Translational studies in pneumococcal meningitis

Mook, B.B.

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PROMOTIECOMMISSIE

Promotor:	Prof. dr. D. van de Beek	Universiteit van Amsterdam
Co-promotor:	Dr. M.C. Brouwer	Universiteit van Amsterdam
Overige leden:	Prof. dr. U. Koedel	Ludwig-Maximilians-Universität München
	Prof. dr. S.L. Leib	University of Bern
	Prof. dr. J.C.M. Meijers	Universiteit van Amsterdam
	Prof. dr. T. van der Poll	Universiteit van Amsterdam
	Prof. dr. I.N. van Schaik	Universiteit van Amsterdam
	Prof. dr. M. Vermeulen	Universiteit van Amsterdam

Faculteit der Geneeskunde

CONTENT

CHAPTER 1

GENERAL INTRODUCTION AND OUTLINE OF THE THESIS

What is bacterial meningitis?
There are few spaces in the human body where bacteria are able to grow in the relative absence of the immune system and in the luxurious presence of sugars and nutrients. The subarachnoid space, filled with cerebral spinal fluid, represents such an unusual "blind spot". Once bacteria invade the cerebral spinal fluid (CSF), and they may do so in various ways, the massive unchecked bacterial multiplication is followed by an equally massive recruitment of white blood cells. The resulting infection and inflammation of the CSF rapidly expands to involve the membranes enveloping the brain and spinal chord resulting in what is known as bacterial meningitis.

As the disease progresses, inflammation can lead to swelling of the brain, hydrocephalus due to impaired CSF resorption, cerebral infarcts and rarely hemorrhages, and seizures caused by the cortical inflammation. In this context, patients invariably died in the period before the introduction of antimicrobial therapy(1). Even now in the 21st century, with antibiotic treatments, the supportive care of the intensive care units, and a greater understanding of the underlying disease pathophysiology, mortality remains high. And of those patients who do survive, almost half suffer from long lasting neurological sequelae, often with great impact on daily activities(2).

Why look at meningitis?
The numbers at the beginning of the 21st century remain compelling: the incidence of bacterial meningitis worldwide is estimated to be 1.2 million cases every year, with *Streptococcus pneumoniae* and *Neisseria meningitidis* accounting for 85% of cases in adults in high income countries, resulting in death in 26% and 10% respectively (2, 3). Of those patients who survive, roughly half suffer long-term neurological sequelae, including hearing loss, focal neurological deficits and cognitive impairment. Clearly there is room for improvement.

Where and how must we look?
Prevention: In many parts of the world, the first step of improvement has consisted/and will continue to consist of effective vaccination strategies. In fact, following the immunization of infants with *Haemophilis influenzae* type b vaccines, the cases in the United States have been reduced by 55% and the incidence of *H. influenzae* meningitis has been nearly eliminated. Pneumococcal vaccines have reduced disease attributable to vaccine serotypes by nearly 90%, though all-age pneumococcal meningitis has decreased by around 25%. Lastly, serotype C meningococcal meningitis has nearly been eliminated following vaccination in high-income countries(4).

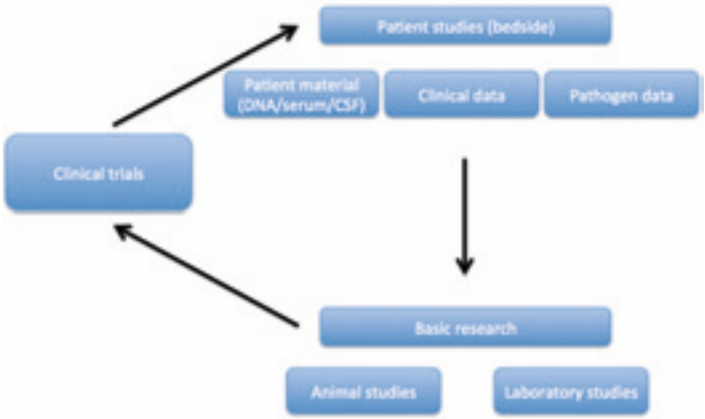
Treatment: Even in an optimized clinical setting, if the treating physician recognizes the urgency of a patient presenting with bacterial meningitis, and appropriate treatment is started immediately, mortality and morbidity remain unacceptably high. Effectiveness of antibiotic treatment has been threatened by the global emergence of multi-resistant pathogens. Future research will have to focus on the development of new antibiotics and adjuvant therapies (such as dexamethasone, glycerol, hypothermia). Tailoring the treatment of an individual patient according to microbial serotype, antimicrobial resistance, underlying bacterial and host genetic profiles will likely play a part in the future.

Translational approach

Bacterial meningitis is a complex disease. Pathophysiologically, the course of the disease is determined by a multitude of factors, such as host genetics and predisposing factors, bacterial genetics, host-pathogen interactions, the inflammatory response and reaction to antimicrobial and adjuvant therapy. Clinically, the recognition of bacterial meningitis can be complicated by an atypical presentation, or atypical CSF examination findings, which may vary with a patient's age, immune status or previous treatment.

To gain insight into the entire spectrum of such disease processes, so-called *translational research* has become the approach of choice. For bacterial meningitis this calls for population based epidemiological studies, genome wide association studies, experimental animal studies, and pathogen characterization/genetic studies, just to name a few. The breakthroughs of the next decades will most likely lie in the integration of these studies.

At the neurology department of the Academic Medical Center in Amsterdam, we have chosen a *translational* approach for our research of bacterial meningitis. The development of a reliable, reproducible animal model forms an integral part of the program. Using animal studies, we hope to gain insight into the pathophysiological processes of bacterial meningitis, and perform preliminary experiments on potential novel (adjuvant) therapies. The research in this thesis provides the foundation of the experimental animal studies that continue to be performed presently in our group.



Background and outline of this thesis

In 2007, the need for the development of an animal model of pneumococcal meningitis resulted in a collaborative effort with the Mayo Clinic, in Rochester Minnesota, USA. Though several animal models had previously been developed, using a variety of techniques, problems of reproducibility, limited disease progression or iatrogenic structural damage, combined with a need for a single model in which most pathological features seen in human pneumococcal meningitis can be measured, fueled our need for a new animal model.

The objective of the research in this thesis is to establish and illustrate the utility of a murine model of pneumococcal meningitis with relevant outcome measures that bridge the gap between animal and human studies, as well as research into the various bacterial strains and potential new (adjuvant) treatment options. **Chapter 2** provides a comprehensive review of the current understandings of the pathophysiological mechanisms involved in pneumococcal meningitis. In **Chapter 3** we describe the development of a mouse model of pneumococcal meningitis as it has been used in subsequent experiments. In **Chapter 4**, the murine model is used in a treatment study in which the effect of daptomycin (a lipopeptide non-lytic antibiotic) is compared to vancomycin in the treatment of pneumococcal meningitis. In **Chapters 5 and 6** we examine the role of inflammasomes (an intracellular multi-protein signaling complexes) in pneumococcal meningitis both in the mouse model as well as a prospective nationwide cohort of patients with pneumococcal meningitis. Using a similar approach **Chapter 7** describes the effects of TAFI (thrombin activatable fibrinolysis inhibitor) on coagulation and inflammation in pneumococcal meningitis. The patterns and differences in complement activation in patients with pneumococcal and meningococcal meningitis are examined in **Chapters 8.** In **Chapter 9**, I summarize the results, place this thesis in context of current study efforts and discuss possible future research directions.

CHAPTERS:

Chapter 1: Introduction

Chapter 2: Pathogenesis and pathophysiology of pneumococcal meningitis

Chapter 3: Characterization of a pneumococcal meningitis mouse model

Chapter 4: Daptomycin in experimental murine pneumococcal meningitis

Chapter 5: Genetic variation in inflammasome genes is associated with outcome in bacterial meningitis

Chapter 6: Inflammasome activation mediates inflammation and outcome in humans and mice with pneumococcal meningitis

Chapter 7: Thrombin-activatable fibrinolysis inhibitor influences disease severity in humans and mice with pneumococcal meningitis

Chapter 8: Cerebrospinal fluid complement activation in patients with pneumococcal and meningococcal meningitis

Chapter 9: Summary and general discussion

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CHAPTER 2

PATHOGENESIS AND PATHOPHYSIOLOGY OF PNEUMOCOCCAL MENINGITIS

Mook-Kanamori BB*, Geldhoff M*, van der Poll T, van de Beek D.

*contributed equally in the writing of this paper.

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INTRODUCTION

Community-acquired bacterial meningitis continues to exact a heavy toll, even in developed countries, despite the implementation of childhood vaccination programs and effective antimicrobial agents (71, 497). The most common etiologic agents are *Streptococcus pneumoniae* and *Neisseria meningitidis*, with the first being responsible for two-thirds of cases in Europe and the United States (18, 70, 496). Today, despite advances in medical care, mortality from pneumococcal meningitis ranges from 16 to 37%, and neurological sequelae, including hearing loss, focal neurological deficits, and cognitive impairment, are estimated to occur in 30 to 52% of surviving patients (231, 496, 500, 526, 528).

During past decades, experimental animal models have shown that the outcome of bacterial meningitis is related to the severity of inflammation in the subarachnoid space and that the outcome can be improved by modulation of the inflammatory response, e.g., with dexamethasone (471). Many randomized clinical trials of dexamethasone in bacterial meningitis have been performed, but the results remain ambiguous (70, 115, 148, 324, 442, 494). An individual patient data meta-analysis of 5 large recent trials showed no effect of dexamethasone (499). However, a prospective cohort study showed a decrease in mortality from 30 to 20% in adults with pneumococcal meningitis after successful nationwide implementation of dexamethasone in The Netherlands (69). Nevertheless, new adjunctive therapies are needed to improve the prognosis of bacterial meningitis.

Previously, we reviewed the epidemiology, diagnosis, and antimicrobial treatment of acute bacterial meningitis (70). In the current review, we focus on current understandings of the pathophysiology and pathogenic mechanisms associated with pneumococcal meningitis. Finally, we discuss targets for future therapeutic strategies.

COLONIZATION

Mucosal Colonization

The human nasopharynx is the main reservoir for *S. pneumoniae*, where it usually leads to asymptomatic colonization. Carriage rates of *S. pneumoniae* are highest among young children (37%) and may rise to up to 58% in crowded situations such as day care centers (50). In adults, crowding may also lead to increased carriage rates, specifically in hospitals, long-term care facilities, shelters, and prisons, where carriage rates of up to 40% have been reported (199, 208), compared to 4% in the general adult population (410). The bacterium is transferred between people mainly by coughing and sneezing. During colonization, adherence, nutrition, and replication are the pneumococcus' main priorities. To reach these objectives, the pneumococcus is confronted with the host's natural barriers at the respiratory mucosa, the host's immune system, and other pathogens colonizing the same niche.

Natural Barrier Evasion

Two important natural barriers preventing pneumococci from binding to the respiratory mucosal surface are the respiratory mucus and lysozyme (98, 350, 449).

The pneumococcus has evolved several strategies to overcome these barriers and reach the respiratory epithelial cell layer.

Mucus entrapment and subsequent clearing may be prevented by the pneumococcus by three ways. First, the capsule of the pneumococcus repulses the sialic acid residues of mucus by its negative charge, thereby decreasing the likelihood of entrapment (350). Second, the pneumococcus expresses several exoglycosidases, including neuraminidase A (NanA), betagalactosidase A (BgaA), beta-*N*-acetylglucosaminidase (StrH), and neuraminidase B (NanB), which are capable of deglycosylating mucus glycoconjugates, thereby decreasing mucus viscosity and preventing mucus entrapment (79, 240, 480). Third, pneumolysin (Ply), a pore-forming toxin, decreases epithelial cell ciliary beating, thereby enabling the pneumococcus to bind to epithelial cells without being removed with the mucus (Fig. 1A) (144, 145).

Lysozyme is a muramidase, which cleaves peptidoglycan, a polymer of sugars and amino acids present in the cell wall of many pathogens, including *S. pneumoniae* (112). Acetylated peptidoglycan molecules of the pneumococcal cell wall (PCW) are specifically prone to lysozyme destruction. The pneumococcus expresses two enzymes, peptidoglycan *N*-acetylglucosamine-deacetylase A (PdgA) and an *O*-acetyltransferase (Adr), which are able to deacetylate peptidoglycan molecules on the pneumococcal surface, rendering the bacterium resistant to lysozyme (Fig. 1B) (101, 112, 515). Both enzymes have been shown to be important during colonization, as PdgA or Adr knockout pneumococci are more prone to exogenous lysozyme and are outcompeted by wild-type (WT) pneumococci in an intranasal model of pneumococcal colonization (112).

Host Mucosal Immune System

At the nasopharyngeal mucosal site, the pneumococcus is targeted by components of the host innate immune system, such as secretory IgA (sIgA), (212), lactoferrin (447), and components of the complement system (51, 390).

sIgA interferes with binding of the pneumococcus to the nasopharyngeal mucosa (223, 274) and facilitates opsonization of bacteria, which enables phagocytosis by antigen-presenting cells (APCs) and neutrophils (212). Pneumococci have several methods to limit opsonization by sIgA. First, the capsule itself prevents binding of sIgA (141). Second, capsule-bound IgA is cleaved by a pneumococcal IgA1 protease. This protease cleaves sIgA at the hinge region, inhibiting IgA-mediated opsonization and promoting binding to the respiratory mucosa (429, 523). The remaining Fab fragment of sIgA binds to the PCW, thereby exposing choline-binding proteins (Cbps) and decreasing the negative charge of the capsule, which also facilitates bacterial adhesion to the epithelial cell (Fig. 1B) (523).

Lactoferrin is an iron scavenger present in multiple human body fluids, including saliva and nasal secretions (405). Lactoferrin acts bacteriostatically by depleting iron necessary for bacterial metabolism. Unbound lactoferrin (apolactoferrin) also has direct bactericidal properties, independent of iron scavenging, toward various pathogens, including *S. pneumoniae* (20, 21, 447). The mechanism by which apolactoferrin destroys bacteria is not completely clear, but it appears to disrupt the bacterial cell, leading to cell lysis (446). Lactoferrin is also present in neutrophils and may enhance bacterial phagocytosis and killing (140). The pneumococcus prevents

apolactoferrin-mediated killing by the expression of pneumococcal surface protein A (PspA), a choline-binding protein expressed on the outer surface of the pneumococcal cell. PspA binds human apolactoferrin at its active site, thereby inhibiting apolactoferrin-mediated bacterial killing (447).

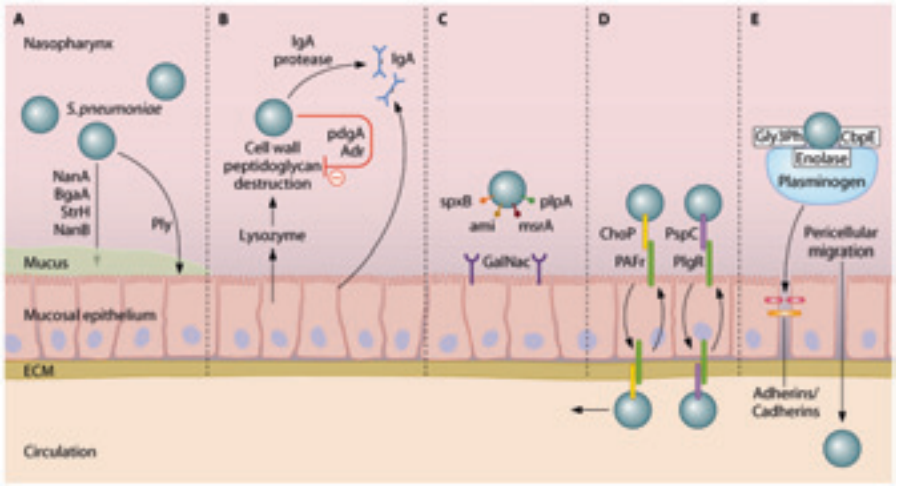

FIGURE 1. (A) Mucus breakdown. S. pneumoniae colonization of the nasopharynx is facilitated by mucus degradation by the enzymes NanA, BgaA, StrH, and NanB. Ply decreases epithelial cell ciliary beating, enhancing bacterial adherence. (B) Evasion of proteolytic enzymes. Pneumococcal cell wall peptidoglycans may be destroyed by lysozyme. PdgA and Adr deacetylate pneumococcal cell surface petidoglycan molecules, rendering them resistant to lysozyme. (C) Epithelial cell binding. S. pneumoniae binds host GalNac by using SpxB, Smi, MsrA, and PlpA. (D) Intracellular translocation. By binding the pIgR with PspC (or PAF receptor [PAFr] with ChoP), pneumococci can use the pIgR or PAF receptor recycling pathway to be transported through the epithelial cell layer. (E) Inter- and pericellular translocation. Plasminogen bound by Gly3Ph, CbpE, and enolase enhances epithelial cell binding and degrades interepithelial adherens junctions, allowing pericellular migration.

A third, important component of the mucosal innate immune system is the complement cascade. Activation of the complement pathway results in cleavage of several complement factors, leading to bacterial opsonization and phagocytosis, leukocyte recruitment, and the assembly of a membrane attack complex (MAC) which forms pores in the pathogen's membrane, inducing cell lysis (211). Complement plays an important role in the immune response against *S. pneumoniae*, since mice as well as humans with complement deficiencies are more susceptible to the transition of pneumococcal colonization to invasive disease (51, 390, 488).

C-reactive protein (CRP) serves as an important innate immune defense mechanism of the respiratory tract (174). CRP is a protein produced by the liver in the acute phase of an infection (211). CRP binds to phosphorylcholine on apoptotic cells (238) and several bacteria, including the pneumococcus (534). Through binding on the bacterial cell surface, CRP can activate the classical complement pathway through complement factor 1q (C1q) (465). Subsequent opsonophagocytosis by the complement system leads to more effective phagocytosis by macrophages. In addition, CRP can bind the Fcγ receptor (FcγR) on macrophages and dendritic cells, thereby enhancing phagocytosis (339, 475) and macrophage cytokine production (334).

The complement cascade is activated in three ways: the classical complement pathway, the alternative complement pathway, and the lectin-induced complement pathway. The classical complement pathway is characteristically activated by

antibody-antigen complexes. Natural IgM, a part of which is directed against pneumococcal C polysaccharides (teichoic acid), contributes to the activation of the classical pathway (335). However, the classical pathway may also be activated through other mechanisms, such as by the binding of acute-phase proteins such as CRP to the pneumococcal surface and subsequent binding of complement component C1q, direct binding of C1q to the bacterium (211), and binding of C1q to the C-type lectin SIGN-R1 (224). When C1q was depleted from human serum, *in vitro* opsonophagocytosis of *S. pneumoniae* was severely affected (544). In addition, C1q-deficient mice showed a severely impaired immune response and worse outcomes in an experimental model of pneumococcal meningitis (428). Furthermore, mice deficient in the pattern recognition receptor SIGN-R1 had reduced activation of the classical complement pathway (224). In this study, C1q was directly activated upon activation of SIGN-R1 by pneumococcal polysaccharides in the spleen, leading to activation of the classical complement cascade and complement component C3 activation, with subsequent pneumococcal opsonization (224). SIGN-R1 is highly abundant on cells of the splenic red pulp and is an important factor in the spleen's function to control invasive pneumococcal disease. Another study showed that splenic macrophages of SIGN-R1 knockout mice were unable to activate splenic B cells to produce pneumococcus-specific IgM (259). Therefore, splenic SIGN-R1-mediated activation of B cells may explain, at least partially, the susceptibility of splenectomized patients to invasive pneumococcal disease.

Activation of C1q by the classical or mannose-binding lectin (MBL) pathway leads to cleavage of complement component C2. In a Swedish cohort, 40 patients with a homozygous C2 deficiency due to a deletion in the C2 gene were described (218). Invasive infections, mainly pneumococcal infections, were found in 23 (58%) of these patients (218).

The alternative pathway is also activated during infection with *S. pneumoniae* and occurs by the direct binding of complement component C3 to the pneumococcal surface (533). The importance of the alternative pathway in pneumococcal opsonization was shown in mice made deficient in factor D, a peptidase involved in activation of the alternative pathway (539). Opsonophagocytosis of *S. pneumoniae* was delayed in factor D-deficient mice compared to wild-type mice, indicating an important role for this complement pathway in the early phase of infection (539). In line with this, a recent study showed that mice deficient in complement factor B, another peptidase involved in activation of the alternative complement pathway, were more susceptible to pneumococcal otitis media (481).

The lectin-induced complement pathway appears to be less important in pneumococcal disease than the classical and alternative pathways. Polymorphisms in MBL, one of the most important activators of the lectin complement pathway, were not associated with increased risk of pneumococcal invasive disease in a genetic association study (331). A larger cohort showed a significant increase in risk for pneumococcal invasive disease, with three codon variants in the MBL locus (426). In a third study, 140 patients with invasive pneumococcal disease, defined by positive blood culture for *S. pneumoniae*, were assessed for three structural variant MBL alleles and one promoter allele (269). In this study, no association was found between susceptibility or outcome of invasive pneumococcal disease and any of the structural MBL variants or promoter alleles. In a subgroup analysis of the 22 patients in the

cohort with pneumococcal invasive disease and meningitis, there was no association between susceptibility or outcome and the MBL genotype (269). However, a meta-analysis combining the results of the above three studies demonstrated an association between susceptibility to invasive pneumococcal disease and homozygosity for one of the three structural variants in the MBL gene, with an odds ratio (OR) of 2.57 (95% confidence interval [CI], 1.38 to 4.80) (68). In a cohort of 57 HIV-positive patients, an increased risk for invasive pneumococcal disease was found to be associated neither with MBL polymorphisms nor with polymorphisms in the downstream molecule MBL-associated serine protease 2 (MASP-2) (203). One genetic association study has been performed regarding outcome and MBL genotypes. This study included only 60 patients with community-acquired pneumococcal pneumonia and did not detect an association between MBL genotype and outcome (138). Experimental studies showed weak to no binding of MBL to *S. pneumoniae* compared to other bacteria (267, 352). Another experimental study showed that although MBL bound to *S. pneumoniae*, it did not increase opsonophagocytosis, and that complement activation by the classical pathway was much more important (73).

Another group of proteins that can activate the lectin-induced complement pathway are ficolins. Two ficolin variants, H-ficolin and L-ficolin, have been studied for the capability of binding to *S. pneumoniae*; only L-ficolin was found to bind some of the pneumococcal strains tested (267). However, no frequency differences were found for polymorphisms in L- ficolin among 290 patients with invasive pneumococcal disease compared to 720 controls from a similar population (89).

The pneumococcus has evolved several strategies to limit complement-mediated opsonophagocytosis. The pneumococcal capsule plays a central role by limiting the amount of complement deposited on the pneumococcal surface and impeding the access to cell-bound complement (205). Furthermore, pneumolysin has been shown to decrease complement opsonization of the pneumococcal cell (400). This is thought to result from the consumption of complement factors by released pneumolysin. In addition, several other pneumococcal outer surface proteins have been shown to affect complement deposition on the pneumococcus, including pneumococcal surface protein C (PspC), PspA, PsaA, and PhpA (111, 213, 232, 356, 399, 400, 411, 547).

