can alter gene copy number, gene expression, mRNA stability, or DNA methylation⁵⁹. Targeted mutations are commonly generated in hybrid mice (C57BL/6 $\times$ 129), resulting in a mixed background. Further inbreeding or backcrossing to other inbred strains may result in a phenotype significantly different from that reported for a mixed genetic background. Therefore, the use of appropriate controls is essential for interpreting the results obtained in a specific mutant.

Future Perspectives

Although the use of genetically engineered animals is afflicted with some limitations, KO mice nonetheless open new avenues to the further understanding of the pathophysiology of bacterial meningitis, and some of the drawbacks will be overcome by the development of new genetical techniques which have emerged recently. Strategies exploiting inducible DNA recombination have been incorporated into gene-targeting procedures to allow temporal, spatial, and cell-specific ablation of gene expression *in vivo*⁹⁸. A large number of site-specific DNA recombinases have been described in different organisms, but due to its relative simplicity and efficiency, the Cre recombinase became the most popular in transgenic mice⁸¹. Cre is a gene of the bacteriophage P1 and recognizes a 34 bp site on the P1 genome called *loxP* and catalyzes DNA recombination between pairs of *loxP* sites. Cre-mediated recombination between two *loxP* sites results in excision of the DNA in between. Therefore, placement of two *loxP* sites between the gene sequence of interest (flanked by *loxP* sites or "floxed") allows the *in vivo* deletion of genes in the presence of Cre. Expression of Cre can be regulated by linking Cre to inducible or cell type-specific promoters. By crossing mice, which express Cre under the control of a specific promoter with floxed mice, temporal or cell type-specific knockouts can be produced. Some cell-specific promoters are of special interest for bacterial meningitis research: driving Cre expression under the control of the lysozyme M promoter (Lys)¹⁸ will give rise to mice deficient in a gene solely in granulocytes and macrophages, whereas crossing floxed mice with mice expressing Cre under control of the *tie2*³⁸ or *GFAP* promoter¹⁰⁹ will KO genes in endothelial cells and astrocytes, respectively.

Nonetheless, even cell-specific ablation of gene expression can be associated with a lethal phenotype if the gene is indispensable for development during embryogenesis in this cell type²⁵. Therefore, a temporal regulation of Cre is needed to arbitrarily control gene expression postnatally. This can be achieved by linking Cre to inducible promoters⁵³. A number of different inducers has been applied, such as interferon⁵³ and tamoxifen¹⁰. However, both inducers are prone to potential side effects which can complicate analyses of gene function. An alternative approach which is becoming more and more popular is the tetracycline-mediated regulation of gene expression^{19, 87}. The Tet-Off and Tet-On systems are binary transgenic systems in which expression of a target gene is dependent on the activity of an inducible transcriptional activator. The transcrip-

tional activator can be regulated both reversibly and quantitatively by exposing the transgenic mice to varying concentrations of tetracycline or its derivate doxycycline. In the Tet-Off expression system, a tetracycline-controlled transactivator protein (*tTA*) regulates expression of a target gene, which is under control of a tetracycline-responsive promotor element (TRE). In the absence of tetracycline (or doxycycline), *tTA* binds to TRE and activates transcription. In the presence of tetracycline, *tTA* cannot bind to TRE and the expression of the target gene is stopped. The Tet-On system is based on a reverse tetracycline-controlled transactivator (*rtTA*). Thus, transcription of the TRE-regulated target gene is stimulated only in the presence of tetracycline. This technique allows the arbitrary turning off and on of genes. Combination with cell-specific promoters which drive *tTA* or *rtTA* expression will result in a cell-specific, quantitatively and temporally controlled expression of the TRE-regulated target gene⁹⁸.

Conclusions

During the last two decades, classical pharmacological approaches using receptor antagonists or enzyme inhibitors have facilitated the investigation of numerous inflammatory factors involved in the pathophysiology of bacterial meningitis. The availability of genetically engineered mice now opens new avenues to further understanding the complex network and interactions of the immune system. In the future, more and more sophisticated techniques will provide precious information on the impact of a particular gene on meningitis-induced brain damage. The research will be focused on three main aspects of the pathophysiology of bacterial meningitis: a) how are pathogens recognized and how is the immune system activated, b) what are mechanisms of the blood-brain barrier disruption and c) how is the inflammatory response regulated? However, a full extrapolation of experimental data from genetically modified mice to humans has to be made with caution and the results derived from these animals have to be validated by pharmaceutical interference to establish new adjuvant therapeutic strategies.

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