PspC, also referred to as CbpA or SpsA, a choline-binding protein attached to the cell wall, is able to bind complement component C3b, thereby preventing opsonization (111, 213, 232, 399). Furthermore, PspC binds human factor H, a factor which inhibits activation of two complement components of the alternative and lectin pathways. By binding and activating factor H, the pneumococcus locally blocks the unfolding of these two complement pathways (110, 348, 399, 543). In addition, PspC binds the complement inhibitor C4b-binding protein, which blocks activation of the classical complement pathway (122). PspA has been shown to interfere with the binding of complement component C3 on the bacterial surface, thereby inhibiting complement-mediated opsonization (356, 400, 411). PhpA is a pneumococcal surface protein with C3-degrading properties (547). Since activation of the complement cascade is crucial in the defense against pneumococcal invasive disease, pneumococcal complement binding proteins are important targets for vaccine development (65, 109, 147, 336).

Binding to Epithelium

The pneumococcal capsule is advantageous in circumventing the host barriers and reaching the respiratory mucosa but covers PCW binding sites for epithelial cell binding. The pneumococcus adjusts its binding properties to its environment through a process called phase variation (106, 296, 522). In this process, the amount of polysaccharide in the capsule varies from an opaque (thick capsule) to a transparent (thin capsule) phase, either covering or exposing binding sites on the pneumococcal surface (522). During colonization, the thick capsule prevents mucus entrapment as well as immunoglobulin and complement binding, thereby preventing opsonophagocytosis (172, 206, 236, 350). Once the pneumococcus has reached the nasopharyngeal epithelium, the transparent phase becomes prominent, unveiling several adhesion molecules for binding to the host epithelium (106, 522).

At the host respiratory epithelium, the pneumococcus binds to glycoconjugates expressed on the epithelial cells of the respiratory mucosa (e.g., N-acetyl-D-galactosamine [GalNac]). Pneumococcal binding molecules interacting with the host glycoconjugates remain elusive. However, several bacterial genes involved in GalNac binding have been identified, including *spxB*, *ami*, *msrA*, and *plpA* (Fig. 1C) (104, 456, 538). Their gene products are involved either directly in binding of glycoconjugates or indirectly by inducing upregulation of their binding molecules on the epithelial lining (16, 105, 207, 268, 538). Binding of the pneumococcus to GalNac is promoted by NanA, a pneumococcal glycosidase that separates sialic acid from mucin, glycolipids, glycoproteins, and oligosaccharides, thereby enhancing the expression of *N*-acetylglucosamine binding sites on host epithelial cells (239, 480). Cleaved sialic acid residues serve as a carbohydrate source for bacterial metabolism (79, 240).

Pneumococcal binding is further enhanced by hydrophobic and electrostatic forces, binding of pneumococcal phosphorylcholine to the platelet activating factor (PAF) receptor, and binding of pneumococcal surface protein C (PspC) to the polymeric immunoglobulin (pIgR) receptor, all facilitating epithelial cell transcytosis (see Bloodstream Survival) (103, 137, 223). Pneumococci also display pili on their surfaces, facilitating adherence to human buccal cells in the nasopharynx; however, which components of the respiratory mucosa interact with the pili are unknown (28, 349, 454).

Cocolonization

The nasopharynx may be colonized by up to 700 different microbial species, including residential flora, transient colonizing microbes, and pathogenic species (1, 75). Microbial survival is therefore dependent on cooperative and competitive strategies, several of which were recently described in the context of pneumococcal infection (113, 372). Pneumococcal intermicrobial interactions include secondary invasive disease following viral infection, prior innate immunity activation following exposure to another pathogen, and the sharing of virulence/ survival factors between pneumococcal serotypes (320).

Viral infection and subsequent bacterial infection have been investigated extensively (76, 320, 337). Prior exposure to influenza virus has been associated with secondary invasive pneumococcal disease (91, 159). The importance of preexposure to influenza virus was recently underlined during the H1N1 pandemic, in which a third of fatal H1N1 cases exhibited evidence of concurrent bacterial pneumonia (88). The

underlying pathogenesis of enhanced susceptibility to invasive pneumococcal disease after influenza virus infection remains unclear but might be related to an altered expression of adhesion molecules. Prior exposure to viral infection has been demonstrated to increase the expression of epithelial cell adhesion molecules both *in vitro* and *in vivo* (25). The exposure of adhesion molecules on the epithelial lining is further aided by influenza virus neuraminidase (NA), which cleaves terminal sialic acid residues, thereby facilitating pneumococcal binding after viral exposure (321). In mice, pneumococcal binding was reduced when NA was blocked pharmacologically or when either the pneumococcus or influenza virus was mutated to be NA deficient (383). Of particular interest has been the PAF receptor, which may be used by pneumococci for adherence to and transcytosis of the epithelium. Though the PAF receptor is upregulated following viral exposure, murine PAF receptor knockout studies yielded conflicting results regarding the contribution of PAF receptor to pneumococcal adherence and subsequent invasion (322, 417, 507). These conflicting results might be explained by variations in pneumococcal serotype, dosing, and timing of coinfection. There are alternative explanations to PAF receptor upregulation for the association of viral and bacterial infections, including mechanical lung epithelium damage, overall impaired pulmonary function, and an altered immune response to secondary infection following viral exposure (320). *Ex vivo* studies in which the tracheal epithelium was severely damaged following viral infection did not show increased binding of *S. pneumoniae* but showed a decreased mucociliary velocity leading to a higher local bacterial burden after secondary infection (392).

Nasopharyngeal interactions between cocolonizing bacteria can lead to growth inhibition, synergism, and exchange of genetic material. Epidemiologic data suggested a negative association between nasopharyngeal colonization of *Staphylococcus aureus* and *S. pneumoniae* (52, 409). *In vitro* studies suggested that *S. aureus* killing was the result of pneumococcal H_2O_2 production, but this effect has not been reproduced invariably *in vivo* (316, 372). Bacteria may also compete or synergize in the nasopharynx by using the host response. Co- colonization of *S. pneumoniae* and *Haemophilus influenzae* led to rapid neutrophil-mediated clearance of *S. pneumoniae* (307). *In vitro* studies revealed that cell components of *H. influenzae* specifically stimulated the complement-dependent phagocytosis of *S. pneumoniae*; depletion of either complement or neutrophils abolished this competitive phenomenon (307).

Finally, multiple pneumococcal strains may cocolonize the nasopharynx, usually leading to intraspecies competition and competitive outgrowth of a single strain (305, 354). One proposed mechanism for this intraspecies competition involves the use of bacteriocins, so-called pneumocins in pneumococci, which are small peptides capable of killing bacteria of the same or closely related species (395). Additionally, *S. pneumoniae* is naturally able to integrate DNA from killed and closely related pathogens into its own genome, thus gaining a competitive advantage (305). In *in vitro* cocultures, pneumococci that were made bacteriocin deficient were rapidly outcompeted by parent strains or pneumococci of other serotypes (113).

INVASIVE DISEASE

Patients at Risk

Invasive pneumococcal disease may take place when two situations coincide: first, the host is colonized with a pneumococcal strain that it has not yet established immunity to, and second, an alteration of the natural barriers or host immune system has occurred (49, 312). Invasive pneumococcal disease is seen during the extremes of age (less than 2 or more than 50 years of age); in patients with underlying conditions, such as splenectomy or asplenic states, sickle cell disease, multiple myeloma, hypogammaglobulinemia, alcoholism, chronic liver or kidney disease, malignancy, malnutrition, Wiskott-Aldrich syndrome, thalassemia major, diabetes mellitus, and basilar skull fracture with leakage of cerebrospinal fluid (CSF); and in children with cochlear implants (3, 19, 42, 71, 161, 265, 329, 341, 364, 422, 497, 498, 524, 527). The use of immunosuppressive drugs, a history of splenectomy, or the presence of diabetes mellitus, alcoholism, or infection with HIV is found in 20% of adults with pneumococcal meningitis (364, 524). Furthermore, damage to the naso- and oropharyngeal mucosae may be elicited by local pneumococcal infection, such as sinusitis or otitis, by viral respiratory infections (specifically by influenza virus [see "Cocolonization"], by smoking, or by allergy (219, 355, 519, 528).

Invading Host Endothelial and Epithelial Cells

Pneumococci are relatively ineffective at invading host endothelial and epithelial cells. However, pressures of the host natural barriers, cocolonization of other micoorganisms, and an activated innate immune response drive pathogens to develop new strategies. Epithelial endoand transcytosis is an important strategy of invasion and also allows intraepithelial bacterial reservoirs and subsequent recolonization of the nasopharynx. Two mechanisms of epithelial transmigration by *S. pneumoniae* have been described (Fig. 1D). First, pneumococcal phosphorylcholine (ChoP) may bind to the PAF receptor on activated epithelial and endothelial cells (103). ChoP is a component of cell wall-associated acids and lipoteichoic acids (LTAs) on the surfaces of transparent pneumococci (221). By binding the PAF receptor, the pneumococcus may enter the PAF receptor recycling pathway, which transports the bacterium to the basal membrane of the host epithelial cell, which may lead to invasive disease (103, 402). Intranasal challenge of mice deficient in the PAF receptor resulted in reduced rates of pneumococcal colonization, pneumonia, and invasive disease (417).

A second mechanism involves the binding of the pneumococcal choline-binding protein PspC (also known as CbpA or SpsA) to the extracellular portion of epithelial pIgR, referred to as "secretory component" (137, 223). Following attachment, the pneumococcus uses the pIgR recycling pathway, analogous to the PAF receptor pathway, to be transported between the apical and basal membranes of the epithelial cell (223, 546). Pneumococcal expression of PspC has been shown to be an important factor for colonization and invasive disease, although its effect on virulence may vary between pneumococcal strains (67, 190, 232, 424, 546). The PspC binding of pIg receptor is observed only in humans, not in mice, rats, or rabbits (223). In addition, PspC also binds sialic acid and lacto-*N*- neotetraose on respiratory epithelial cells, further facilitating colonization (424). The level of pIg receptor directly correlates with the degree of pneumococcal attachment and epithelial invasion (546). pIg receptors are expressed in a decreasing gradient from the upper to the lower respiratory tract, while the opposite pattern is observed for the PAF receptor (325,

546). Therefore, it has been suggested that where pIg receptor serves mainly as a pneumococcal receptor in the nasopharynx, the PAF receptor acts as a ligand for attachment and invasion of the pulmonary epithelium (546).

Inter- or pericellular migration is another mechanism by which bacteria may cross epithelial or endothelial cell layers (Fig. 1E) (371). Plasminogen, bound by the pneumococcal receptors enolase, Gly3Ph, and CbpE, plays a central role in this process and has been shown to serve two purposes (24, 35, 36). First, plasminogen increases adhesion of pneumococci to the epithelial surface (23). Second, bound plasmin is able to cleave proteins involved in the intercellular adherens junctions, which bind epithelial cells together to form a mechanical barrier to underlying tissues (23). This disruption is mediated by the degradation of cadherin, an essential component of interepithelial adherens junctions (23). Murine pneumococcal nasopharyngeal colonization studies demonstrated that epithelial barrier function was diminished through the downregulation of cadherins in a Toll-like receptor (TLR)-dependent manner (32). Third, epithelial permeability is also modulated by the innate immune system in a transforming growth factor beta (TGF-)-dependent manner, possibly to allow for adequate migration of immune cells and inflammatory mediators into infected areas (31). Thus, the breakdown of the tight junctions, though necessary for an adequate immune response, may allow for pneumococcal access to the basal membrane and subsequent invasive disease.

Extracellular Matrix

At the basal side of the epithelium or endothelium lies the basement membrane, which is comprised mainly of a network of collagen type I, laminin, and proteoglygans (9). Like many bacteria, pneumococci use hyaluronan lyase to degrade major components of the extracellular matrix (ECM), hyaluronan, and certain chondroitins, thereby facilitating invasive disease (215). The importance of hyaluronan lyase for the development of invasive pneumococcal disease was demonstrated in mice, as intranasally administered hyaluronidase adjuvant enhanced the development of invasive disease after an otherwise noninvasive intranasal inoculation of pneumococci (552). Moreover, pneumococci isolated from patients with pneumococcal meningitis expressed higher levels of hyaluronidase than pneumococci isolated from asymptomatic carriers (263).

Fibronectin, a large multidomain ECM glycoprotein, is found in nearly every human tissue environment that the pneumococcus is likely to encounter and is bound by several pneumococcal adhesins, among which the most important are the pneumococcal adhesion and virulence A (PavA) and B (PavB) proteins (200, 216). In murine infection models, PavA-deficient pneumococci had impaired adherence to murine epithelium and endothelial cells and were unable to sustain long- term nasopharyngeal colonization (220, 394). Furthermore, although pneumococci lacking PavA showed similar growth to WT pneumococci in a sepsis model, PavA mutants were rapidly cleared from the central nervous system (CNS) after intracranial infections (220). Possibly, PavA not only serves to directly bind fibronectin but also plays a role in the effective adherence and virulence mediated by other, so far unknown determinants (394).

BLOODSTREAM SURVIVAL

Complement System

Once in the bloodstream, pneumococci are confronted with additional host defense mechanisms. Complement represents the first step of innate immunity against bacteremia. The classical complement pathway plays a dominant role in pneumococcal clearance, although the classical and alternative complement pathways are also activated by streptococcal species (214, 374). Pneumococci have developed two ways to minimize complement-mediated opsonization and phagocytosis. First, pneumococci undergo a second phase variation and become encapsulated. The polysaccharide capsule serves as a nonspecific barrier, significantly reducing complement deposition on the bacterial surface and limiting subsequent interaction with phagocytes (2, 221). In murine studies, systemically administered unencapsulated pneumococci were shown to be avirulent (395).

Second, pneumococcal surface proteins PspA, PspC, and pneumolysin target specific complement components, thereby reducing complement-mediated bacterial clearance. PspA, which is expressed ubiquitously among pneumococci, inhibits C1q and subsequent C3b deposition (214). PspC binds human factor H, thereby blocking the formation of C3 convertase (C3bBb), leading to lower C3b production and limiting opsonophagocytosis (292, 548). Pneumococci can also attach to erythrocytes through a process called immune adherence, which is dependent on the binding of complement components C3b, C4b, C1q, and MBL to both the pneumococcus and erythrocyte receptor CR1 (189, 292, 351). Immune complexes containing pneumococci, bound by complement to erythrocytes, are then transferred to macrophages, after which the erythrocytes are returned to the circulation (99). Recent *in vitro* studies showed that PspA and PspC work synergistically to limit complement-mediated adherence and transfer to phagocytes (292).

Pneumolysin, released during pneumococcal autolysis, readily binds the Fc portion of IgG, thereby potently activating the classical complement pathway, increasing bacterial virulence by independently depleting complement factors away from the bacterium, and limiting opsonophagocytosis (13). Murine bacteremia studies showed that pneumolysin-deficient pneumococci are either cleared from the bloodstream or allowed to develop into chronic bacteremia (359). Furthermore, serum complement depletion may be particularly important in circumstances of overall limited complement availability, such as liver cirrhosis (12), and may further increase pneumococcal virulence at sites of limited complement presence, such as the nasopharynx (318).

Lastly, the acute-phase CRP binds phosphorylcholine (Chop) on the PCW (4, 514) and subsequently interacts with C1q, leading to the activation of the classical complement pathway (93, 451). In mice, CRP is not an acute-phase protein, and treatment with human CRP reduced mortality following pneumococcal infection (467, 468). *In vitro* studies showed that CRP reduced pneumococcal binding to the epithelial cell PAF receptor (175).

Recognition by the Host Immune System

Pneumococci are recognized by APCs through the binding of pattern recognition receptors, which are specifically directed toward general motifs of molecules expressed by pathogens that are essential for pathogen survival. Pattern recognition

receptors involved in sensing pneumococci include TLR2 (192, 286, 333, 441, 511, 541), TLR4 (59, 278, 311), TLR9 (10, 278, 333), and nucleotide oligomerization domain 1 (Nod1) (357, 549). Upon activation of these receptors, APCs release various cytokines, which induce a cascade of inflammatory reactions, including the recruitment of neutrophils (211). The most important cytokines released by phagocytic cells are tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), and IL-6 (419). IL-1β and TNF-α act on local vascular endothelial cells, increasing vascular permeability and vasodilatation and upregulating adhesion molecules such as E-selectin, P-selectin, and vascular cell adhesion molecule 1 (VCAM-1) to enable the influx of neutrophils and other lymphocytes from the blood to the site of infection (Fig. 2) (142, 470).

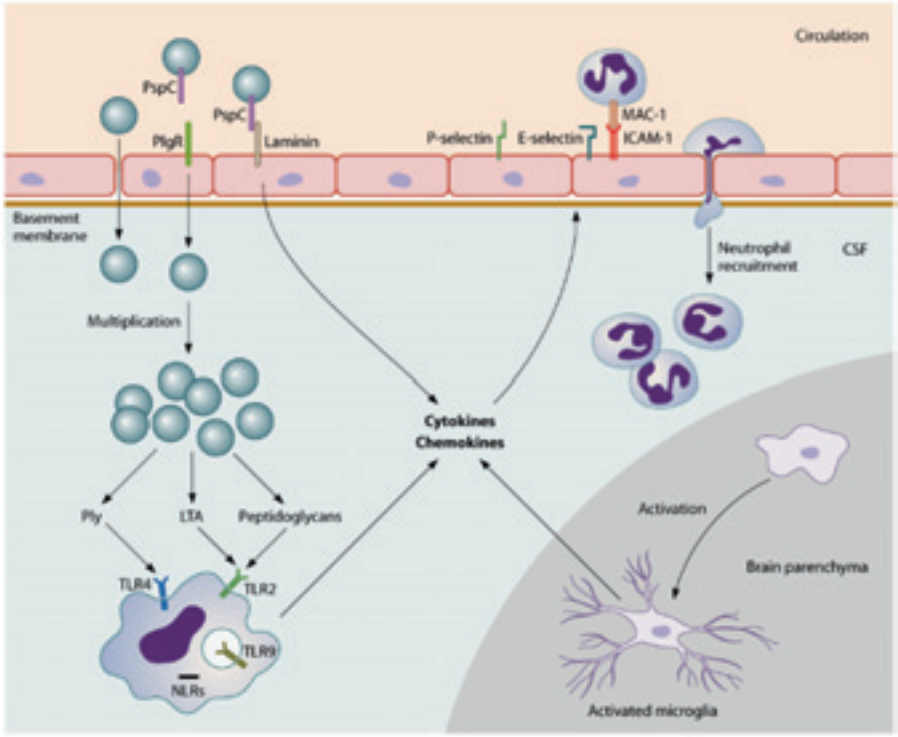

FIGURE 2. S. pneumoniae adheres to endothelial cells by using PspC, which binds laminin and pIgR, enabling transcytosis across the endothelium. Once in the CSF, pneumococci multiply freely and release bacterial products such as LTA and Ply, which are recognized by TLR2 and TLR4 on circulating APCs. The subsequent release of proinflammatory cytokines and chemokines from macrophages and microglial cells results in upregulation of endothelial cell P- and E-selectin and ICAM (which binds MAC-1 on leukocytes), leading to increased neutrophil recruitment into the CSF.

Initiation of Coagulation

Most patients with invasive pneumococcal disease show evidence of coagulation activation (288, 313). Inflammation-induced thrombin generation is not dependent on direct interaction of bacteria and the coagulation cascade but rather on the exposure of blood to tissue factor (TF) (290). TF is expressed primarily on cells outside the vasculature (128, 149) and is exposed to coagulation factors during vascular damage. Low levels of circulating TF have been detected in healthy individuals (167), in

whom the role of TF in thrombin generation remains uncertain (81, 195, 407). The expression of TF in blood cells is limited to monocytes and can be elevated considerably during inflammation or sepsis (370). The upregulation of TF is largely IL-6 dependent, as studies have shown abrogation of TF-dependent thrombin generation when IL-6 is blocked (506).

Upon exposure to blood, TF forms a complex with factor VII and catalyzes the conversion of factor X into factor Xa. Factor Xa allows prothrombin conversion to thrombin, although this reaction occurs to a significant extent only after thrombin-induced feedback activation of factor VIII and factor V, nonenzymatic cofactors in the tenase and prothrombinase complexes, respectively (81, 290). The prothrombinase and tenase complexes convert prothrombin (factor II) into thrombin (factor IIa), which then leads to the conversion of fibrinogen to the clot-forming fibrin protein (289). The activity of prothrombinase and tenase complexes is markedly enhanced by the presence of activated platelets, which become activated during inflammation but may also be activated directly by thrombin itself (427).

Inflammation-mediated thrombin formation is regulated by three anticoagulant mechanisms: antithrombin (AT), the protein C system, and tissue factor pathway inhibitor (TFPI), all of which may be impaired during systemic infection (290). Antithrombin inhibits thrombin and factor Xa, though during severe infection antithrombin levels are markedly lower due to impaired synthesis, degradation, and consumption during thrombin generation (291). Circulating protein C, which upon conversion to activated protein C by the thrombin-thrombomodulin complex degrades the essential coagulation factors Va and VIIIa, is hampered during severe inflammation by enzymatic degradation by neutrophil-derived elastase and by impaired synthesis as well as decreased activation by depressed levels of thrombomodulin (135, 143). Lastly, the importance of TFPI has been demonstrated in studies in healthy human volunteers injected with endotoxin, in whom administration of TFPI induced a marked inhibition of coagulation (117). Animal studies showed that rabbits deficient in TFPI were more susceptible to severe disseminated intravascular coagulation (DIC), and primates infused with TFPI were able to survive exposure to otherwise lethal amounts of *Escherichia coli* (404).

The degradation of fibrin clots is mediated by plasmin, the active form of plasminogen, which is activated by tissue-type plasminogen activator (tPA) and urokinase-type plaminogen activator (uPA), both of which are stimulated by the inflammatory cytokines TNF-α and IL-1β (505). During severe infection, these cytokines subsequently induce plasminogen activator inhibitor type 1 (PAI-1), thereby limiting fibrinolysis and resulting in a net procoagulant state (505). Higher levels of PAI-1 in patients with meningococcal septicemia or disseminated intravascular coagulation have been shown to be associated with poor outcomes and mortality (326, 530).

At relatively high concentrations, thrombin forms a complex with thrombomodulin and activates thrombin-activatable fibrinolysis inhibitor (TAFI; also known as plasma carboxypeptidase B, carboxypeptidase U, and carboxypeptidase R) (48, 518). Activated TAFI inhibits fibrinolysis by limiting plasmin formation through the inhibition of plasminogen and tPA incorporation into fibrin clots (338). Furthermore, TAFI is able to inhibit several proinflammatory substrates, such as bradykinin and

complement components C3 and C5a (84). The importance of TAFI and C5a was first demonstrated in a mouse model in which TAFI knockout mice showed a higher mortality when challenged with sublethal doses of lipopolysaccharide (LPS) and cobra venom factor (22).

CENTRAL NERVOUS SYSTEM INVASION

Intracellular Translocation across the Blood-Brain Barrier

Cerebral vascular endothelial cells show marked differences from their systemic counterparts. They exhibit very tight junctions, low rates of pinocytosis, and relatively large numbers of mitochondria (398). In human brain microvascular endothelial cell cultures, the pneumococcus was able to adhere to the vascular endothelial PAF receptor, allowing transmigration through the endothelial cell to the basolateral site (418). This mechanism of transcytosis is similar to that seen at the pulmonary epithelium (see Invasive Disease) and is mediated by binding of pneumococcal phosphorylcholine to the PAF receptor (103, 417). Pneumococci in the transparent phase are more efficient at invading the brain endothelial cell layer than opaque variants, which are dependent on the expression of phosphorylcholine (418). Concordantly, PAF receptor-deficient mice showed less translocation of pneumococci across the blood-brain barrier and, therefore, a decreased incidence of pneumococcal meningitis after intravenous challenge (402). Many of these studies have been performed with brain vascular endothelial cells. However, another important site of entry might be the choroid plexus epithelium, as shown for *Streptococcus suis*, which induces epithelial cell death and blood-brain barrier disruption in porcine choroid plexus epithelium (473) but may also translocate intracellularly across the plexus epithelium (474).

Nasopharyngeal colonization models demonstrated binding of pneumococcal PspC to pIgR on local epithelial cells, facilitating pneumococcal invasion (546). However, in a cell line of human brain microvascular endothelial cells, the pIgR was not expressed (546). *In vitro* and animal experiments showed that pneumococcal PspC may bind the laminin receptor on brain microvascular endothelial cells (360). This receptor, by which endothelial cells are bound to the major component of basement membranes, laminin, was also shown to be a ligand for neurotropic viruses and prions (6, 158, 360). Laminin appears to be involved in binding of bacteria that may cause meningitis, such as *S. pneumoniae*, *N. meningitidis*, and *H. influenzae*, to brain microvascular endothelial cells (360). Pneumococcal PspC binds to laminin, and in a mouse model of pneumococcal sepsis, a pneumococcal PspC mutant caused a decreased frequency of pneumococcal meningitis (360). These results indicate that the interaction between laminin and pneumococcal PspC plays a role in intracellular translocation of pneumococci across the blood-brain barrier.

Intercellular Translocation across the Blood-Brain Barrier

Pneumococci may translocate into the CSF intercellularly, by disruption of the interepithelial tight junctions. In an animal model of pneumococcal meningitis, tight junctions between brain microvascular endothelial cells became disrupted in the course of the disease (398). This may be due to damage caused by the pneumococcus or by factors of the host immune response (153, 448, 558). Analogous to the nasopharyngeal setting, pneumolysin was capable of disrupting an endothelial cell

layer in an *in vitro* endothelial cell culture, which may enhance blood-brain barrier disruption *in vivo* (558).

After crossing the dense vascular endothelial cell lining, pneumococci have several methods of disrupting and invading the basement membrane. The first involves binding of plasminogen to the bacterial surface, which may subsequently be activated by tPA (129). In patients with bacterial meningitis, levels of uPA correlated with breakdown of the blood-brain barrier and pleocytosis (536). *In vitro* models showed that pneumococcus-mediated activation of plasminogen resulted in damage of extracellular matrix components and the basement membrane (129), although conversely, an *in vivo* mouse model failed to demonstrate an effect of tPA or uPA receptor on pneumococcal transmigration across the blood-brain barrier (379). Finally, pneumococci may bind fibronectin (502), vitronectin, and collagen in the extracellular matrix, which may enhance blood-brain barrier disruption (34, 262).

CENTRAL NERVOUS SYSTEM IMMUNE RESPONSE Immune Activation

During multiplication, pneumococci concurrently undergo autolysis, which eventually leads to a stationary phase where multiplication and autolysis rates are similar (479). The released bacterial products are highly immunogenic and may lead to an increased inflammatory response in the host (489). Bactericidal antibiotics causing bacterial lysis may also induce a similar effect and lead to a temporarily increased host inflammatory response and increased disease severity (344, 345, 483).

A variety of pneumococcal compounds are proinflammatory. The pathophysiological aspects of the different compounds may be reproduced by intracisternal inoculation of heat-killed unencapsulated pneumococci, purified PCW, cell wall lipoteichoic acid, or cell wall peptidoglycan (490). Heat-killed encapsulated pneumococci or purified pneumococcal capsular polysaccharides inoculated intracisternally into rabbits did not cause meningitis, indicating that the pneumococcal capsule is not immunogenic in the CSF (490). Inoculation with knockout pneumococcal strains is another way to study the immunogenicity of pneumococcal compounds. In a murine model of pneumococcal meningitis, intracisternal inoculation with pneumolysin-deficient pneumococci resulted in lower bacterial loads, better clinical scores, and longer survival of the host (529). However, histological inflammatory changes in this study were similar to those induced by wild-type pneumococci (529).

Anatomical Localization of Blood-Brain Barrier Invasion by Leukocytes

Neutrophils are thought to cross the blood-brain barrier mainly at the venous side of the penetrating cerebral blood vessels (182). Here they migrate to the perivascular space, which is continuous with the subarachnoid space. However, some neutrophils penetrate the brain parenchyma. Neutrophilic infiltrates in the brain have been seen primarily in spaces adjacent to CSF, such as the corpus callosum, periventricular space, and the meninges (482). Neutrophils mediate bacterial killing by phagocytosis of opsonized bacteria (211). Phagocytosis is initiated by recognition and binding of bacteria by a neutrophil and is facilitated by opsonization of the bacteria by complement and antibody. Following binding, the neutrophil engulfs the bacteria, after which the cell membrane closes around the pathogens and is cut off, forming a free membranecovered entity within the cell called an endosome (211). In the activated neutrophil, the endosome containing the pathogens is fused with a lysosome

present in the cell, which contains several bactericidal mediators, including nitric and oxygen species, but also activated lysozymes, and the bacteria are killed. In addition to intracellular killing, neutrophils also secrete nitric and oxygen species, establishing a bactericidal milieu around the cell (211). Adversely, these nitric and oxygen species may damage the surrounding tissue when they are present in large amounts and may be responsible, at least in part, for the neuronal damage seen in pneumococcal meningitis. This topic is discussed further in Neuronal Damage and Histopathology.

Pattern Recognition Receptors

Immune activation in the cerebrospinal fluid is initiated by the recognition of different bacterial pathogen-associated molecular patterns (PAMPs) by APCs (Table 1) (7, 440). These APCs are present at low levels in the CSF, (116) or are situated in the meninges, choroid plexus, perivascular space, or brain parenchyma as astrocytes and microglial cells (92, 160). Major pattern recognition receptors involved in initial sensing of pneumococci in the CNS are TLR2 (133, 248), TLR4 (245), TLR9 (10), and Nod-like receptors (NLRs) (Fig. 3) (300).

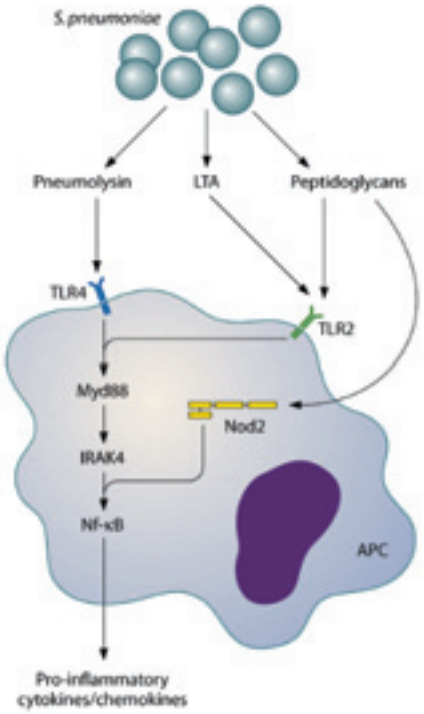

FIGURE 3. Host pattern recognition receptors involved in sensing S. pneumoniae. TLR2 is activated by pneumococcal cell wall peptidoglycan and LTA. Nod2 is activated by cell wall peptidoglycans and TLR4, which in turn is activated by Ply. TLR2 and -4 activate the transcription factor NF-_B via MyD88 and IRAK-4. Nod2 also activates NF-_B, inducing transcription of several proinflammatory cytokines.

TLR2 recognizes PCW LTA (441, 443). TLR2 signaling is enhanced by the TLR2 coreceptor, CD14, and by LPS binding protein (LBP) (441, 541). In a model of pneumococcal meningitis, TLR2-deficient mice showed increased disease severity

TABLE 1. Effects of pattern recognition receptor knockout or deficiency

Model/Setting	*Outcome*	*Reference*
TLR2 KO mice	Higher cerebellar and blood bacterial titers, increased disease severity, no difference in cytokine response	(248)
TLR2 KO mice	Significantly increased disease severity, higher CSF bacterial loads and earlier death	(133)
CD14 KO mice	Significantly increased disease severity, higher CSF bacterial loads and earlier death	(133)
TLR2/CD14 double KO mice	Significantly increased disease severity, higher CSF bacterial loads and earlier death	(133)
TLR4 KO mice	No difference with WT mice	(245)
TLR2/TLR4 double KO mice	Decreased inflammatory response and increased disease severity in TLR2 and TLR4 double mutants	(245)
TLR2/TLR4/TLR9 triple KO mice	No differences in immune response, bacterial load or survival as compared with TLR2/TLR4 double deficient mice	(245)
Nod2 deficient microglial and astroglial cell line	Reduced levels of TNF-α and IL-6 production	(300)
Nod2 KO mice	Decreased MIP-1a and TNF-α production and decreased cerebral demyelination and gliosis	(300)
SIGN-R1 on primary mouse and rat microglial cells	Involved in the uptake of pneumococcal capsular polysaccharides into the cell	(373)
Caspase-1 KO mice	Less severe inflammation and improved survival in a pneumococcal meningitis mouse model	(258)
IRAK-4 deficiency in children	Increased susceptibility to invasive pneumococcal infections, including meningitis	(272)
MyD88 deficiency in children	Increased susceptibility to invasive pneumococcal infections, including meningitis	(516)
NEMO deficiency in patients	Increased susceptibility to invasive pneumococcal infections, including meningitis	(270, 517)
MyD88 KO mice	Increased mortality due to pneumococcal sepsis and meningitis, accompanied by decreased symptoms of infection and inflammatory parameters	(11, 256)

with increased blood-brain barrier disruption and intracranial complications and increased bacterial loads (133, 248). Cytokine production was similar in TLR2-deficient and wild-type mice with pneumococcal meningitis, except for that of TNF-α, which was significantly higher in TLR2-deficient mice (133, 287). Since the phenotype of TLR2-deficient mice with pneumococcal meningitis was not as severe as that seen with mice lacking MyD88, an important general adaptor molecule for TLR signaling, it was proposed that other TLRs besides TLR2 may play a role in sensing pneumococci in the CNS (248, 256).

TLR4 recognizes pneumococcal pneumolysin (311). TLR4- deficient mice did not differ significantly from wild-type mice in their host immune response, cerebrovascular changes, or outcome during pneumococcal meningitis (245). However, in mice deficient in both TLR2 and TLR4, a marked reduction in inflammatory mediators, increased bacterial replication in the CNS, and reduced survival were seen compared to those for wild-type mice or mice with a single TLR deficiency (245). Thus, in meningitis, both TLR2 and TLR4 are important receptors in detecting the pneumococcus and initiating a robust inflammatory response to the pathogen, and one receptor may compensate for the absence of the other (245).

TLR9 is an intracellular pattern recognition receptor and is activated by CpG repeats in bacterial DNA (196). *In vitro*, *S. pneumoniae* was able to activate alveolar and

peripheral macrophages through TLR9 and induced IL-8 production in TLR9-transfected human embryonic kidney cells (10, 333). *In vivo*, TLR9-deficient mice showed reduced resistance to *S. pneumoniae* after intranasal challenge (10). However, in a model of pneumococcal meningitis, triple mutant TLR2/ TLR4/TLR9-deficient mice did not show significant differences in immune response, bacterial load, or survival compared with TLR2/TLR4-deficient mice (245). Therefore, TLR9 appears to play a minor role in pneumococcal meningitis, although this was assessed only in TLR triple-knockout mice.

NLRs are a second group of intracellular pattern recognition receptors involved in detecting pneumococci (357). NLRs belong to a family of receptors which, upon activation, induce activation of NF-B or mitogen-activated protein kinase (MAPK) pathways and inflammatory caspases (357). In human embryonic kidney 293 cells, Nod2 was activated by internalized pneumococci through sensing of *meso*-diaminopimelic acid (*meso*-DAP) motifs of the bacterial peptidoglycan (151, 357). *In vitro* experiments showed that microglial and astroglial cells are activated by *S. pneumoniae* through Nod2 (300). Murine microglial and astroglial cells deficient in Nod2 showed reduced levels of TNF-α and IL-6 production (300). With *in vivo* experiments using a pneumococcal meningitis model, Nod2 activation of primary murine glial cells induced macrophage inflammatory protein 1α (MIP-1α) and TNF-α production and enhanced cerebral demyelination and gliosis (300). Thus, activation of Nod2 appears to be one of the contributing factors leading to cerebral damage in bacterial meningitis.

Another group of NLRs are the inflammasomes, which include a complex of various pattern recognition receptors sharing the caspase adaptor apoptosis-associated speck-like protein (ASC) and leading to caspase-1 activation when triggered (150). Cleavage and activation of caspase-1 lead to cleavage of different procytokines into their active forms, including IL-1β and IL-18 (8, 123, 408). In addition, inflammasome activation may lead to a specific form of controlled cell death, different from apoptosis, called pyroptosis (146). Inflammasomes are intracellular pattern recognition receptors and can be activated by several endogenous and exogenous ligands, including bacteria (486), bacterial DNA (237), bacterial toxins (187), endogenous reactive oxygen species (ROS) produced by macrophages in response to infection (431), and uric acid released through cell injury during inflammation (157). Little is known about the role of inflammasomes in bacterial meningitis. In patients suffering from bacterial meningitis, cerebrospinal fluid levels of caspase-1 were increased (258). In children with bacterial meningitis, as well as a rat model of pneumococcal meningitis, increased IL-1β levels were measured in the CSF (30, 86). Koedel et al. showed that mice lacking caspase-1 displayed less severe inflammation and improved survival in a pneumococcal meningitis mouse model (258). Similar results were found in a pneumococcal meningitis model with IL-18 knockout mice (554), indicating a role for inflammasome activation in the pathophysiology of pneumococcal meningitis.

A fourth group of pathogen recognition receptors involved in sensing *S. pneumoniae* are the C-type lectins, which are highly expressed on splenic dendritic cells and also on peritoneal macrophages (276). A member of this group, SIGN-R1, was shown to facilitate phagocytosis by recognition of the pneumococcal capsular polysaccharide (225, 276). Mice lacking functional SIGN-R1 fail to effectively phagocytose *S.*

pneumoniae, leading to an inability to clear the infection and resulting in increased inflammatory parameters and reduced survival in both a model of pneumococcal peritoneal sepsis (276) and one of intranasally induced pneumonia (260). Furthermore, SIGN-R1 plays a role in the activation of the classical complement pathway by binding C1q (224). Park et al. showed the presence of SIGN-R1 on microglial cells in mouse and rat brains, which was functionally active in taking up pneumococcal capsular polysaccharides into the cell (373). Therefore, SIGN-R1 may be an important pathogen recognition receptor in the brain during pneumococcal meningitis.

Downstream Signaling Molecules

Upon stimulation of one of the above pattern recognition receptors, an intracellular cascade is activated and leads to the production of inflammatory molecules, usually cytokines or chemokines, which modulate the immune response by activating or attracting specialized immune cells. Deficiencies and polymorphisms in the pathogen recognition receptor downstream signaling cascade in humans have been associated with invasive pneumococcal disease, including meningitis.

The most extensively characterized TLR downstream signaling protein in pneumococcal invasive disease is IRAK-4 (Fig. 3) (391). This adaptor protein is one of the links in TLR- and IL-1 receptor (IL-1R)-induced activation of MyD88 and NF-kB, which ultimately results in cytokine production (420, 545). Specifically, children with IRAK-4 deficiency are susceptible to (recurrent) invasive pneumococcal infections, which are associated with high mortality (272). In a group of pediatric patients with normally expressed IRAK-4 but with recurrent invasive pneumococcal disease, deficiencies in the common adaptor molecule of TLR and IL-1R pathways, MyD88, were found (516). Deficiencies in IRAK-4 and MyD88 give indistinguishable phenotypes. Both patient groups are unresponsive to all TLR1, -2, -5, -6, -7, and -8 agonists (516), TLR9 agonists (323), and IL-1R agonists (271). In IRAK-4- or MyD88-deficient patients, the TLR3 signaling pathway is not affected, and the TLR4 pathway is affected only partially. Both TLR3 and -4 can still signal through the MyD88-independent TRIF pathway, leading to cytokine production (516). Stimulation of whole blood of IRAK-4- or MyD88-deficient patients with several different TLR agonists showed impaired production of IL-1β, IL-6, IL-8, IL-10, IL-12, monocyte chemoattractant protein 1 (MCP-1), MIP-1α, and MIP-1β (516). Stimulation with a TLR3 or TLR4 agonist showed impaired production of IL-6, IL-10, and IL-12, as well as that of IL-8 in the case of TLR3 stimulation and IL-1β in the case of TLR4 stimulation (516). Among patients with an IRAK-4 or MyD88 deficiency, 68% suffer from invasive pneumococcal disease, and *S. pneumoniae* is responsible for 53% of all episodes of infectious episodes in these patients (391). Invasive bacterial disease in these patients consists of meningitis in 41% of IRAK-4-deficient patients and 52% of MyD88-deficient patients (391). IRAK-4 and MyD88 appear to be specifically important at a young age, as no fatal disease has been reported after the age of 8 years, with no invasive infections after the age of 14 years (391). Two patients have been described as having a homozygous mutation in the gene encoding NEMO, an adaptor molecule of the MyD88- dependent TLR, IL-1R, and TNF receptor (TNF-R) signaling pathways, and this mutation is associated with invasive pneumococcal disease (270, 517).

In mice, MyD88 deficiency resulted in increased susceptibility to systemic infection after colonization and increased mortality due to pneumococcal sepsis and meningitis (11, 256). Pneumococcal infection in MyD88$^{-/-}$ mice was accompanied by decreased symptoms of infection and inflammatory parameters (256), similar to the phenotype seen in patients lacking functional MyD88 or IRAK-M (391, 517). Deficiencies in the TLR and IL-1R signaling pathways have been associated with recurrent pneumococcal disease (68), illustrating the importance of these pathways in controlling pneumococcal infection.

Proinflammatory Cytokines

The early response cytokines IL-1, TNF-α, and IL-6 are produced after pneumococcal recognition (472, 508). Several cells have been found to be capable of sensing pneumococci and produce proinflammatory cytokines: perivascular and meningeal macrophages (393, 557), vascular endothelial cells (153), astrocytes (154), and microglial cells (193, 413). These early-phase cytokines induce upregulation of several adhesion factors on the vascular endothelium, mediating leukocyte influx (see above) (142, 470). The majority of leukocytes recruited to the CSF are polymorphonuclear neutrophils, and influx occurs largely in the first 6 h of infection (557).

TNF-α is an important early proinflammatory response cytokine. Patients with bacterial meningitis have increased CSF TNF-α levels early in the course of disease (66, 169, 285, 448, 493). Intrathecal levels of TNF-α correlated with severity of blood-brain barrier disruption, disease severity, and neurologic sequelae in a study including 48 patients with bacterial meningitis (448). In this study, TNF-α levels decreased within 24 h after the onset of antibiotic treatment (448). In animal models of pneumococcal meningitis, TNF-α was produced mainly in the first 6 to 24 h of the immune response (29, 363). One hour after intrathecal injection of recombinant TNF-α, CSF leukocyte recruitment was observed in a rabbit model (433). Intrathecal administration of anti- TNF-α antibody together with *S. pneumoniae* reduced CSF leukocytosis, protein content, and brain edema in these experiments (433). TNF-α administered intravenously also mediated blood-brain barrier opening, facilitating bacterial traversal into the CSF (484). However, TNF-α production is also essential for defense, as TNF-α-deficient mice showed decreased survival in a pneumococcal meningitis model (163). Thus, TNF-α has been shown to be a marker of the acute inflammatory response and is associated with inflammation-related complications of bacterial meningitis but is also essential for an adequate host response to the infection.

IL-1β is a proinflammatory cytokine produced by, e.g., perivascular and meningeal macrophages (557). CSF IL-1β levels are increased in the first 18 h of infection (438). Pro- IL-1β is cleaved into its active form by caspase-1, which is regulated by a group of different receptors called the inflammasome (408). Reported data on the role of IL-1β in bacterial meningitis are somewhat contradictory. Levels of IL-1β were not associated with the degree of blood-brain barrier disruption in patients with bacterial meningitis (448). However, a pneumococcal model using caspase-1 knockout mice showed decreased levels of IL-1β and decreased intracranial pressure (ICP), leukocyte recruitment, and brain edema compared to those in WT mice (258). IL-1β administered intrathecally did not lead to CSF pleocytosis or brain edema in a rabbit model of pneumococcal meningitis (433). However, antibodies against IL-1β

decreased leukocyte influx induced by TNF-α (433). Mice deficient in the receptor for IL-1α and IL-1β (IL-1R) showed impaired survival and decreased cytokine responses without alterations in CSF pleocytosis (551). Thus, although IL-1β did not influence CSF pleocytosis in pneumococcal meningitis, other caspase-1-cleaved cytokines may be responsible for the reduced pleocytosis observed in caspase-1 knockout mice.

IL-6 is a proinflammatory as well as anti-inflammatory cytokine and has been shown to be upregulated in the acute phase of many infection models (155). In a mouse pneumococcal meningitis model, IL-6 knockout mice displayed increased CSF pleocytosis but decreased cerebral edema, blood- brain barrier disruption, and intracranial pressure (376). This was also described for a model of pneumococcal pneumonia where IL-6 was shown to downregulate multiple proinflammatory as well as anti-inflammatory cytokines (504). Thus, in pneumococcal meningitis, IL-6 attenuates CSF leukocyte recruitment but does not inhibit complications related to fluid shift.

Gamma interferon (IFN-γ) is one of the major cytokines of the T-helper 1 (Th1) pathway. IFN-γ was increased in the CSF of patients with pneumococcal meningitis (170, 261). IFN-γ was also expressed in brain tissue of rats with pneumococcal meningitis (121). The exact role of IFN-γ in pneumococcal meningitis remains unclear. IL-12p70, an important stimulus for IFN-γ production, could be detected in patients with pneumococcal meningitis (261) and in animal models of pneumococcal meningitis (121). Macrophage inflammatory factor (MIF) was found to be increased in the CSF of patients with pneumococcal meningitis and has also been associated with disease severity (361), suggesting a role for MIF in the pathophysiology of pneumococcal meningitis (162).

Anti-Inflammatory Cytokines

Anti-inflammatory cytokines include IL-10 and TGF-β (120, 277, 466, 476). IL-6 may act partially as an anti-inflammatory cytokine and has been discussed earlier (504). IL-10 is an anti-inflammatory cytokine with multiple effects, including downregulation of proinflammatory cytokines and costimulatory molecules on macrophages (120, 476) and impairment of neutrophil phagocytosis and killing (275). IL-10 has been shown to downregulate TNF-α, IL-6, and keratinocyte-derived chemokine (KC), thereby reducing CSF pleocytosis in pneumococcal meningitis (555). Nonetheless, in experimental pneumococcal meningitis, IL-10 knockout mice did not have altered bacterial loads or survival (555). This anti-inflammatory cytokine has been described as an important repressor of sepsis-associated neuronal damage. Its pathophysiology is unclear, but it appears that inflammatory mediators as well as bacterial components cross the blood-brain barrier and induce a local inflammatory response (358, 492, 509). In mice overexpressing IL-10, the development of sepsis-associated neuronal damage as a result of pneumococcal sepsis has been shown to be decreased (358). In line with this, Koedel et al. showed that intravenously administered recombinant IL-10, as opposed to intracisternally administered IL-10, reduced the levels of CSF proinflammatory cytokines, CSF pleocytosis, cerebral edema, and intracranial pressure in a rat model of pneumococcal meningitis (249). Interestingly, intracisternally administered IL-10 had the opposite effect, as it increased CSF pleocytosis in rats with pneumococcal meningitis and induced an

inflammatory response in uninfected rats (249). Thus, systemic IL-10 reduces cerebral inflammation and secondary complications in pneumococcal meningitis.

TGF-β is an anti-inflammatory cytokine with multiple functions, including differentiation and maintenance of regulatory T cells (Tregs), differentiation of Th17 T cells, and inhibition of Th1 and Th2 T-cell maturation and differentiation (295), but TGF-β also suppresses macrophage activation and production of several proinflammatory cytokines, such as IL-1β, IL-6, and TNF, by microglial cells (277, 466). Activated Tregs produce TGF-β in an autocrine fashion and are thought to modulate the immune response in such a way that the host's tissues are minimally damaged while the invading pathogen is effectively eliminated, by downregulating the acute inflammatory response. In a mouse model of pneumococcal meningitis, TGF-β was associated with cerebral vasculitis, a frequent complication in patients with meningitis (231, 310). Mice with leukocytes deficient in TGF-β receptor II (TGF-βRII) showed increased neutrophil influx into the subarachnoid space, which was accompanied by increased bacterial clearance and survival of the host (310). In addition, TGF-β RII knockout mice showed decreased blood-brain barrier disruption, intracranial pressure, and cerebral vasculitis (310). However, when TGF-β2 or TGF-β1 was administered intraperitoneally in a rat model of sterile meningitis induced by a PCW lysate, cerebral edema, intracranial pressure, and cerebral blood flow (CBF) decreased (387). Thus, leukocyte TGF-βRII signaling has an unfavorable effect on the course of pneumococcal meningitis, although systemic TGF-β production appears to decrease the complications of meningitis.

Chemokines

Chemokines are a subgroup of cytokines with chemotactic activity recruiting effector immune cells to the site of infection (211). Multiple chemokines have been reported to be upregulated in the CSF of patients with pneumococcal meningitis, including MIP-1delta (CCL15), NAP-2 (CXCL7), MIF, MCP-2 (CCL8), PARC (CCL18), MIP-3α (CCL20) (226), ENA-78 (CXCL5), GRO-α (CXCL-1) (455, 553), IL-8 (CXCL-8) (201, 363, 455, 493, 553), MCP-1 (CCL2), MIP-1α (CCL3), and MIP-1β (CCL4) (455). In animal models of pneumococcal meningitis, additional chemokines have been identified by protein arrays for brain tissue, including MIP-1γ (CCL9), MIP-2 (CXCL-2), lymphotactin (XCL-1), TCA-3 (CCL1), eotaxin (CCL11), MCP-5 (CCL12), eotaxin-2 (CCL24), TECK (CCL25), PF-4 (CXCL4), CRG-2 (CXCL10), SDF-1α (CXCL12), BLC (CXCL13), and CXCL16 (246). The role in pneumococcal meningitis of many of these chemokines has not been elucidated yet.

IL-8 is one of the well-characterized chemokines involved in pneumococcal meningitis. IL-8 was found to be chemotactic for neutrophils in the CSF of patients with bacterial meningitis (455). Furthermore, CSF IL-8 levels increased as a result of blocking leukocyte recruitment in rabbits with pneumococcal meningitis, indicating local production of chemotactic cytokines (368). In patients with bacterial meningitis, no correlation was found between the CSF white blood cell (WBC) count and IL-8 (455). Ostergaard et al. showed that not intracisternal but rather systemic IL-8 levels induced CSF pleocytosis in a rabbit model of pneumococcal meningitis (369). Thus, IL-8 appears to regulate CSF pleocytosis from the systemic compartment, comparable with the proinflammatory cytokine TNF-α and the anti-inflammatory cytokines IL-10 and TGF-β.

The CCL chemokines MCP-1, MIP-1α, and MIP-2 were produced in vitro by astrocytes (193) and microglial cells in response to PCW structures (397). In vitro, antibodies against MCP-1, MIP-1α, and MIP-1β inhibited monocyte chemotactic properties of CSF from patients with pneumococcal meningitis (455). Furthermore, intracisternal inoculation of recombinant MIP-1 or MIP-2 induced blood-brain barrier disruption, CSF leukocytosis, and cerebral edema in a rabbit model of pneumococcal meningitis; blocking MIP-1 or MIP-2 delayed these inflammatory alterations by 2 h (433). Another experiment showed that blocking the receptor for MIP-1, i.e., CCR2, specifically reduced the influx of monocytes into the subarachnoid space in a mouse model of pneumococcal meningitis, while not changing bacterial clearing (328). Thus, both MIP-1 and MIP-2 are produced by immune cells resident in the brain and attract monocytes and neutrophils from the bloodstream into the CSF in the acute stage of infection. The role of MCP-1 in pneumococcal meningitis has not been studied extensively.

Of the CXCL chemokines, ENA-78 was found to be upregulated in patients with bacterial meningitis and exhibited specifically neutrophil chemotactic properties together with IL-8 (553). GRO-α was also found at high levels in the CSF of patients with bacterial meningitis (553), as well as in a rat model of pneumococcal meningitis, but it did not exert any chemotactic activity (30, 553).

In summary, multiple chemokines have been shown to be upregulated in pneumococcal meningitis. Most of them have a role in attracting leukocytes to the CSF. However, the roles of many other chemokines have not been investigated extensively.

Leukocyte Migration Adhesion Molecules

In response to proinflammatory cytokines, selectins and integrins are upregulated on the blood endothelium and leukocytes are attracted from the bloodstream (85, 182). Cerebral perivascular and meningeal macrophages play a key role in attracting leukocytes across the blood-brain barrier into the CSF (393). About 90% of the attracted leukocyte population consists of neutrophilic granulocytes, with the other 10% being predominantly monocytes (287, 328, 393). Rats depleted of perivascular and meningeal macrophages by use of clodronate showed decreased leukocyte recruitment into the CSF despite increased expression of MIP-2, IL-6, and VCAM-1 (393). Furthermore, these depleted rats showed increased bacterial outgrowth in the CSF and poorer clinical scores than those for control rats with pneumococcal meningitis (393). Thus, leukocyte attraction to the subarachnoid space seems to be crucial for efficacious clearing of *S. pneumoniae* from the subarachnoid space and dependent on perivascular and meningeal macrophage activation but appears to be mediated by cytokines other than IL-6 and MIP-2.

Other cytokines and chemokines attracting leukocytes to the subarachnoid space are TNF-α, IL-8 (systemic), MIP-2, and ENA-78 (see above) (433, 553). Monocytes are also attracted from the bloodstream into the CSF but appear to play a minor role in the pathogenesis of pneumococcal meningitis (328).

Leukocytes cross the blood-brain barrier by binding to selectins on the endothelium (182). Binding to P- and E-selectin promotes leukocyte rolling across the endothelium (182). Blocking L-selectin by fucoidin treatment reduced leukocytosis and disruption

of the blood-brain barrier in rabbits challenged intrathecally with pneumococcal antigen (182). Integrins are also upregulated on the vascular endothelium, facilitating binding of leukocytes and subsequent blood-brain barrier migration (85). An important integrin involved in leukocyte recruitment in pneumococcal meningitis is ICAM-1, which is known to bind MAC-1 (CD11b/CD18) on the leukocyte surface (82, 491). Rabbits treated intravenously with antibodies against CD18 showed decreased CSF leukocytosis (82), blood- brain barrier permeability, and brain edema and improved survival after intracisternal challenge with PCW or *S. pneumoniae* (491). Interestingly, antibodies directed against CD11b did not alter CSF leukocytosis in the same rabbit model of pneumococcal meningitis (491), which may implicate a role for CD11a/CD18 or CD11c/CD18. In line with this, CD11a/CD18- deficient mice showed increased rates of meningitis and otitis media following intraperitoneal infection with *S. pneumoniae* (396).

The integrin ICAM-1 was shown to be expressed on brain vascular endothelial cells in response to PCW, through an autocrine loop involving TNF-α (153). In a rat model of meningitis induced by PCW, antibodies against ICAM-1 reduced the increase in CBF, increase in ICP, brain edema, and CSF leukocyte counts observed in the first hours after induction of meningitis (520). In an infant mouse model of pneumococcal peritonitis, ICAM-1 deficiency did not reduce the incidence of meningitis, and histopathologically there was no difference in the severity of inflammation (469). Thus, ICAM-1 is not solely responsible for leukocyte recruitment to the brain.

Other Chemoattractants

PAF is a protein produced by neutrophils and endothelial cells in response to inflammatory stimuli, and it facilitates adhesion of leukocytes to the vascular endothelium (43, 308, 460). In rabbits, PAF administered intrathecally induced blood-brain barrier permeability and cerebral edema at doses much lower than those at which it induced leukocytosis (82). Antibodies against CD18 blocked these effects (82).

In response to pneumococci, endothelial cells and neutrophils are stimulated to produce reactive nitrogen species (RNS), such as NO, by endothelial nitric oxide synthetase (eNOS) and inducible nitric oxide synthetase (iNOS), respectively (153, 537). The cerebral vasculature appears to be the main location where ROS are active (434), and increased levels of ROS are associated with blood-brain barrier disruption (254). In patients with meningitis, positive correlations were found between CSF derivatives of NO production and CSF leukocyte counts and protein concentrations in a group of 27 children with bacterial meningitis; however, only 2 of these children had confirmed pneumococcal meningitis (340). Mice deficient for iNOS showed decreased blood-brain barrier disruption and decreased IL-1β, IL-6, TNF-α, MIP-1α, and MIP-2 mRNA levels in the brain (537). The opposite was true for eNOS-deficient mice, which showed more profound leukocyte infiltrates, increased cytokine levels, and decreased survival due to pneumococcal meningitis (253). A third form of NOS, neuronal NOS (nNOS), appears to play a minor role in fluid balance-related complications of pneumococcal meningitis (375). In addition to RNS, ROS such as O_2^- are produced by the enzyme NADPH oxidase in neutrophils, macrophages, and endothelial cells in response to infection (435). In mice deficient for the subunit of NADPH oxidase in nonphagocytic cells, such as endothelial cells (p47), detrimental

effects on blood-brain barrier permeability, subarachnoid space inflammation, and bacterial outgrowth were found (435). Mice deficient for the subunit of NADPH oxidase in phagocytic cells (gp91) did not show any inflammatory differences from WT mice in the course of pneumococcal meningitis (435). Thus, RNS/ROS produced specifically by cerebral endothelial cells, as opposed to granulocytes and macrophages, contribute to the blood-brain barrier damage and associated complications observed during pneumococcal meningitis.

The fibrinolysis factor uPA is also implicated in leukocyte recruitment to the brain in pneumococcal meningitis. In a group of 12 patients with bacterial meningitis (67% of cases were caused by *S. pneumoniae*), CSF uPA levels were associated with leukocyte recruitment and blood-brain barrier disruption (536). In this study, serum uPA levels correlated with unfavorable clinical outcomes for these patients with bacterial meningitis (536). Mice deficient in uPA showed reduced CSF leukocytosis, although blood-brain barrier permeability, ICP, expression of chemokines, bacterial killing, and clinical outcomes were not different from those for WT mice (379). Interestingly, deficiency in tPA did not have any implications in a mouse pneumococcal meningitis model (379).

The Complement System

A fourth chemoattractant factor for CSF leukocytes is the complement system. Low or undetectable CSF levels of C3, C4, and B were found in uninfected control subjects (478). In response to infection, the liver produces an array of acute- phase proteins which includes several complement components (188, 461). Circulating monocytes, macrophages (95, 461), and epithelial cells of the pulmonary and gastrointestinal tracts (96, 462) also produce substantial amounts of complement components. In addition, brain resident macrophages and monocytes recruited to the CSF during meningitis may also locally produce complement components. C3 was also found to be produced by astrocytes and neurons in response to HIV or proinflammatory cytokines (74, 314, 457). Cultured human brain pericytes from a patient with Alzheimer's disease have been shown to produce C1q (512), and activated astroglial cells can produce C1q, which has been associated with increased blood-brain barrier damage in a rat model of neurotoxicity (306). Microglial cells have been shown to upregulate C1q, C3, C4, and C5a production in response to injury (183). Thus, levels of complement components are increased in the peripheral blood but may also be produced locally in the brain during infection or inflammation.

During infection or inflammation, the immune response in the brain compartment may do more harm than good. Under normal circumstances, the brain expresses multiple inhibitory factors for complement activation. One of these, factor H, was found to be expressed constitutively in neurons, brain endothelial cells, microglial cells, and astrocytes in mice (184). In a mouse model of antibody-mediated inflammation, expression of factor H in these cells was suppressed; when recombinant factor H was administered, complement opsonization, axonal injury, and leukocyte infiltration decreased (184). Thus, in addition to monocytes and macrophages, brain resident cells may contribute to the production of complement factors leading to leukocyte influx during inflammation.

In a rabbit model of pneumococcal meningitis where cobra venom (known to consume complement factor 3) was administered systemically, treated mice showed

decreased survival accompanied by increased bacterial outgrowth in the CSF (488). CSF pleocytosis was similar between the treated and untreated groups, but neutrophils were severely impaired in phagocytosis and killing of bacteria (488). In line with these results, mice deficient in C1q or C3 also showed increased bacterial outgrowth in the CSF in a pneumococcal meningitis model, which was accompanied by decreased survival (428). C1q and C3 knockout mice displayed a tempered inflammatory response, which was reflected by a decreased leukocyte count in the CSF, decreased brain cytokine and chemokine levels, and fewer meningitis-associated intracranial complications. However, survival was decreased in this model as a result of more fulminant sepsis accompanied by systemic complications (428). Similar results were found in mice deficient for the C3b receptor (CR3), which is also an integrin involved in binding of leukocytes to the endothelium (378). $CR3^{-/-}$ mice showed increased bacterial outgrowth compared to WT mice, with decreased survival, as a result of decreased neutrophilic superoxidase production in the $CR3^{-/-}$ mice leading to ineffective bacterial killing, while CSF pleocytosis was not different between groups (378). In rabbits, intracisternally administered C5a resulted in rapid CSF pleocytosis and increased CSF protein levels which peaked 1 h after injection (222). Furthermore, the CSF of rabbits with pneumococcal meningitis lost its chemotactic activity to neutrophils after incubation with an antibody against C5a (139).

MMPs

Matrix metalloproteinases (MMPs) are Zn^{2+}- and Ca^{2+}- dependent endopeptidases capable of breaking down and remodeling extracellular matrix components such as fibronectin, laminin, proteoglycans, and type IV collagen (423, 540). MMPs are produced mainly by activated neutrophils and, to a lesser extent, also by macrophages, monocytes, and possibly TNF-α-stimulated endothelial cells (194, 233). Furthermore, constitutive MMP expression is found on microglial cells and astrocytes and may be modulated during neuroinflammation and meningitis (102, 173, 464).

The action of MMPs was initially believed to be limited to the breakdown of ECM during leukocyte migration across the subendothelial layer (319). However, experimental bacterial meningitis models revealed that inhibition of MMPs did not result in a reduction of CSF pleocytosis, although blood-brain barrier permeability disruption was attenuated (377). More recent studies have revealed a wide range of MMP substrates, such as chemokines, growth factors, and adhesion molecules, as well as cytokines and cytokine receptors, allowing MMPs to influence the course of various inflammatory conditions (319, 510).

In patients with bacterial meningitis, CSF levels of MMP-8 and MMP-9 were elevated (285). Moreover, higher levels of MMP-9 were detected in children with meningitis who developed hearing impairment or secondary epilepsy than in those who recovered without neurological deficit (285).

To avoid unwanted proteolytic activity, the activity of all MMPs is tightly regulated by binding to inhibitory proteins called tissue inhibitors of metalloproteinases (TIMPs). MMP-9, for instance, forms complexes with and is inactivated by TIMP-1, both of which are upregulated during pneumococcal meningitis (377). In a murine

model of pneumococcal meningitis, the induction of TIMP-1 was delayed in relation to that of MMP-9, favoring increased collagen IV degradation and subsequent increased blood-brain barrier permeability (445). Thus, treatment options including drugs specifically targeting MMPs are being investigated (281). Interestingly, in a rat model of pneumococcal meningitis, adjuvant treatment of pneumococcal meningitis with dexamethasone resulted in lower MMP-9 mRNA expression, suggesting a possible mechanism of corticosteroids as an adjuvant treatment for bacterial meningitis (301).

Oxidative Stress

One of the characterizing features of pneumococcal meningitis is the marked recruitment of leukocytes into the CSF (364, 524). The subsequent release of large amounts of RNS and ROS has been documented for patient populations as well as in animal models and plays a central role in the development of intracranial complications and brain damage (119, 229, 283). In the past decade, ROS and RNS have been investigated extensively as potential targets for adjuvant treatment (reviewed by Klein et al. [242]).

During pneumococcal meningitis, RNS are produced by iNOS and eNOS (537). NOS inhibition studies with experimental pneumococcal meningitis models have yielded contradictory results, at least in part due to a lack of drug specificity for single isoforms of NOS (436). Moreover, iNOS and eNOS knockout studies show that the source of RNS is pivotal in determining its function during disease progression. Thus, NO derived from iNOS appears to contribute to blood-brain barrier disruption and to production of proinflammatory mediators (such as IL-1β, TNF-α, and MIP-2), whereas eNOS- derived NO plays a largely protective role (253, 537).

Reactive oxygen species, such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals, are produced by brain resident immune cells as part of the host response to invasive bacterial infections (27). Pneumococci themselves are also an important source of H_2O_2, which not only is able to cause direct cytotoxic damage but also interacts with host NO to form the highly reactive species peroxynitrite ($ONOO^-$) (37, 63, 198). When present in large quantities, ROS overwhelm the resident antioxidant mechanisms (such as superoxide dismutase and glutathione), leading to tissue exposure to oxidative stress (27). Interventions aimed at scavenging ROS or enhancing antioxidant activity have generally resulted in reductions of intracranial complications such as elevated ICP, increased CBF, brain edema, and neuronal injury (242, 436).

Peroxynitrite, which is formed by the combination of superoxide radicals and NO, is a very reactive, short-lived molecule whose direct detection has proven difficult; the compound nitrotyrosine (which is formed by the reaction of NOO^- with tyrosine) is widely used as a marker (401). In patients with bacterial meningitis, elevated CSF levels of nitrotyrosine were associated with an unfavorable outcome and with lower CSF concentrations of the antioxidant ascorbic acid, suggesting antioxidant depletion by the RNS (229). Furthermore, in autopsy studies, nitrotyrosine was detected in the leptomeninges, subarachnoid granulocytes, and penetrating cortical and leptomeningeal vasculature.

Peroxynitrite can damage neurons and glial cells in two ways. First, it causes damage by means of lipid peroxidation and cell membrane destabilization, which occurs by peroxynitrite attack on lipid peroxidation and is consistently seen in brain homogenates of rats with pneumococcal meningitis (242, 257). Blocking of lipid peroxidation with aminosteroids limits neuronal damage (257). Alternatively, peroxynitrite can cause DNA fragmentation and subsequent poly(ADP-ribose) polymerase (PARP) activation, which leads to cell energy depletion and cell death (436). PARP knockout mice, as well as mice treated with a PARP inhibitor, demonstrated lower levels of inflammation and a better clinical course during pneumococcal meningitis (257, 436).

Klein et al. (242) have reviewed the mechanisms of oxidative damage (activation of cytokines and chemokines, neutrophil activation, lipid peroxidation, DNA and mitochondrial damage, tyrosine nitration, MMP activation/TIMP inactivation, K^+ channel activity alterations, and prostaglandin synthesis) as well as the resulting pathophysiologic alterations in pneumococcal meningitis (242).

Coagulation

The importance of coagulation and fibrinolytic dysregulation during pneumococcal meningitis is illustrated by the large number of cerebrovascular complications, which occur in up to one-third of patients (524). Analysis of CSF in patients with bacterial meningitis revealed increased levels of both coagulation and fibrinolytic factors (Table 2) (535, 536). More recently, PAI-1 was shown to be associated with the occurrence of brain infarctions (525), though no causal relationship was determined. Furthermore, in a recent autopsy series of patients who died of pneumococcal meningitis, fibrin thrombi and cerebral infarctions were found in the absence of inflammatory vessel wall infiltrates, suggesting that disseminated cerebral intravascular coagulation might be an additional explanation for ischemic stroke in pneumococcal meningitis (513). The precise mechanism of cerebral infarction remains unclear but may include mechanisms such as vascular endothelial swelling, local vascular inflammation, and cerebral intravascular coagulopathy (386, 513).

Microhemorrhages are also frequently observed in the leptomeninges, cortex, and white matter and are located mostly around congested small veins and capillaries (513). It may be hypothesized that the massive clotting process results in local depletion of clotting factors, thereby inducing the local formation of microhemorrhages. In severe cases of disseminated cerebral intravascular coagulation, these microhemorrhages might potentially lead to clinically manifested intracerebral macrohemorrhages, which are rarely observed in patients with bacterial meningitis.

TABLE 2. Coagulation studies

Setting (human studies)	*Material*	*Factor*	*Concentration*	*Reference*
38 patients with bacterial meningitis: GCS <9 vs. >9	Serum	PLT/dPLT PTr INR D-dimer	↑ ↓ ↑ ↑	(264)
92 patients with bacterial meningitis vs. controls and patients with viral meningitis.	CSF	sTF TaT pT fragment F1+2 (F1+2), t-PA PAI-1 D-dimer	↑ ↑ ↑ ↑ ↑ ↑	(525)
12 patients with bacterial meningitis vs. 10 patients with GBS and 10 controls.	Serum/CSF	uPA uPAR PAI-1 PA-dependent Pl activation	↑/↑ =/↑ =/↑ ↑	(536)
12 patients with bacterial meningitis vs. 10 patients with GBS and 10 controls.	Serum/CSF	tPA	↑↑/↑	(535)
Setting (murine studies)	***Material***	***Factor***	***Concentration***	***Reference***
C57BL/6 tPA-/- uPAR-/-	Brain Frozen Section	tPA/uPA PAI-1/2 uPAR MIP-2 KC Albumin	↑/= ↑/↑ ↑ ↑ ↑ ↑	(379)

NEURONAL DAMAGE AND HISTOPATHOLOGY

Neuronal Damage/Histopathology

Human observational studies have repeatedly found long-term sequelae after pneumococcal meningitis, including sensomotor deficit, hearing loss, and cognitive impairment, which may occur in up to 30% of surviving patients (202, 241, 496, 497, 500, 526). Human histopathological data showed that the parenchymal damage was caused by increased ICP, cytotoxic and vasogenic edema, herniation, and local leukocyte infiltration or abscess formation, as well as by cortical necrosis and hippocampal neuronal loss (346, 513). Experimental animal models of pneumococcal meningitis have demonstrated large variations in histopathological features (Fig. 4 and Table 3), most likely due to different combinations of bacterial strains, infected animal species, methods of inoculation, and stages of infection (64, 178).

Microgial Activation

Microglial cells, a specific subset of cells related to monocytes and dendritic cells, form the initial line of defense of brain parenchyma against damage, injury, or infection and play a pivotal role in tissue repair, removal of dead debris, and

recruitment of other immune cells to the site of infection (90, 353, 403). Together with meningeal and perivascular macrophages, microglia express a wide palette of TLRs and become activated during bacterial infections (343). Once activated, microglia are capable of producing large amounts of proinflammatory cytokines as well as reactive oxygen and reactive nitrogen intermediates, thus potentially playing both neuroprotective and neurotoxic roles (97, 209, 317).

In vitro studies have demonstrated that microglial cells express TLRs 2, 4, and 9, which upon activation by specific TLR agonists induce proinflammatory cytokine and NO production, as well as increased phagocytic activity (130, 413). More specifically, the neurotoxic effects of CpG DNA, which is released into the subarachnoidal space in large amounts by pneumococci during bacterial growth and following antibiotic treatment, were shown to be mediated largely via stimulation of TLR9 on microglial cells (209). Furthermore, pneumolysin, which is a ligand for TLR4, was also found to be a potent activator of microglial cells, causing microglial cytotoxicity at high concentrations (130).

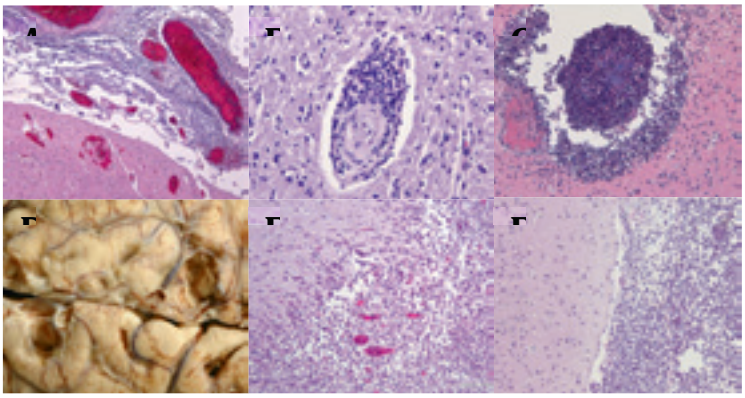

FIGURE 4. Neuronal damage and histopathology in humans with pneumococcal meningitis. The images show the histopathology of patients with bacterial meningitis, including parenchymal and meningeal hemorrhages (A), neutrophilic infiltration and arteritis obliterans (B), abscess formation and venous thrombosis (C), recent infarctions (D and E), and meningitis without cortical infiltration (F).

Activin A, a member of the TGF-β superfamily, is a neuroprotective cytokine which has been shown to be expressed constitutively in CSF and elevated in patients during bacterial meningitis (131, 532). Elevated activin A levels in CSF have also been detected in a rabbit meningitis model and were produced by cultured microglial cells following treatment with agonists of TLRs 2, 4, and 9 (132, 327). Furthermore, *in vitro*- cultured murine microglial cells stimulated with LPS showed that cotreatment with activin A increased microglial proliferation and negatively regulated production of NO, IL-1β, IL-6, and TNF-α (532).

TABLE 3. Neuronal damage

Subject	*Outcome*	*Reference*
Microglial activation	Micorglial activation is induced by pneumococcus pneumoniae and mediated by TLR's 2, 4, and 9	(130, 177, 209, 300)
	Activin A may mediate microglial proliferation and activation	(132, 327, 532)

Neuronal apoptosis	The early phase of hippocampal apoptosis is caspase dependent and mediated by H_2O_2, pneumolysin, and apoptosis inducing factor (AIF)	(60, 62, 63, 266, 318, 330, 521)
	The late phase of hippocampal apoptosis is caspase independent and mediated by pneumococcal cell wall products, in a microglial TLR-2 dependent manner	(37, 279, 280, 330)
Sepsis mediated neuronal damage	Neuronal damage may result sepsis without concurrent bacterial growth in the CNS compartment	(358, 365, 366, 380)
Cochlear damage	Cochlear damage is correlated to CSF inflammation, and is mediated by TLR and MyD88 signaling pathways	(15, 39, 40, 247)
	NO may contribute to cochlear damage. iNOS and eNOS are upregulated during PM and damage can be attenuated using NO-inhibitors/O_2 scavengers	(14, 227, 241, 243)

Although no studies have been performed regarding the effects of dexamethasone on microglial activation during pneumococcal meningitis, *in vitro* studies using LPS-stimulated glial cells demonstrated that microglial activation after bacterial exposure is limited by corticosteroid treatment, providing a possible explanation for the observed beneficial effects of dexamethasone treatment *in vivo* (115, 148, 197, 495).

A recently published hypothesis suggested that microglia may respond differently to a stimulus preceded by another stimulus, a phenomenon called □priming□(509). Aging itself may result in enhanced microglial activation following single or repeated stimulation (171, 385). Thus, the usually tightly controlled microglial activation may become self-amplifying and even neurotoxic (509). Whether this dysregulation of microglial function plays a role in pneumococcal meningitis seems plausible but has not yet been investigated.

Neuronal Apoptosis

Neuronal damage is caused by the dual effects of an overwhelming inflammatory reaction and direct effects of bacterial toxins (164). Though the hippocampus is not exposed to pneumococci or infiltrating leukocytes directly, it is surrounded by interstitial fluid which is contiguous with the CSF, allowing secreted bacterial toxins and immune system mediators to diffuse into the parenchyma (63, 412). Recent research with murine models has shown that pneumococcus-mediated hippocampal apoptosis occurs in at least two phases, separated by both time and mechanism (330, 521).

The early phase is initiated by the pneumococcal toxins H_2O_2 and pneumolysin and results in caspase-independent apoptosis-like pyknotic cell death of both mature and immature neurons throughout the dentate gyrus of the hippocampus (63, 330). This is subsequently followed by caspase-dependent apoptosis of the immature neurons in the subgranular region of the dentate gyrus, triggered primarily by bacterial cell wall stimulation of leukocytes (178, 330).

Pneumolysin, which is also implicated in apoptosis of microglial and brain microvascular endothelial cells, was shown to colocalize to dying neurons in the dentate gyrus in a rabbit model of pneumococcal meningitis (63, 318). Animals infected with either pneumolysin- or H_2O_2-deficient pneumococci showed only partial attenuation of early neuronal apoptosis (63). Additional blocking of H_2O_2 in animals infected with pneumolysin-deficient pneumococci led to a marked further

reduction of cell death, suggesting that H_2O_2 and pneumolysin together are responsible for early hippocampal apoptosis (63).

Because pneumococci do not express catalase, they are capable of producing high levels of H_2O_2, which can diffuse freely through the cellular membranes of target cells to damage intracellular structures (384). H_2O_2 triggers the release of Ca^{2+} from the endoplasmic reticulum and its influx from the extracellular space. This results in a loss of mitochondrial membrane stability and in further increases of Ca^{2+} and ROS production. Pneumolysin possibly contributes directly to mitochondrial permeabilization through pore formation (60), after which apoptosis inducing factor (AIF) is cleaved from the mitochondrial membrane by calpein and cathespin is transported through the cytosol into the nucleus (62). There, AIF causes chromatin condensation and large-scale DNA fragmentation, leading to cell death (266). Paradoxically, AIF may also have antiapoptotic properties through the regulation of ROS through peroxide scavenging (266).

The late, caspase-dependent phase of neuronal apoptosis is PCW and TLR2 dependent (37, 330). The observation that *in vitro* exposure of isolated cultured neurons to PCW products does not lead to cell death led to the hypothesis that late-phase apoptosis may be dependent on the host inflammatory response (330). Neurons themselves do not express TLR2 or -4 and are not sensitive to exposure to the corresponding bacterial ligands. However, when cocultured with microglial cells, neurons revealed caspase-dependent, TLR-mediated late apoptosis when they were exposed to PCW or LPS (279, 280). Therefore, the inflammatory response of microglial cells and invading neutrophils may underlie the caspase-dependent hippocampal apoptosis during pneumococcal meningitis.

Several experimental treatments aimed at reducing hippocampal apoptosis have been studied in animal models of pneumococcal meningitis. Much attention has gone to nonbacteriolytic antibiotic therapies, such as rifampin, daptomycin, and clindamycin (see Targets for Adjunctive Therapy). In experimental rabbit models, rifampin given either alone or as a pretreatment before ceftriaxone resulted in a reduction of hippocampal apoptosis, though no reduction in mortality was observed (54, 165, 458). Likewise, both daptomycin and clindamycin treatments yielded less hippocampal neuronal damage than that with ceftriaxone treatment in rabbit and rat models, respectively (55, 177). Finally, inhibition of MMP and TNF-α converting enzyme (TACE) was also shown to reduce cortical damage and neuronal apoptosis, as well as preserving learning performance in rats with pneumococcal meningitis (281).

Sepsis and Hippocampal Damage

Recent findings with experimental animal models have suggested that neuronal damage may also be caused by pneumococcal growth outside the CNS compartment (365). Mice exposed intravascularly to purified PCW developed hippocampal apoptosis at 6 h postinoculation, an effect that was not reproduced in TLR2- or NOD2-deficient mice and was limited in mice overexpressing IL-10 (358). These findings suggest a possible third, earlier, IL-10-repressible mechanism of neuronal damage, which may precede pneumococcal invasion of the CNS (358). Additionally, in the setting of experimental meningitis, bacteremia has been shown to contribute not only to increased hippocampal apoptosis but also to dysregulation of CBF

autoregulation, reduced meningeal inflammation, and attenuated CSF pleocytosis (366, 380). The mechanisms involved remain largely unclear.

Cochlear Damage and Hearing Loss

Hearing loss is a common long-term complication in survivors of bacterial meningitis. Up to 30% of survivors of pneumococcal meningitis experience unior bilateral hearing loss, which is often permanent and may be quite severe (125, 273, 415, 524).

The underlying pathophysiology has been studied in several animal models (39, 40, 45, 244, 421, 477) Cochlear involvement may result from direct spread of pneumococcal infection from the meninges, CSF, and cochlear perilymphatic system (40, 227, 244). Alternatively, a hematogenous route may occur following bacteremia or sepsis (227, 244).

The resulting cochlear infiltration of pneumoccoci and neutrophils results in a severe granulocytic inflammation of the perilymphatic spaces and in the release of proinflammatory cytokines and cytotoxic mediators (56). Hearing loss was correlated with the level of CSF inflammation in a rabbit model of pneumococcal meningitis (40), and intrathecal administration of TNF-α alone was sufficient to induce cochlear injury similar to that observed with bacterial meningitis (15). Furthermore, TLR and MyD88 knockout mice demonstrated less hearing loss following pneumococcal meningitis (247). In a rat model, cochlear expression of iNOS and eNOS was upregulated following pneumococcal meningitis, and RNS-mediated cochlear damage could be attenuated both electrophysiologically and histopathologically by RNS scavengers (227, 241, 243). Earlier studies with guinea pigs showed that local perfusion of the scala tympani with NO donor compounds resulted in cochlear damage and could be attenuated by NO inhibitors or O_2 scavengers (14).

Cerebrovascular Complications

Cerebrovascular complications are very common during pneumococcal meningitis (497, 528). Arterial stroke occurs in up to 30% of patients, cerebral venous thrombosis in 9%, and intracerebral hemorrhage in up to 9% (231, 524, 531). Autopsy studies in the 1930s through 1960s showed inflammatory infiltrations of cerebral arteries and veins (77, 83, 126). Taking these together with angiographic descriptions of segmental arterial narrowing in patients with ischemic stroke complicating pneumococcal meningitis, the general assumption has been that infarctions during bacterial meningitis are caused by vasculitis.

In a recent human histopathological analysis of patients with pneumococcal meningitis, among whom half of patients had evidence of cerebral infarctions and 67% showed microhemorrhages, there was no evidence of large-vessel vasculitis (513). Moreover, the observed small-vessel vasculitis did not colocalize with areas of infarction, and in this series, no evidence of disseminated intravascular coagulation in the systemic compartment was observed (513). These results suggest the possibility of cerebral intravascular coagulation, independent of systemic coagulopathy or cerebral vasculitis, as the cause for both cerebral infarctions and hemorrhages.

The pathogenesis of cerebral infarction remains unclear and is the subject of ongoing research, which has focused largely on two areas: first, the dysregulation of the

coagulation and fibrinolytic pathways, not only systemically but also locally, as exemplified by the upregulation of PAI-1 and elevated levels of prothrombin fragments F1 and -2 and soluble tissue factor in the CSF of patients with pneumococcal meningitis (264, 525, 536); and second, endothelial cell dysfunction, which may lead to localized swelling and release of procoagulant factors and proinflammatory cytokines. Also, endothelin, which is one of several potent vasoactive peptides, has been shown to be elevated in CSF during acute stages of infection (33, 251, 389). In a rat model, treatment with bosentan (an endothelin antagonist) normalized otherwise reduced CBF. Although endothelin inhibition lowered cortical necrosis, no effect on hippocampal damage was observed (389).

TARGETS FOR ADJUNCTIVE THERAPY

Inhibition of Complement Activation

Complement activation is crucial in the early phases of host defense against pneumococcal disease. Generally, complement activation leads to formation of a membrane pore (the MAC) in the pathogen, leading to cell lysis. However, complement components C3a, C4a, and C5a (204) are cleaved in the activation of the complement cascade and serve as anaphylatoxins. They recruit leukocytes to the site of infection, enhance neutrophil survival, and inhibit neutrophil oxidative burst (107, 186, 459). In a murine sepsis model, a C1 inhibitor improved survival through complement inhibition (299). In experimental pneumococcal meningitis, inhibition of C1 resulted in reduced meningeal inflammatory responses, decreased cytokine levels, decreased bacterial outgrowth, and improved survival in rats (550). Interference in the final common complement pathway may present a promising future target for adjunctive therapy (Table 4).

TABLE 4. Therapeutic/adjuvant treatments in experimental settings

Subject	*Target/Treatment*	*Outcome*	*Reference*
Inhibition of Leukocyte migration	L-selectin/ Fucoidin	Lowers CSF pleocytosis, protein content and CBF, ICP and cytokine production (IL-1 and TNF-α).	(17, 57, 179, 181, 182, 368, 491)
	ICAM-1/ABs CD-18/ABs	Reduced CSF leukocyte count, CBF, ICP and brain edema . Reduction of CSF pleocytosis, BBB permeability, brain edema, and increased survival.	(520) (491)
	G-CSF	(Pre-)treatment with G-CSF leads to lower levels of CSF pleocytosis, pro-inflammatory cytokines.	(58, 118, 362)
	P-selectin/ Pertussis E-selectin	Lowers CSF pleocytosis.	(432)
Inhibition of pattern recognition receptors	Kinase inhibitor AG126/ ERK1/2 inhibition.	Decreased microglial production of pro-inflammatory cytokines and chemokines, pleocytosis, CBF, and brain edema.	(193)
Inhibition of pro-inflammatory cytokines	TNF- α / thalidomide	Decreased TNF- α levels (but not IL-1β), decreased CSF pleocytosis.	(53)

	TNF- α / TACE	Increased survival in murine model in both WT and TLR2 KO mice.	(134)
	IL-6/ ABs	Reduced CSF pleocytosis and CSF protein content in rat model	(315)
	IL-10/ ABs	Reduced CSF pleocytosis and CSF protein content as well as IL-6 levels .	(249)
Nonbacteriolytic antibodies	Daptomycin	Reduced levels of inflammatory cytokines and cortical damage.	(100, 176, 177)
	Rifampicin	Reduced bacterial protein synthesis, lowered mortality in murine model Reduces release of PCW products, inflammation and neuronal damage in combination with ceftriaxone.	(463) (165, 347, 458)
	Moxifloxacin	Reduced bacterial cell wall components LTA and TA in CSF in rabbits. No reduction in mortality over cefalosporin therapy	(463) (124)
Radical Scavenging	iNOS inhibitors/NAC	Reduction of proinflammatory cytokine production, cortical damage and CSF pleocytosis. and hearing loss.	(228, 230, 242, 243, 302)
Inhibition of caspases	Caspase-3/BDNF	Reduction of neuronal apoptosis	(44, 293)
	All Caspases/BAF	Reduction in cognitive decline	(210)
Inhibition of complement	C1	Reduction in meningeal inflammation response	(550)
Inhibition of MMPs	BB-94	Reduction of BBB permeability, lowers ICP.	(377)
	GM6001	Decreased TNF- αlevels, reduction of neuronal apoptosis	(284)
	MMP and TACE/ TNF484	Reduction of cortical necrosis No reduction of hippocampal apoptosis.	
	MMP and TACE/ BB-1101	Reduction of both cortical necrosis and hippocampal apoptosis. Preserved learning performance	(281)

Inhibition of Proinflammatory Cytokines

TNF-α is essential for a robust inflammatory response but may also elicit inflammation-related complications (163, 433). Thalidomide is a TNF-α inhibitor which is used in the treatment of multiple myeloma (452). In a rabbit model induced by intrathecally administered heat-killed pneumococci, intraperitoneally administered thalidomide was associated with decreased CSF TNF-α levels (but not IL-1β levels) and decreased CSF pleocytosis, but there was no effect on blood-brain barrier permeability (80). When TNF-α was blocked by a TACE inhibitor in an experimental mouse model of pneumococcal meningitis, survival was increased in WT and $TLR2^{-/-}$ mice (134). Thus, TACE inhibition may improve survival, even in a host with deficient TLR2 signaling (134).

Blocking IL-6 intravenously in a rat model of pneumococcal meningitis reduced CSF pleocytosis and protein content (315). Similar results were found with antibodies

against IL-10 administered intravenously, which also decreased CSF IL-6 levels (249). Administration of recombinant TGF-β2 intraperitoneally in the acute phase of pneumococcal meningitis in rats reduced the subarachnoid inflammatory response by inhibiting the increase in CBF and brain water content (387).

Inhibition of Pattern Recognition Receptors

Inhibiting TLR signaling with the aim of decreasing subsequent cytokine responses presents a promising strategy. The kinase inhibitor tyrphostin AG126 was shown to inhibit phosphorylation of the signaling molecule extracellular signal-regulated kinase 1/2 (ERK1/2) in microglial cells (193). ERK1/2 is activated in blood monocytes in response to LPS through activation of CD14 and TLR4 (185). In microglial cells, treatment with this kinase inhibitor resulted in decreased production of proinflammatory cytokines and chemokines (193). When the kinase inhibitor was administered intraperitoneally in a mouse pneumococcal meningitis model, leukocyte recruitment to the CSF, CBF, brain edema, and TNF-α production were reduced (193). This provided evidence that blocking the TLR signaling pathway may reduce the severity of disease in pneumococcal meningitis.

Inhibition of Leukocyte Influx into the CNS

One of the strategies to prevent brain damage is to limit leukocyte recruitment to the CSF or to increase leukocyte apoptosis. Recruitment of leukocytes (mainly polymorphonuclear leukocytes [PMNs]) to the subarachnoid space results in the clearance of bacteria, which is accompanied by the production of several toxic mediators that may induce damage not only to the bacteria but also to the brain.

The first step in extravasation of PMNs involves binding of the leukocytes to selectins on the vessel endothelium. Fucoidin is a polysaccharide that blocks the leukocyte receptor L-selectin. Intravenous treatment with fucoidin in several animal models of pneumococcal meningitis reduced CSF pleocytosis (17, 179, 182), in association with reduced CSF protein content (179, 182), modestly decreased CSF lactate (179, 182), and decreased CBF and ICP, without influencing blood-brain barrier permeability, cerebral edema, and outcome (17). Furthermore, fucoidin prevented the increase in CSF TNF-α and IL-1 levels in response to intrathecally administered PCW in rabbits (181); however, when live bacteria were administered intrathecally and the rabbits were treated with ampicillin, fucoidin had no effect on cytokine production (181). A similar study without antibiotic treatment reported a reduction in IL-1 and an increase in CSF IL-8 in fucoidin-treated rabbits with pneumococcal meningitis (368). In contrast to these studies, in a rat model of pneumococcal meningitis, fucoidin treatment led to decreased survival (57). The leukocyte concentration in CSF was lower in fucoidin-treated rats, but systemic leukocytosis was increased, as was systemic bacterial outgrowth. However, rats in this study were pretreated with fucoidin, which may have led to the differences in the systemic immune response (57).

After binding of leukocytes to selectins, firm adhesion to the vascular endothelium is mediated by ICAM-1. In a rat model of meningitis with PCW component-induced inflammation, antibodies against ICAM-1 reduced CSF leukocyte counts, CBF, ICP, and brain edema in the first 6 h (520). In experimental pneumococcal meningitis, an intravenously administered antibody against CD18, a subunit on leukocytes for

binding ICAM-1, induced either by live bacteria or by PCW, reduced CSF leukocyte counts, blood-brain barrier permeability, and brain edema and increased survival (491). However, anti-CD18 antibodies tended only to inhibit CSF leukocyte counts in a rabbit model of PCW-induced inflammation (180).

Vascular endothelial growth factor (VEGF) is a peptide involved in angiogenesis, but it has also been described to function as a macrophage and granulocyte chemoattractant (485). Levels of VEGF were associated with CSF leukocyte counts in experimental meningitis induced by heat-killed pneumococci (503). However, blocking of VEGF in rabbits with pneumococcal meningitis did not reduce the extent of brain edema, leukocyte influx, or blood-brain barrier permeability (503).

A different approach involves the induction of an increased systemic proliferation of leukocytes, which would lead to better control of systemic infection and, subsequently, to enhanced control of CNS infection. In patients with meningitis, granulocyte colony-stimulating factor (G-CSF) and macrophage colony-stimulating factor (M-CSF) have been found to be elevated in the CSF (156, 450). In these studies, G-CSF and M-CSF levels correlated with CSF leukocytosis (450). In mice with pneumococcal meningitis, expression of granulocyte-macrophage colony-stimulating factor (GM-CSF) in the brain was increased (376). Rabbits pretreated with G-CSF intravenously 1 h before intrathecal inoculation with *S. pneumoniae* showed increased peripheral but not subarachnoid leukocytosis and increased CSF levels of TNF and IL-1 but no reduction of subarachnoid bacterial outgrowth or neuron-specific enolase, an indicator of neuronal cell damage (438). A similar experiment showed no influence on subarachnoid bacterial killing, but systemic pleocytosis and bacterial killing were increased (362). CSF leukocytosis and protein content and levels of IL-8, TNF-α, and IL-1β were decreased in G-CSF-pretreated animals, indicating a decreased subarachnoid inflammatory response (362). Similar results were found in a study with rats (58). However, late administration of G-CSF (28 h after infection) did not have any influence on disease parameters (58). In 22 patients with pneumococcal meningitis, adjunctive treatment with recombinant G-CSF was performed together with standard treatment with ceftriaxone and dexamethasone. G- CSF was continued for 4 days unless leukocyte counts exceeded $40x10^9$ cells/liter. Lactate and glucose levels returned to normal more quickly in G-CSF-treated patients than in historical controls, and no adverse events were recorded. However, this was not a randomized controlled study (118).

Another approach to limit neutrophil-mediated damage in pneumococcal meningitis is to induce apoptosis in neutrophils by use of roscovitine. Mice treated with a combination of antibiotics and roscovitine showed increased resolution of inflammation, decreased cerebral hemorrhages, and faster recoveries (250).

In meningitis caused by *Cryptococcus neoformans*, the fungal capsular polysaccharide glucuronoxylomannan (GMX) inhibited leukocyte extravasation despite high IL-8 levels in the CSF (297). The mechanisms by which GMX inhibits leukocytosis are unknown; however, when GMX was administered intravenously in an experimental rabbit model of meningeal inflammation with heat-killed pneumococci, CSF TNF-α levels and leukocytosis decreased, in association with reduced brain edema and inflammation on brain histopathology (298).

A similar mechanism has been described for pertussis toxin, which interferes with the binding of PMNs to P-selectin and E-selectin, the first steps of diapedesis. Intravenous treatment with pertussis toxin in a rabbit model of meningeal inflammation induced by heat-killed pneumococci altered CSF pleocytosis compared to that in untreated animals (432).

Inhibition of Caspases

Caspase activation has been implicated in the activation of proinflammatory cytokines (258) as well as in the mediation of programmed cell death of cerebral endothelial cells and neurons in the hippocampal dendate gyrus (37, 330). Experimental models of pneumococcal meningitis using mice deficient in caspase-1 or after pharmacological blocking of caspase-1 demonstrated lower levels of IL-1β and subsequent diminished proinflammatory cytokine production, as well as fewer meningitis-induced complications (258). The protective effect of caspase-3 inhibition on the development of neuronal damage was demonstrated in a rat model of pneumococcal meningitis and was independent of cytokine modulation (166). Also, the broad-spectrum caspase inhibitor z-VAD-fmk was shown to reduce hippocampal neuronal apoptosis in a rabbit model of pneumococcal meningitis compared to that in untreated controls (61). Moreover, rats inoculated with group B streptococci demonstrated less cognitive decline following adjunctive treatment with the pan-caspase inhibitor bocaspartyl (OMe)-fluoromethylketone (210). An interesting observation is that exogenous administration of brain-derived neutrotrophic factor (BDNF), which has been shown to block caspase-3 (191), reduced neuronal apoptosis in both rat and murine models (44, 293). BDNF was found to be upregulated naturally during bacterial meningitis and after treatment with antibiotics with adjunctive dexamethasone yet lowered during standard antibiotic treatment, suggesting a possible mechanism of corticosteroid therapy (294). So far, no clinical trials have been performed with caspase inhibitors as adjunctive therapy.

Adjunctive Dexamethasone Therapy

Dexamethasone is a widely used anti-inflammatory drug. The mechanisms by which dexamethasone inhibits inflammation are not clear, but it decreases proinflammatory cytokine production in monocytes, dendritic cells, astroglial cells, and neutrophils (108, 154, 217, 425), increases the production of anti-inflammatory cytokines such as IL-10 (108), inhibits ROS production by leukocytes (108), and decreases leukocyte adherence (153, 303). Dexamethasone acts on multiple molecules of the TLR downstream signaling cascade, including TAK-1, ERK1/2, MAPK, NF-kB, and STAT3 (41, 542). Astroglial cells stimulated with PCW components produced decreased amounts of NO and TNF-α when they were treated with dexamethasone (38, 154, 444). Brain microvascular endothelial cells also showed reduced levels of TNF-α and IL-1 and decreased expression of ICAM-1 (153). On the molecular level, in peripheral blood mononuclear cells dexamethasone was shown to inhibit *S. pneumoniae*-induced IkBk phosphorylation and degradation and binding of NF-kB to DNA, both of which are downstream effector mechanisms of TLR signaling (332). Dexamethasone induced increased levels of IkBk mRNA, which may bind and inhibit the p65 subunit of NF-kB. These effects resulted in decreased IL-8 production by peripheral blood mononuclear cells (332).

In experimental pneumococcal meningitis, adjunctive dexamethasone reduced TNF-α, lactate (304), and NO (119a) levels when it was administered together with antibiotics. Furthermore, dexamethasone decreased ICP, brain edema, and CSF pleocytosis in rats with PCW-induced meningeal inflammation (255, 388). In a rabbit model, neuron-specific enolase, a marker of overall neuronal damage, was reduced in animals treated with ceftriaxone and dexamethasone compared to those treated with ceftriaxone alone, though an increase in hippocampal apoptosis was also observed (556). Moreover, in rats treated with adjunctive dexamethasone, the observed increase in hippocampal apoptosis was accompanied by impaired learning performance (282), and uninfected rats treated with dexamethasone also demonstrated increased hippocampal damage (47). In an analysis of several prospective multi-center trials in which patients with bacterial meningitis were treated with either adjunctive dexamethasone or placebo, the use of dexamethasone was not associated with cognitive impairment (202, 526).

Adjuvant treatment with dexamethasone resulted in a reduction of hearing loss in rabbit and gerbil models of pneumococcal meningitis (235, 406) but did not have a significant effect in infant rats (94). A recent meta-analysis of human trials evaluating adjuvant dexamethasone treatment suggested that dexamethasone may reduce hearing loss among survivors (499). Clearly, further trials are necessary to assess the effects of dexamethasone on cochlear injury and hearing loss.

In children with bacterial meningitis, CSF TNF-α and IL-1 levels were decreased if patients had been treated with adjunctive dexamethasone therapy (342). In a large randomized controlled trial in Vietnam investigating the efficacy of dexamethasone addition to conventional antibiotic regimens in adults with bacterial meningitis, CSF samples from a large group of patients were examined (309). For 195 of a total of 341 patients included in this study, CSF was available at baseline (when therapy was started) and at a follow-up puncture 1 to 4 days later. Of these 195 patients, 88 had received dexamethasone along with antibiotics starting at baseline, and 107 received placebo and antibiotics. For 24% of patients, *S. pneumoniae* was confirmed as the causative agent by CSF culture. Other causative agents included *S. suis* (43%) and *N. meningitidis* (8%). CSF samples from these patients were analyzed for IL-6, IL-8, IL-10, IL-12, IL-1β, and TNF-α. Cytokine levels in CSF from the first lumbar puncture at baseline were similar between both groups. Dexamethasone treatment reduced the levels of IL-6 and IL-8 and increased the levels of IL-10 (median, 37 pg/ml versus 33 pg/ml; *P* value 0.01) in the CSF at follow-up compared to those with placebo (309). Levels of IL-12, IL-1β, and TNF-α were similar between both groups at follow-up. In addition to the differences in anti-inflammatory cytokine profiles, opening pressure of the follow-up lumbar puncture was reduced and the CSF glucose level and CSF/plasma glucose ratio were restored sooner in patients receiving dexamethasone. CSF lactate and protein levels as well as leukocyte counts were similar at follow-up in the dexamethasone and placebo groups (309).

Many randomized clinical trials of dexamethasone for treatment of bacterial meningitis have been performed, but the results have remained somewhat ambiguous (70, 115, 494, 495, 499). An individual patient data meta-analysis of 5 large recent trials showed no effect of dexamethasone (499). A prospective cohort study showed a decrease in mortality from 30 to 20% for adults with pneumococcal meningitis after nationwide implementation of dexamethasone therapy in the Netherlands (69).

Dexamethasone treatment has been implemented as routine therapy for patients with suspected or proven pneumococcal meningitis in many countries (487, 497).

Adjunctive Glycerol Therapy

Glycerol (glycerine 1-2-3-propanetriol), a hyperosmolar compound, is an essential component of cell membranes. It has been used in neurosurgery, neurology, and ophthalmology to decrease raised tissue pressures (26, 78, 87, 168, 416, 501). Toxicological data show that it is safe and is associated with few, rare, mild, mostly gastrointestinal side effects (152). Furthermore, glycerol is inexpensive and readily available, facilitating widespread implementation if it is effective. The effects of glycerol in the neurological/neurosurgical setting have been hypothesized to lie in the resulting increase of plasma osmolality, which was shown to reduce the excretion of CSF by some 20 to 30%, leading to increased cerebral blood flow and improvement of brain oxygenation (453). For acute stroke, glycerol has been shown to confer only a short-term advantage, without demonstrating benefits in the long-term outcome (416).

The first clinical trial evaluating glycerol as a potential adjuvant treatment for bacterial meningitis was conducted with 122 Finnish children with bacterial meningitis and suggested a reduction in hearing impairment as well as in long-term neurological sequelae (234). More recently, a large South American trial using adjuvant glycerol for the treatment of children with bacterial meningitis provided additional support for its efficacy in the reduction of neurological sequelae, although hearing loss and mortality were not diminished (381, 382). However, reliable interpretation of the findings was compromised by several methodological problems (430). Nevertheless, the results fueled additional trials, most recently a study in Malawi where 256 adults with bacterial meningitis were randomized to receive either placebo or glycerol as adjuvant treatment (5). The trial was halted prematurely when an interim analysis after 100 deaths showed increased death in the glycerol group (5). The discrepancies in the various studies most likely lie in the variation in study populations, such as age, comorbidity, and causative pathogen, as well as in the variation in treatment regimens and methods of assessing outcome parameters.

Relatively few animal studies have been performed with glycerol, and they have not been able to demonstrate clinical or histological benefits of adjuvant glycerol therapy (46). Moreover, in a rabbit model, pneumococcal meningitis treated with glycerol alone was associated with an increased level of hippocampal neuronal apoptosis (439). A study in which healthy mice were treated with high doses of glycerol (much higher than trial dosages) showed that treatment was associated with the occurrence of seizures (127), which were also seen more in the glycerol-treated patients in the Malawi trial (5). This finding was not in concurrence with the South American trial and may be explained by either the shorter glycerol regimen (2 days instead of 4 days) or increased disease severity in the Malawi group.

Though glycerol seemed attractive as a potential adjuvant treatment for bacterial meningitis, the lack of effectiveness in experimental models, combined with its harmful effects in the Malawi trial, question the value of additional studies on adjunctive glycerol for the treatment of adult meningitis (72).

Nonbacteriolytic Antibiotics

The massive inflammatory response following pneumococcal meningitis has been shown to play a key role in the development of brain damage and subsequent poor outcomes (252). In part, the inflammatory response is determined by bacterial lysis products, as shown in experimental pneumococcal meningitis models where inoculation with PCW led to massive inflammation and neuronal damage (489, 490). Although bacteriolytic antibiotic regimens may limit the overall amount of release of bacterial products, temporary increases in the release of bacterial components have been documented following treatment (344). These observations have fueled the study of nonbacteriolytic antimicrobials as future therapy options.

Specifically, recent research of nonbacteriolytic antimicrobials has focused on the potential use of daptomycin, rifampin, and moxifloxacin. Daptomycin, a lipopeptide antibiotic, has been shown to effectively clear *S. pneumoniae* in experimental rat, mouse, and rabbit meningitis models (100, 177). Treatment with daptomycin led to lower levels of inflammatory cytokines and possibly to less cortical brain damage and neuronal apoptosis than those for treatment with ceftriaxone alone (176, 177). Although adjunctive treatment with dexamethasone did not result in a reduction of overall pneumococcal clearance from the CSF, daptomycin penetration into the inflamed meninges was reduced in the presence of adjunctive corticosteroid therapy (136).

Rifampin inhibits bacterial protein synthesis and was shown to lead to diminished levels of PCW product release *in vitro* (463). In a murine model of pneumococcal meningitis, a decrease in mortality was observed in mice treated with rifampin versus ceftriaxone, an effect which was most notable during the first hours following antibiotic therapy (347). Moreover, recent studies with a rabbit model demonstrated that short-term pretreatment with rifampin before ceftriaxone reduced the release of PCW products, inflammation, and neuronal damage compared to those with treatment with ceftriaxone alone (54, 165, 458).

In a rabbit model of pneumococcal meningitis, treatment with moxifloxacin, a relatively novel quinolone, was shown to result in lower levels of the proinflammatory cell wall components LTA and TA in CSF than those obtained by treatment with ceftriaxone (463). Pneumococcal clearance from the CSF was comparable after treatment with either moxifloxacin or ceftriaxone, and drug levels were not reduced following adjunctive treatment with dexamethasone (367, 437). However, in a murine model, moxifloxacin failed to reduce mortality compared to standard cephalosporin therapy (124).

Radical Scavenging

Investigations of potential adjunctive antioxidant therapies have been limited to animal studies and so far lack sufficient evidence for clinical testing. A recent comprehensive review by Klein et al. summarizes the efforts and findings in this area of potential treatment options (242). Among the most promising candidates for future clinical applications are the iNOS inhibitors, the peroxynitrite scavenger Mn(III)tetrakis(4-benzoic acid)-porphyrin (MnTBAP), uric acid, alpha-pheny-*tert*-butyl-nitrone (PBN), and *N*-acetyl-L-cysteine (NAC) (242). In spite of some beneficial effects, such as reductions of proinflammatory cytokine pleocytosis (230), reductions in cortical damage (302), attenuated CSF pleocytosis (228, 230), and a diminished incidence of hearing loss (243), adverse outcomes have been reported,

such as impaired bacterial killing, increased neuronal apoptosis, and impaired learning function (302). Of all potential antioxidant agents, only NAC is currently used routinely in clinical practice (for the treatment of acetaminophen intoxications), and therefore it is a likely candidate for evaluation in a clinical setting.

CONCLUSIONS

Despite significant advances in treatment and vaccinations, pneumococcal meningitis remains one of the most important infectious diseases worldwide and continues to be associated with high mortality and morbidity. The growing emergence of drug resistance as well as shifts in serotype incidence is fueling further development of novel antibiotic and adjuvant treatment strategies. In addition to the widespread introduction of dexamethasone, other options for adjuvant drugs may lie in modulating ROS/RNS-mediated damage, in caspase inhibition, or in drugs targeting specific mediators in the inflammatory, complement, or coagulation cascades. Extensive research in this area is being performed using experimental animal meningitis models, though so far no clinical treatment trials with humans have been performed. Although the limitations of animal models of meningitis lie in the great variability between species, inoculation methods, and ages of infected animals, experimental medicine continues to provide the backbone for both intervention and pathophysiology studies and will hopefully pave the way to new knowledge and treatment of this deadly disease.

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CHAPTER 3

CHARACTERIZATION OF A PNEUMOCOCCAL MENINGITIS MOUSE MODEL

Mook-Kanamori B*, Geldhoff M*, Troost D, van der Poll T, van de Beek D.

*contributed equally in the writing of this paper.

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ABSTRACT

Background: *S. pneumoniae* is the most common causative agent of meningitis, and is associated with high morbidity and mortality. We aimed to develop an integrated and representative pneumococcal meningitis mouse model resembling the human situation.

Methods: Adult mice (C57BL/6) were inoculated in the cisterna magna with increasing doses of serotype 3 colony forming units (CFU; n = 24, 10^4, 10^5, 10^6 and 10^7 CFU) and survival studies were performed. Cerebrospinal fluid (CSF), brain, blood, spleen, and lungs were collected. Subsequently, mice were inoculated with 10^4 CFU *S. pneumoniae* serotype 3 and sacrificed at 6 (n = 6) and 30 hours (n = 6). Outcome parameters were bacterial outgrowth, clinical score, and cytokine and chemokine levels (using Luminex®) in CSF, blood and brain. Meningeal inflammation, neutrophil infiltration, parenchymal and subarachnoidal hemorrhages, microglial activation and hippocampal apoptosis were assessed in histopathological studies.

Results: Lower doses of bacteria delayed onset of illness and time of death (median survival CFU 10^4, 56 hrs; 10^5, 38 hrs; 10^6, 28 hrs; 10^7, 24 hrs). Bacterial titers in brain and CSF were similar in all mice at the end-stage of disease independent of inoculation dose, though bacterial outgrowth in the systemic compartment was less at lower inoculation doses. At 30 hours after inoculation with 10^4 CFU of *S. pneumoniae*, blood levels of KC, IL6, MIP-2 and IFN-γ were elevated, as were brain homogenate levels of KC, MIP-2, IL-6, IL-1β and RANTES. Brain histology uniformly showed meningeal inflammation at 6 hours, and, neutrophil infiltration, microglial activation, and hippocampal apoptosis at 30 hours. Parenchymal and subarachnoidal and cortical hemorrhages were seen in 5 of 6 and 3 of 6 mice at 6 and 30 hours, respectively.

Conclusion: We have developed and validated a murine model of pneumococcal meningitis. Keywords: Meningitis, Critical care, Neurology, Animal model, Infectious diseases

BACKGROUND

Bacterial meningitis is a life threatening infectious disease of the central nervous system (CNS). The annual incidence is estimated to be up to 2.6 to 6.0 cases per 100 000 in Europe and may be up to ten times higher in developing countries (1, 2). The most common pathogen beyond the neonatal period is *Streptococcus pneumoniae* (1, 3), causing 70% of cases. Despite advances in medical care, mortality from pneumococcal meningitis remains between 16% and 37% and neurological sequelae affect 30-52% of survivors (4-6). There is a continuing need for the development of new treatment strategies. Complications associated with pneumococcal meningitis include cerebral infarction, hemorrhages, motor and sensory deficit, seizures, memory and learning impairments, and hearing loss (2, 7, 8). Autopsy studies of patients who died following pneumococcal meningitis revealed cerebral edema, cerebral infarctions and hemorrhages, apoptosis and necrosis of the hippocampal dentate gyrus (9-11). Many of these pathological features have been reproduced in animal models, which provide the setting for novel drug development and pathophysiological studies (12, 13). Several murine models have been developed, using intracerebral (14, 15), intraperitoneal (16), intravenous (16), intranasal (17) or intracisternal inoculation methods (18, 19), and have recently been reviewed (12). Problems with

reproducibility, limited disease progression or iatrogenic structural damage, combined with a need for a single model in which most pathological features seen in human pneumococcal meningitis can be measured, has fueled the development of new animal models. Here we describe the development of an adult mouse model of pneumococcal meningitis in which many of the human pathological features are demonstrated.

METHODS

A clinical isolate of *S. pneumoniae* serotype 3 was obtained from ATCC (catalog number 6303), and was grown to mid log phase in 4 hours at 37□C in Todd-Hewitt broth supplemented with 0.5% yeast extract. At an OD_{620} of 0.8 to 1.0 the *S. pneumoniae* were centrifuged and washed twice by resuspension in sterile 0.9% NaCl and recentrifugation. Finally, the bacteria were resuspended in sterile NaCl 0.9% to yield an approximate concentration of 1 x 10^9 colony forming units (CFU)/ml. The exact number of CFUs was subsequently determined for inoculates by serial dilution method and on blood agar plates (overnight at 37□C). Animal experiments were approved by the Institutional Animal Care and Use Committee of the Academic Medical Center, Amsterdam. To determine the inoculation dose and optimal time points of sacrifice, 24 8-10 week old male C57BL/6 mice (Charles River Laboratories, Germany) received 0.1 mg/kg s.c. buprenorphine and short-term anesthesia using 1.5-2.0% isoflurane during inoculation. The mice were divided into 4 groups, each receiving a different concentration of bacterial inoculum (10^4, 10^5, 10^6 and 10^7 CFU *S. pneumoniae* per mouse; n = 6 per dose). Inoculation was conducted by injecting 10 µL of bacterial suspension into the cisterna magna using a 32- gauge needle. All animals were evaluated directly following inoculation and subsequently at 4 hour intervals. The following scoring was used (Table 1): range: 0-41 pts; each scoring parameter ranging from 0, corresponding to no deficit, to a variable maximum score. The maximum score was determined by the estimated contribution of the variable to overall health of the mouse): weight loss (0-4 pts), activity (0-4 pts), time to return to upright position (0-6 pts), state of skin/fur (0-3 pts), posture (0-2 pts), eye discharge or protrusion (0-4 pts), respiration rate (0-4 pts), irregular/labored breathing (0-4 pts), epilepsy, limb paresis or ataxia (0-10pts). The clinical course was divided into a pre-symptomatic period (from time of inoculation until clinical score ≤ 10) and symptomatic period (clinical score > 10 until death/sacrifice). Survival studies were performed and cerebrospinal fluid (CSF), brain, blood, spleen, and lungs were collected post mortem. After determining inoculation dose and time-points of sacrifice, the model was further characterized using 12 additional mice inoculated with 10^4 CFU *S. pneumoniae* serotype 3 and sacrificed at 6 (n = 6) and 30 hours (n = 6). Bacterial titers were determined in samples of lung, brain, and spleen (diluted 1:4 in sterile NaCl 0.9% and homogenized). Blood was heparinized in a 1:4 dilution and CSF was diluted 1:100 in sterile NaCl 0.9%. All bacterial titers were determined by plating serial dilutions on blood agar plates and incubating overnight at 37°C. Cytokine and chemokine measurement were performed on the left cerebral hemisphere diluted 1:4 in sterile NaCl 0.9%, homogenized and lysed in lysis buffer (150 mM NaCl, 15 mM Tris, 1 mM $MgCl(H2O)_6$, 1 mM $CaCl_2$ $(H_2O)_2$, 1% Triton, AEBSF 4 µg/ml, EDTA-NA2 50 µg/ml, pepstatin 10 ng/ml, leupeptin 10 ng/ml, pH 7.4). Samples of brain homogenate, serum and CSF were then centrifuged and supernatant stored at -80 C. Cytokine concentrations were determined with luminex¨ technology using a mouse cytokine and chemokine Bioplex kit (Bio-Rad

Laboratories, Veenendaal, The Netherlands). All mice were perfused by cardiac puncture with PBS prior to harvesting tissue. Histopathology was performed on the right cerebral hemisphere fixed in 4% paraformaldehyde and paraffin embedded. Coronal 10 µm sections of the entire hemisphere were cut for subsequent staining. Hematoxylin and eosin (HE) and Nissl staining were performed to visualize hemorrhages, cortical necrosis, vasculitis and abscess formation. To determine neuronal apoptosis in the dentate gyrus of the hippocampus, four 10 µm sections of the anterior, middle and posterior portion of the hippocampus were stained with Caspase-3 antibodies (polyclonal rabbit-anti-mouse, 1:100; Cell Signalling, Danvers, MA). In each section, the total number of caspase-3 positive cells was counted in both the dentate gyrus (DG) and cornu ammonis (CA) regions. Scoring was independently conducted by two investigators. Microglial activation was evaluated by immunohistochemistry using Iba-1 antibody (polyclonal rabbit-anti-mouse, 1:2000; ABcam, Cambridge, UK) staining of frontal lobe 10 µm sections. No quantitative analysis was performed. Comparisons of cytokine levels between groups were calculated using the Mann-Whitney U test. A Kruskal-Wallis one-way ANOVA was used to compare clinical scores of pre-symptomatic and symptomatic periods. Histopathological scores of neuronal apoptosis were compared using Student' s t-test. For all analyses a p-value < 0.05 was considered significant.

TABLE 1 Clinical score parameters, assessed values and weighted scores

Parameter	Value	Weighted score	Maximum score
Weight loss from baseline	5%	0	
	10%	1	
	15%	2	
	20%	3	
	25%	4	4
Activity	normal	0	
	increased/decreased	1	
	mildly diminished	1	
	diminished	2	
	severely diminished	3	
	coma	4	4
Time to return to upright	normal	0	
position	upright < 5 sec	2	
	upright < 30 sec	4	
	No turn upright	6	6
Coat	normal	0	
	diminished grooming	1	
	soiled	1	
	piloerection	1	3
Posture	normal	0	
	slight hunched back	1	
	severe hunched back	2	2
Eyes	normal	0	
	protruding	1	
	sunken eyes	1	
	closed eyelids	1	
	discharge	1	4

Respiration rate (per min)	>150	0	
	<150	1	
	<100	2	
	<75	3	
	<50	4	4
Breathing	irregular	2	
	laboured	2	4
Neurologic exam	normal	0	
	ataxia	2	
	limb paresis/paralysis	2	
	epileptic seizure	2	
	status epilepticus	6	10
Total			41

RESULTS

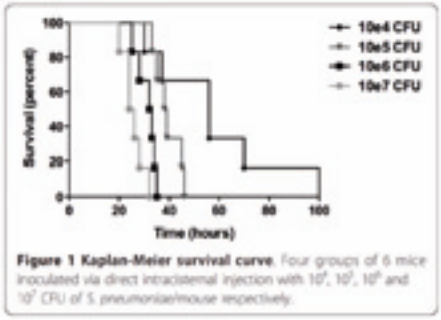

Figure 1 Kaplan-Meier survival curve. Four groups of 6 mice inoculated via direct intracisternal injection with 10^4, 10^5, 10^6 and 10^7 CFU of *S. pneumoniae*/mouse respectively.

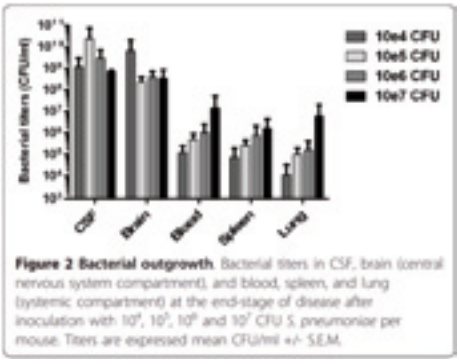

Figure 2 Bacterial outgrowth. Bacterial titers in CSF, brain (central nervous system compartment), and blood, spleen, and lung (systemic compartment) at the end-stage of disease after inoculation with 10^4, 10^5, 10^6 and 10^7 CFU *S. pneumoniae* per mouse. Titers are expressed mean CFU/ml +/- S.E.M.

Mortality occurred in nearly all inoculated mice (Figure 1); one mouse inoculated with 10^4 CFU survived beyond the study window of 216 hours after inoculation and was sacrificed. The median survival time was dose dependent (10^4 CFU, 56 hrs; 10^5, 38 hrs, 10^6, 28 hrs; 10^7, 24 hrs). To approximate a physiological setting, we selected 10^4 CFU as the lowest concentration of bacterial inoculum in which most animals would die if left untreated. Furthermore, for future experimentation we chose 30 hours post inoculation as the latest time point for sacrifice, at which all animals were still alive and the natural course of the infection could be followed as long as possible. Clinical scoring was performed on all mice in the survival study. The average duration of the pre-symptomatic period (clinical score $\leq$ 10) was dose dependent, and increased approximately 1.5-fold with each successive 10-fold increase of bacterial inoculum concentration. The duration of the symptomatic period did not differ significantly between inoculation doses (mean, 11.7 hrs, SD 4.8; Table 2). Bacterial meningitis was confirmed in all 23 mice in the survival study by way of culture of CSF and brain homogenate following death or sacrifice. The average pneumococcal concentration in the CSF and brain homogenates was 2.0 x 10^9 CFU/ml and 7.9 x 10^8 CFU/ml respectively. Bacterial titers in de CNS compartment (CSF and brain) did not increase with higher inoculation doses (Figure 2). In comparison with the CNS compartment, in the systemic compartment (blood, spleen, lung) bacterial concentrations were much lower

TABLE 2 Duration of pre-symptomatic (time from inoculation to clinical score ≤ 10) and symptomatic (time from clinical score > 10 to death/sacrifice) periods

Inoculation dose (CFU/mouse)	*10e4*	*10e5*	*10e6*	*10e7*	*p-value*
Pre-symptomatic period (hrs/st.dev)	40.7 (14.9)	26.1 (6.1)	18.7 (3.9)	12.0 (2.1)	0.001
Symptomatic period (hrs/st.dev)	9.1 (3.2)	13.0 (6.3)	10.8 (3.6)	13.6 (5.3)	0.557

(means 1.0 x 10^6, 4.0 x 10^5 and 2.0 x 10^5 CFU/ml, respectively), and an increasing bacterial titer was observed with each successive 10-fold increase of bacterial inoculum concentration. Mice with pneumococcal meningitis showed increased plasma levels of KC at 6 hours (Figure 3; median 62 versus 213 pg/ml, P = 0.004) and 30 hours (median 62 versus 2031 pg/ml, P < 0.0001) compared to saline inoculated mice. Furthermore, at 30 hours IL-6 (median 2 versus 202 pg/ml, P < 0.001), MIP-2

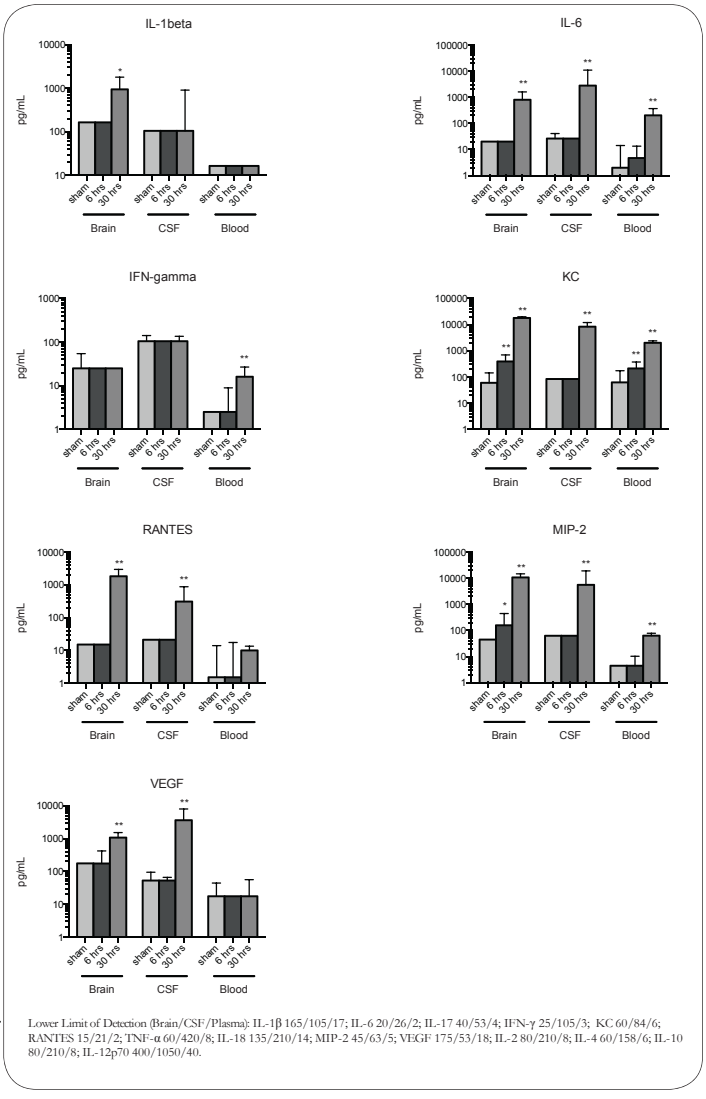

Figure 3 Cytokine levels in plasma, brain homogenates and CSF. Median concentrations (expressed in pg/ml) of cytokines in mice inoculated with either NaCl or 104 CFU/mouse of S. pneumoniae and sacrificed after 30 hours, or 6 and 30 hours respectively. Comparisons of cytokine levels between groups were calculated using the Mann-Whitney U test. (*P < 0.05; **P < 0.01).

Lower Limit of Detection (Brain/CSF/Plasma): IL-1β 165/105/17; IL-6 20/26/2; IL-17 40/53/4; IFN-γ 25/105/3; KC 60/84/6; RANTES 15/21/2; TNF-α 60/420/8; IL-18 135/210/14; MIP-2 45/63/5; VEGF 175/53/18; IL-2 80/210/8; IL-4 60/158/6; IL-10 80/210/8; IL-12p70 400/1050/40.

(median 5 versus 63 pg/ml, P =0.002) and IFN- γ (median 3 versus 16 pg/ml, P = 0.002) were elevated in plasma of *S. pneumoniae* compared to sham inoculated mice. IL-1β, IL-2, IL-4, IL-10, IL-12p70, IL-17, RANTES, TNF-α, IL-18 and IL-33 were not significantly altered in the plasma of mice with pneumococcal meningitis compared to sham controls. In brain homogenates, mice with pneumococcal meningitis compared to saline inoculated mice showed elevated levels of KC and MIP-2 at both 6 hours (Figure 3; KC median 60 versus 393 pg/ml, P < 0.0001; MIP-2 median 45 versus 159 pg/ml, P = 0.003) and 30 hours (KC median 60 versus 18116 pg/ml, P < 0.0001; MIP-2 median 45 versus 10637 pg/ml, P < 0.0001) time points. IL-6 (median 20 versus 795 pg/ml, P < 0.0001), IL-1β (median 165 versus 939 pg/ml, P = 0.014), and RANTES (median 15 versus 1823 pg/ml, P < 0.0001) were increased at 30 hours post infection in mice inoculated with pneumococcal meningitis as compared to saline inoculated mice. IL-2, IL-4, IL-10, IL-12p70, IL-17, IFN-γ, TNF-α, IL-18 and IL-33 were not altered in brain homogenates of mice with pneumococcal meningitis compared to sham controls. In CSF of mice with pneumococcal meningitis compared to saline inoculated mice, IL-6 (median 26 versus 2772 pg/ml, P = < 0.001), KC (median 84 versus 8369 pg/ml, P =0.002), MIP-2 (median 63 versus 5542 pg/ml, P = 0.002) and RANTES (median 21 versus 309 pg/ml, P = 0.005) are elevated 30 hours post infection. Histopathology at 6 hours after

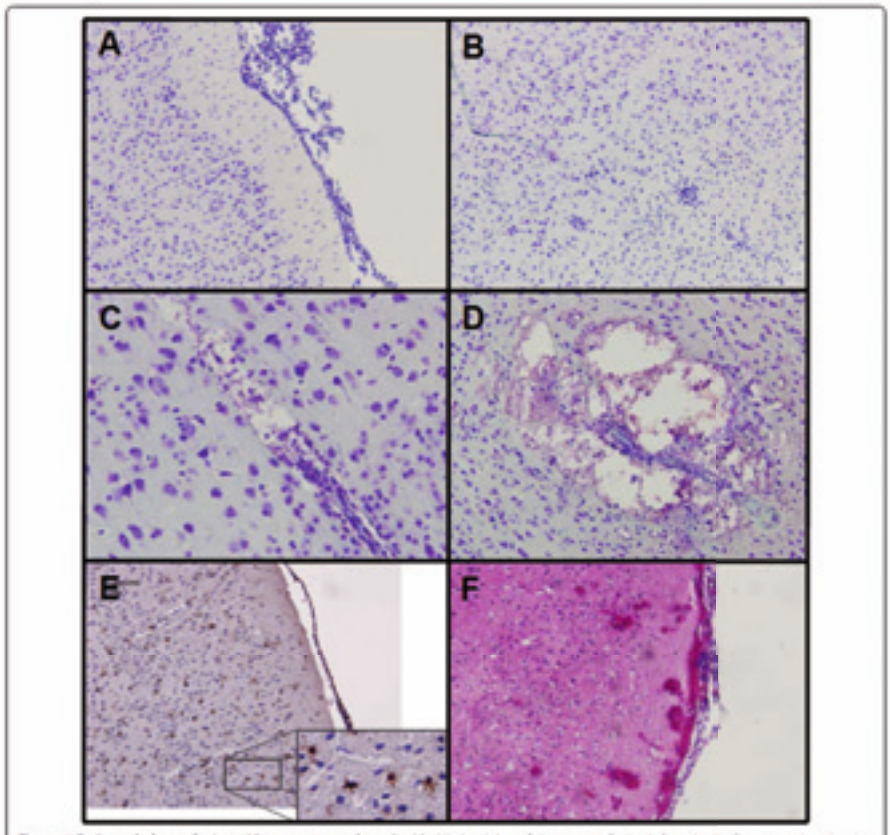

Figure 4 Brain pathology of mice with pneumococcal meningitis. Nissl staining of the cortex of mice infected with *S. pneumoniae*, showing extensive leptomeningeal inflammatory infiltrate (A; 100x magnification), perivascular lymphocytic cuffing (B; 100x magnification), perivascular lymphocytic infiltration combined with bacterial overgrowth (C; 200x magnification) and further bacterial overgrowth combined with perivascular necrosis (D; 100x magnification). Iba-1 immunohistochemistry revealed microglial activation (E; 100x magnification). Hematoxylin and eosin staining showed subarachnoidal and cortical hemorrhages (F; 100x magnification).

infection showed high levels of meningeal inflammation in both peripheral and ventricular CSF compartments, but none of the mice had parenchymal lymphocytic infiltration, hemorrhages, microglial activation, or hippocampal apoptosis (Figure 4A). However, 30 hours after inoculation 3 of the 6 mice showed parenchymal lymphocytic infiltration and pockets of bacteria were seen in 2 of 6 mice, located in the perivascular spaces of the penetrating vasculature (Figure 4B). At 30 hours, 5 of 6 mice had one or more parenchymal, mainly cortical, hemorrhages. Three mice demonstrated subarachnoidal hemorrhages. Extensive diffuse microglial activation was observed 30 hours after infection and at end stage-stage of disease at all inoculation doses (Figure 4E), although no quantitative analyses were performed. Neuronal apoptosis in the dentate gyrus of the hippocampus was scored independently by two investigators with a kappa of 0.75. A significant increase in hippocampus neuronal apoptosis was observed at 30 hours post infection and was significantly higher than saline infected mice (0.6 vs. 2.8 cells, $P < 0.001$; Figure 5).

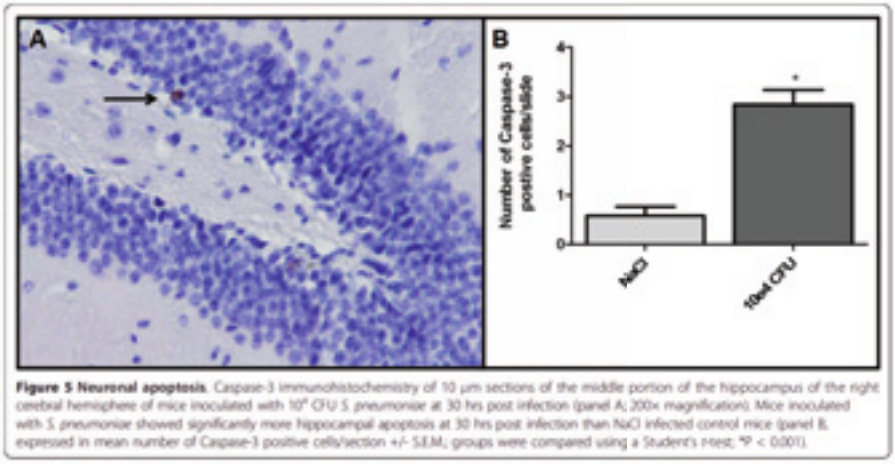

Figure 5 Neuronal apoptosis. Caspase-3 immunohistochemistry of 10 µm sections of the middle portion of the hippocampus of the right cerebral hemisphere of mice inoculated with 10^4 CFU *S. pneumoniae* at 30 hrs post infection (panel A; 200× magnification). Mice inoculated with *S. pneumoniae* showed significantly more hippocampal apoptosis at 30 hrs post infection than NaCl infected control mice (panel B; expressed in mean number of Caspase-3 positive cells/section +/- S.E.M.; groups were compared using a Student's *t*-test; *P < 0.001).

DISCUSSION

We developed a murine model of pneumococcal meningitis in which the histopathological and inflammatory features as well as observed complications resemble clinical and pathological findings in humans following bacterial meningitis (1, 20). The most important features of this model lie in the possibility of combining a relatively low dose of inoculum and long period of disease progression, allowing for a reproducible setting to examine clinical features as well as sufficient time to develop the histopathological features seen in a human setting. In previous murine models, pneumococcal meningitis was established by either 1) direct bacterial inoculation into the CNS, which generally very short survival times and thus limited use for the study of inflammation processes, or 2) intranasal or intraperitoneal inoculation routes, which more closely models the longer physiological inflammatory mechanisms (12). Unfortunately, mice inoculated via the intranasal or intraperitoneal route often died as the result of sepsis or pneumonia, and only 50% actually developed meningitis (17). In this model the comparison of clinical score progression between mice with different inoculation doses lead to the following conclusions: first, although the pre-symptomatic period was dose dependent (onset of symptoms were later at lower doses of bacterial inoculation), the duration of the symptomatic period was approximately

11.5 hours and similar between groups. This dose-dependent delayed onset provides a model in which direct inoculation in the CNS results in nearly 100% of mice developing meningitis, combined with a prolonged presymptomatic period in which various inflammatory mechanisms may be studied. Second, the clinical features contributing to deterioration were largely similar between the 4 different inoculation groups. For example, at the beginning of the symptomatic period (clinical score > 10) the most important contributing factors of clinical deterioration in all four inoculation groups were weight loss, diminished activity and deficits during neurological examination. At the final clinical assessment during the survival experiment, the most important additional factors of clinical deterioration in all 4 groups were the time to turn to upright position and increasing respiratory problems. TNF-α, IL-1 and IL-6 are considered to be the early response proinflammatory cytokines that are upregulated early in during pneumococcal meningitis (13). Surprisingly, TNF-α was not elevated at any time-point in our model. Previous animal models demonstrated that TNF-α was mainly increased during the first 6 to 24 hours of the immune response (21, 22). However, human studies show increased CSF levels of TNF-α but only early in the course of the disease (23, 24). This discrepancy may be explained by the lack of measurements that were performed between 6 and 30 hours after infection. IL-1β, which in humans is increased in the first 18 hours of infection (25), was also markedly increased in brain homogenates, but not in blood in our mice 30 hours after infection. The IL-6 concentrations did significantly increase CSF, brain homogenate and plasma 30 hours after infection. This is consistent with other infection models, in which IL-6, a cytokine displaying both pro- and anti-inflammatory properties (26), has been shown to be upregulated early during infection. In previous pneumococcal meningitis models IL-6 was shown to be involved in CSF leukocyte recruitment and possibly in the regulation of blood brain barrier disruption (27). The anti-inflammatory cytokine IL-10, which has been shown to downregulate TNF-α, IL-6 and KC was not measurably increased at any time point (28). Of the chemokines, the functional murine IL-8 homologue KC/CXCL1 and MIP-2/CXCL2 were both markedly increased in CSF, brain homogenate and blood at 30 hours after infection. Furthermore, early upregulation of KC and MIP-2 was also observed as early as 6 hours in brain homogenate, but not in plasma, where only KC was significantly increased. In humans, IL-8 has been shown to be elevated in CSF during pneumococcal meningitis (29), yet in a rabbit meningitis model it was systemic IL-8 that appeared to regulate CSF pleiocytosis (30). MIP-2, which is produced by astrocytes and microglial cells, but also by monocytes and macrophages, has been shown in vitro to be a chemoattractant for monocytes and neutrophils recruitment (29). Brain histopathology in our model resemble the human situation in pneumococcal meningitis, We found meningeal and parenchymal infiltration, (micro)hemorrhages, perivascular lymphocytic cuffing and perivascular bacterial overgrowth, the beginning of abscess formation, microglial activation, and neuronal apoptosis in the dentate hippocampal gyrus. Parenchymal (micro)hemorrhages were frequently observed (83%) and varied in size and location. These results reflect findings in a recent autopsy series in which microhemorrhages were found in 10 of 16 (67%) patients who died of pneumococcal meningitis (10). In the clinical setting clinical setting only 1-9% of all patients are documented to have intracranial hemorrhagic complications (4), which is likely to be an underestimation of the actual number of hemorrhages as only radiological evidence was included. In our model no cortical necrosis was observed at any time point, including in mice that died in the survival studies. Cerebral infarctions occur in approximately 30% of patients with

pneumococcal meningitis (5, 20, 31), and cortical necrosis has been modeled successfully in several rat and infant mouse meningitis models (19, 22). Possible reasons for the absence of necrosis may lie in the duration from inoculation until sacrifice, the choice of animal, age of the mice used, and antibiotic treatments used in other models. The underlying mechanisms for both ischemic stroke and hemorrhages remain unclear, though human CSF studies have suggested dysregulation of local coagulation cascade, complement activation, and diffuse cerebral intravascular coagulopathy (10, 13, 32). The observations of microglial activation at 30 hours after infection reflect in vitro findings in which microglial cells are activated after exposure to *S. pneumoniae* (33). Similarly, the delayed activation of microglial cells supports the results of a previous study in a rabbit model of pneumococcal meningitis in which increased levels of the microglial derived immunomodulatory protein activin A was found at 12 hours after inoculation (34). Microglia represent a specific subset of cells related to monocytes and dendritic cells and form the initial line of defense of brain parenchyma against damage, injury and infection and become activated in a toll-like receptor dependent fashion upon pneumococcal exposure (35, 36). Upon activation, microglia produce large amounts of proinflammatory cytokines, as well as reactive oxygen and nitrogen intermediates, thereby possibly playing both neuroprotective and neurotoxic roles (13, 37-39). The role of microglia during pneumococcal meningitis is largely unknown at present, but interest has been fueled by the observation that microglial activation in vitro is limited by corticosteroids treatment (40), which has become the standard adjuvant therapy in the treatment of bacterial meningitis in many countries (2, 41). Neuronal apoptosis was first observed in the human autopsy studies of patients who died of bacterial meningitis and was situated in the dentate gyrus of the hippocampus (9). Cognitive impairments and more specifically learning difficulties have been attributed to hippocampal apoptosis which has been modeled in mice, rats and rabbits (18, 42, 43). Furthermore, the adjuvant treatment of corticosteroids has been suggested as a possible factor aggravating hippocampal apoptosis and reducing learning capacity (42, 44). The process of apoptosis most likely occurs in an early caspase independent and a late caspase dependent mechanism (45). In this model we were able to detect the late stage caspase-3 dependent apoptosis at 30 hours post infection, providing an additional outcome parameter for further pathophysiological and therapeutic investigations.

CONCLUSIONS

The value of this mouse model is that it provides an experimental setting of pneumococcal meningitis which is highly reproducible, and provides several of the most valuable outcome parameters such as bacterial titers, meningeal and parenchymal infiltration, cytokine profiles, microglial activation, neuronal apoptosis in the hippocampus, perivascular infiltration and (micro)hemorrhages. We feel that the integration of these pathological features, which are characteristic of what is observed in human autopsy studies into a single model, is a valuable tool in the further investigation of both pathophysiological and therapeutic intervention studies.

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CHAPTER 4

DAPTOMYCIN IN EXPERIMENTAL MURINE PNEUMOCOCCAL MENINGITIS

Mook-Kanamori BB, Rouse MS, Kang C-I, van de Beek D, Steckelberg JM, Patel R.

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ABSTRACT

Background: Daptomycin, a lipopeptide antibiotic, could be an alternative to vancomycin for treatment of pneumococcal meningitis. We determined the activity of daptomycin versus vancomycin, with dexamethasone as an adjuvant, in a murine model of pneumococcal meningitis.
Methods: Ninety-six 25–30 gram mice were inoculated intracisternally with serotype 3 *Streptococcus pneumoniae* modified by the integration of a luminescent lux operon. All mice were treated with either dexamethasone 1 mg/kg intraperitoneally every 6 hours alone or in combination with either vancomycin or daptomycin, also administered intraperitoneally. Serum antimicrobial concentrations were selected to approximate those achieved in humans. Following treatment, bioluminescence and cerebrospinal fluid (CSF) bacterial concentrations were determined. Caspase-3 staining was used to assess apoptosis on brain histopathology.
Results: Sixteen hours post intracisternal inoculation, bacterial titers in CSF were 6.8 log10 cfu/ml. Amongst the animals given no antibiotic, vancomycin 50 mg/kg at 16 and 20 hours or daptomycin 25 mg/kg at 16 hours, CSF titers were 7.6, 3.4, and 3.9 log10 cfu/ml, respectively, at 24 hours post infection (p-value, < 0.001 for both vancomycin or daptomycin versus no antibiotic); there was no significant difference in bactericidal activity between the vancomycin and daptomycin groups (p-value, 0.18). CSF bioluminescence correlated with bacterial titer (Pearson regression coefficient, 0.75). The amount of apoptosis of brain parenchymal cells was equivalent among treatment groups.
Conclusion: Daptomycin or vancomycin, when given in combination with dexamethasone, is active in the treatment of experimental pneumococcal meningitis.

BACKGROUND

Pneumococcal meningitis is associated with high mortality and morbidity rates (1). Patients with suspected or proven pneumococcal meningitis should receive immediate empirical therapy consisting of a combination of a third-generation cephalosporin and vancomycin, plus dexamethasone (1). Daptomycin, a lipopeptide antibiotic which has excellent activity against a broad range of Gram-positive microorganisms, including penicillin and cephalosporin resistant pneumococci (2, 3), could be an alternative to vancomycin for treatment of pneumococcal meningitis. Rapid bactericidal activity, without lysis and limited inflammatory response, makes daptomycin an attractive possibility for the treatment of multidrug-resistant pneumococcal meningitis (2, 3). No published animal studies have evaluated the combination of daptomycin plus dexamethasone for treatment of experimental pneumococcal meningitis. In this study, we used a murine model of pneumococcal meningitis to compare the activity of daptomycin with that of vancomycin, when given in combination with dexamethasone.

METHODS

Bacteria

Streptococcus pneumoniae A 66.1 serotype 3 rendered bioluminescent by integration of a modified lux operon into its chromosome (*S. pneumoniae* Xen 10, Xenogen Corporation, Alameda, CA) was studied. This strain has been previously shown to be as virulent as its parent strain (4). Serotype 3 is one of the most common

pneumococcal serotypes causing community-acquired bacterial meningitis in adults (5). Bacteria were incubated in Todd-Hewitt broth (THB) at 37□C in 5% CO2 to an OD620 of 0.4 and subsequently rinsed in phosphate buffered saline (PBS) and centrifuged to yield a final bacterial concentration of 5 x 10^{10} colony forming units (cfu)/ml. The exact number of cfu in the inoculum was determined retrospectively by growth of serial dilutions of the inoculum on blood agar plates.

In vitro studies

The MBC and MIC of daptomycin and vancomycin (with and without dexamethasone), and of penicillin, against *S. pneumoniae* Xen10 were determined using methods described by the Clinical Laboratory Standards Institute. Time kill studies to determine the bactericidal activity of daptomycin 1 μg/ml or vancomycin 2 μg/ml alone and in combination with dexamethasone 100 μg/ml were performed as described by Anhalt and Washington (6).

Pharmacokinetics of vancomycin and daptomycin

The 30 minute serum concentration of vancomycin or daptomycin in 25□30 gram immunocompetent hairless mice (Charles River Laboratories, Wilmington, MA) was determined following intraperitoneal injection of 40, 50 and 60 mg/kg vancomycin or 20, 30, 40 and 50 mg/kg daptomycin, respectively. Pharmacokinetic profiles were determined by injecting 50 mg/kg vancomycin or 25 mg/ kg daptomycin intraperitoneally and obtaining serum samples 30, 60, 120, 180 and 240 minutes post injection. Drug activity was measured using a microbiological assay technique. Standard curves for serum drug level determination were performed by making drug dilutions in pooled mouse serum (Innovative Research, Incorporated, Southfield, MI). The reporter bacteria for the vancomycin and daptomycin bioassays were Bacillus subtilis and Micrococcus luteus, respectively. Zones of growth inhibition were measured to the nearest millimeter. The detection limit for both drugs was 0.5 μg/ml.

Murine meningitis model

This study was approved by the Institutional Animal Care and Use Committee of the Mayo Clinic, Rochester, Minnesota. Ninety-six 25□30 gram immunocompetent hairless mice were divided into three treatment groups. Sixteen hours prior to treatment, mice were anesthetized using ketamine and xylazine (45 mg/kg and 5 mg/kg I.M., respectively) and 3 x 10^4 cfu of bacteria in a 20 μL volume were inoculated into the cisterna magna. The time of treatment was selected as the latest time point at which antimicrobial treatment could realistically lead to recovery. Treatment was initiated by dividing all inoculated mice into three groups and treating with 1 mg/kg dexamethasone alone, or in combination with 25 mg/kg daptomycin or 50 mg/kg vancomycin. At 22 hours post infection an additional dose of dexamethasone 1 mg/kg was administered. At 20 hours post infection an additional dose of vancomycin 50 mg/kg was given to the vancomycin treated animals. Mice not receiving vancomycin at 20 hours were injected with an equal volume of saline. At 16, 20, and 24 hours post infection, 8, 40 and 48 animals, respectively were re anesthetized and cerebrospinal fluid (CSF) was collected by way of puncture from the cisterna magna (using a 28 G 1/2 needle 0.3 x 13 mm from BD Microlance). CSF white-blood cell count was determined and bacterial burden was quantified in CSF by plating serial 10-fold dilutions in sterile isotonic saline onto blood agar plates. Results were expressed as log10 cfu/ml; the detection limit was 20 cfu/ml of CSF. Following collection of CSF, animals were euthanized with a lethal dose of pentobarbital, their

brains collected and placed in 10% neutral buffered formalin. There was no mortality (prior to tissue harvest).

Imaging studies
In vivo bioluminescence imaging was performed using the Lumazone Imaging System (1002 FE series; Roper Scientific, Tucson, Arizona) at 16, 20 and 24 hours post infection. Animals were sedated with ketamine plus xylazine, placed in an imaging box without restraint, and imaged for a maximum of 10 minutes at 4 x 4 binning resolution. Luminescence was quantified in photons/sec; correlation analysis with bacterial titers was performed.

Histopathology studies
Serial coronal sections of formalin-fixed whole brain were prepared. Brain was routinely processed, embedded in paraffin, sectioned at approximately 5□6 µm, and stained with hematoxylin and eosin (H&E), cresyl echt violet for Nissl substance, and a rabbit anti-human cleaved caspase- 3 HRP antibody (Seventh Wave Laboratories, Chesterfield, MO), for apoptosis. The whole brain sections were scored for inflammation, neuronal necrosis, and parenchymal apoptosis by an independent veterinary pathologist (Dr. Ewing, Genzyme Corporation, Cambridge, MA).

Statistics
Bacterial concentrations between treatment groups at each time point were compared using the student t-test. Correlation analysis between log10 bacterial CSF titers and log10 photons/second was calculated using SPSS statistical software. For inflammation, neuronal necrosis, and apoptosis scores, statistical analysis included a nonparametric Kruskal-Wallis test followed by a Dunn's test comparing all treatment groups using a confidence level of 95%.

RESULTS

In vitro studies
The MIC/MBC of daptomycin, vancomycin, penicillin and dexamethasone were 0.06/0.125, 0.125/0.25, 0.03/ 0.06 and > 128/> 128 respectively. The in vitro bactericidal activity of daptomycin and vancomycin was not affected by 100 µg/ml of dexamethasone (Figure 1).

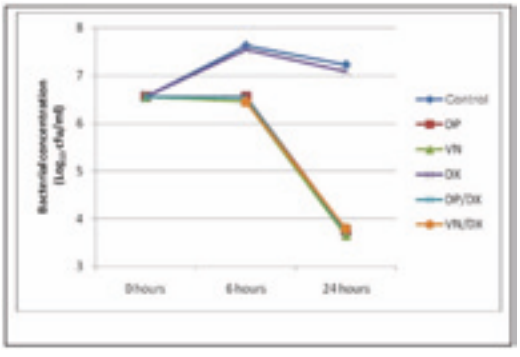

FIGURE 1: Time kill assay. Bactericidal activities of daptomycin (1 µg/ml) or vancomycin (2 µg/ml) are not affected by dexamethasone (100 µg/ml). DP, daptomycin; VN, vancomycin; DX, dexamethasone.

Pharmacokinetics of vancomycin and daptomycin
The highest dose of daptomycin currently approved by the FDA is 6 mg/kg q.d.

intravenously, corresponding to a serum Cmax of 90□100 µg/ml. In mice, 25 mg/kg of daptomycin intraperitoneally was found to best approximate the human Cmax and used for the remainder of the experiments (Figure 2). A 30 minute daptomycin concentration of 127 µg/ml decreased to 16 µg/ml after 4 hours. Vancomycin was given at a dose of 50 mg/kg, which yielded a level of 55 µg/ml at 30 minutes, thereafter rapidly declining to < 0.5 µg/ml at 3 hours (Figure 2). Because of its short half-life, vancomycin was given every 4 hours, whereas daptomycin was given once. Dexamethasone was given every 6 hours.

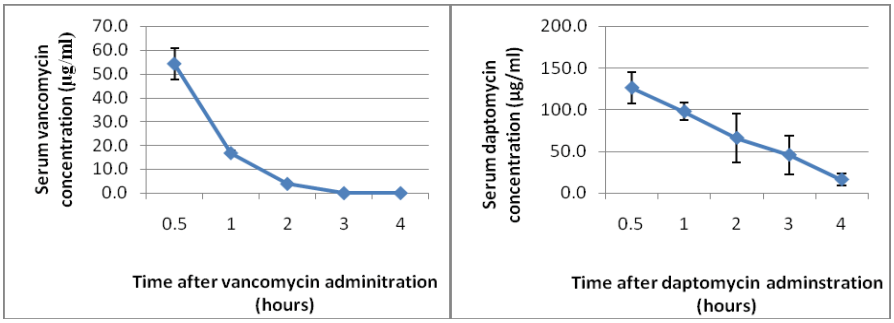

FIGURE 2: Serum antibiotic concentration after a single intraperitoneal dose of vancomycin (50 mg/kg) or daptomycin (25 mg/kg).

Murine meningitis model

Daptomycin and vancomycin, given in combination with dexamethasone, resulted in similar bactericidal activity in vivo. Mice treated with dexamethasone alone showed an increase in CSF pneumococcal concentration after 8 hours (+ 1.31 log10 cfu/ml), but not after 4 hours (- 0.02 log10 cfu/ml) of treatment (Table 1, Figure 3). Four hours after a single treatment with either daptomycin or vancomycin accompanied by 1 mg/kg of dexamethasone, bacterial titers dropped by 1.33 and 0.43 log10 respectively (p-value: 0.004 and 0.36, respectively, versus no antibiotic). After 8 hours, daptomycin-/dexamethasone- and vancomycin-/ dexamethasone-treated mice showed a reduction of bacterial titer of 2.33 log10 and 2.82 log10 versus no antibiotic (p-value < 0.001 for both groups). The antibiotic treatment groups differed significantly at 4 (p-value 0.02 daptomycin versus vancomycin), but not at 8 (p-value 0.18, daptomycin versus vancomycin) hours. At 8 hours, 2/16 of the daptomycin-/dexamethasone-treated mice and 4/ 16 of the vancomycin-/dexamethasone-treated mice had bacterial concentrations below the limit of detection. White blood cell quantification in the CSF appeared to be influenced by blood contamination during sample collection. No significant difference between treatment groups was observed at any time point (data not shown).

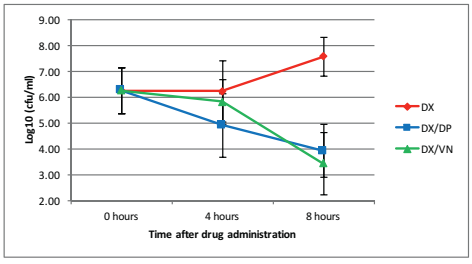

FIGURE 3: Concentrations of *S. pneumoniae* in cerebrospinal fluid. Concentration of *S. pneumoniae* in cerebrospinal fluid after 0, 4 and 8 hours of intraperitoneal injection of dexamethasone (administered at 0 and 6 hours) alone (DX), daptomycin (administered at 0 hours) and dexamethasone (administered at 0 and 6 hours)

(DX/DP), or vancomycin (administered at 0 and 4 hours) and dexamethasone (administered at 0 and 6 hours) (DX/VN).

Treatment	0 hours (log_{10} cfu/ml)	4 hours (Δlog_{10} cfu/ml)	8 hours (Δlog_{10} cfu/ml)
Dexamethasone	6.24 ± 0.86	-0.02 ± 1.49	1.31 ± 1.16
Dexamethasone/daptomycin*		-1.33 ± 1.24	-2.33 ± 1.51
Dexamethasone/vancomycin#		-0.43 ± 1.49	-2.82 ± 1.36

*Daptomycin 25 mg/kg intraperitoneally, administered at 0 hours.
#Vancomycin 50 mg/kg intraperitoneally, administered at 0 and 4 hours.

TABLE 1. Mean bacterial concentration and change, after 0, 4 or 8 hours of treatment respectively, expressed in log_{10} cfu/ml. Concentrations at 0 hours are expressed as mean ±SD.

Imaging studies

Due to technical problems with the imaging system, 70 of 96 (73%) mice were imaged. Forty-six mice had luminescence lower than the detection limit and were not included in the correlation analysis. Amongst the 24 animals with luminescence above the detection limit, imaging findings were consistent with meningitis without infection of other sites (Figure 4). Imaging studies revealed a correlation (Pearson's R) of 0.75 between CSF bacterial titer and photons per second ($P < 0.0001$). The luminescence detection limit corresponded to 4.0 log10 cfu/ml or 5.0 log10 photons/sec, when imaging for 10 minutes at 4 x 4 binning resolution (Figure 5).

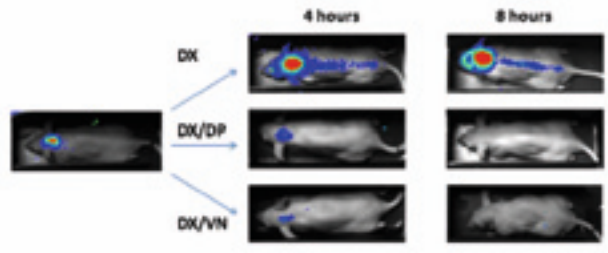

FIGURE 4: Representative bioluminescence images. Representative bioluminescence images taken using the Lumazone Imaging System after 0, 4 and 8 hours of treatment with dexamethasone (administered at 0 and 6 hours) alone (DX), daptomycin (administered at 0 hours) and dexamethasone (administered at 0 and 6 hours) (DX/DP), or vancomycin (administered at 0 and 4 hours) and dexamethasone (administered at 0 and 6 hours) (DX/VN). Photons/second are displayed on a calibrated color overlay (blue = low, through red = high).

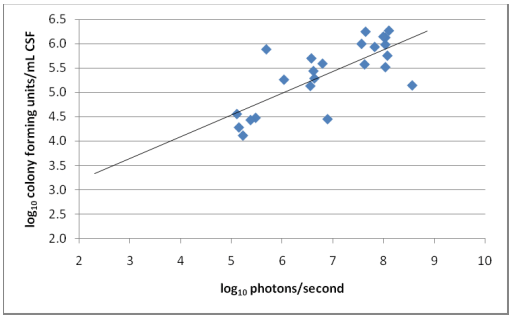

FIGURE 5: Correlation between bacterial cerebrospinal fluid concentration and chemiluminescence (Pearson's R, 0.75; P < 0.0001).

Histopathology studies

Mice intracisternally inoculated with *S. pneumoniae* had minimal to mild, focal to multifocal meningeal infiltrates of predominantly neutrophils admixed with small numbers of macrophages and small lymphocytes at one or more levels of the brain at

all time points examined (Figure 6). Inflammatory cell infiltrates displayed mild multifocal apoptosis. The amounts of inflammation and apoptosis of brain parenchymal cells were equivalent among treatment groups (data not shown). Some of the mice had involvement of the brain parenchyma primarily limited to the brainstem. The involvement featured focal neutrophilic encephalitis or abscess formation associated with variable numbers of bacterial cocci, neuronal necrosis or loss, hemorrhage, and vacuolation of the neuropil. The density of bacterial cocci in some of the foci of encephalitis of antibiotic-treated mice appeared to be lower than that of the pre-treatment or dexamethasone only treated mice. Neuronal necrosis was not observed in any other areas of the brain. The incidence of encephalitis in the dexamethasone alone, daptomycin and vancomycin groups at 20 hours was 4/8 (50%), 4/14 (29%), and 3/15 (20%), respectively ($P > 0.05$). The incidence of encephalitis in the dexamethasone alone, daptomycin and vancomycin groups at 24 hours was 5/10 (50%), 2/15 (13%), and 5/14 (36%), respectively ($P > 0.05$).

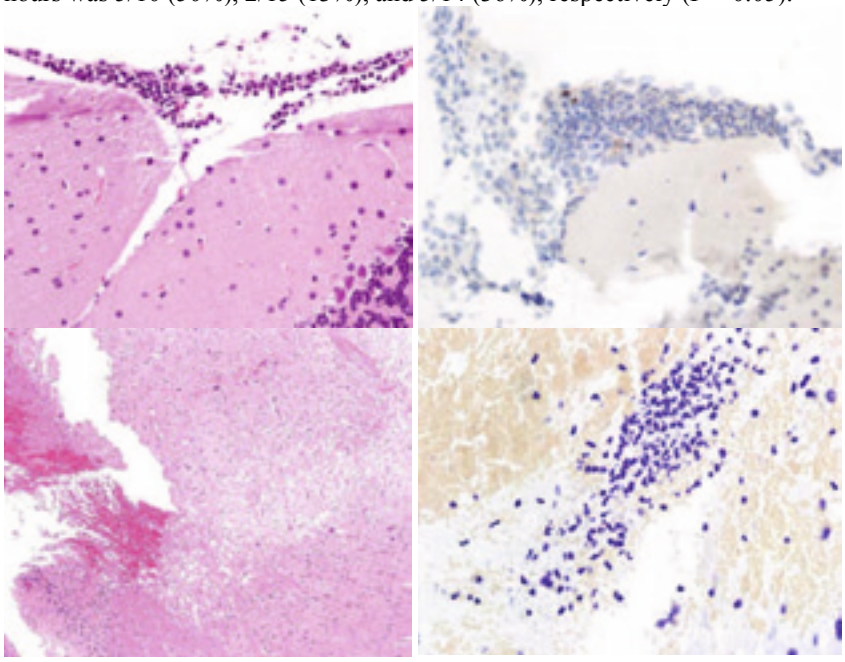

FIGURE 6: Photomicrographs of brain from pre-treatment (16 hours) mice. Upper panels; H&E 400x left, caspase-3 400x right, and lower panels; H&E 400x left; cresyl echt violet 500x right. Meninges of the cerebellum showed a mild infiltrate of neutrophils (upper left panel), exhibiting multifocal positivity with the apoptosis marker (upper right panel). Foci of necrosis with hemorrhage in the brain stem (lower left panel), focally infiltrated by neutrophils and associated with bacterial cocci (lower right panel), were noted.

Discussion

At drug serum concentrations approximating the human Cmax, daptomycin and vancomycin were both highly effective at killing *S. pneumoniae* in CSF, when given in combination with dexamethasone. Daptomycin was more rapidly bactericidal than vancomycin, resulting in a significantly lower bacterial CSF titer after just four hours of treatment, although after eight hours, CSF bacterial counts did not differ significantly. In vitro time-kill studies demonstrated equivalent bactericidal activity of

daptomycin and vancomycin. Four studies of daptomycin treatment of experimental meningitis have been reported (6-9). Using a rabbit meningitis model, Cottagnoud et al. showed a 6% (of serum drug levels) penetration of daptomycin across inflamed meninges, and better activity than ceftriaxone plus vancomycin against penicillin-resistant pneumococcal strains (6). A follow-up study showed that daptomycin was associated with negligible release of [3H]choline (a marker of cell wall lysis) compared with ceftriaxone treatment (8). Similarly, Gerber et al. reported bactericidal activity in a rabbit model of Staphylococcus aureus meningitis; they also observed that the degree of meningeal inflammation affected the penetration of daptomycin across the bloodbrain- barrier (7). Finally, Grandgirard et al. compared daptomycin with ceftriaxone in an experimental model of rat pneumococcal meningitis; daptomycin more efficiently cleared pneumococci from CSF than did ceftriaxone, reduced the concentration of matrix metalloproteinase-9 concentration in CSF 40 hours after infection, and prevented development of cortical injury (9). None of these studies used dexamethasone as adjuvant treatment, which has become standard practice in the treatment of human bacterial meningitis in adults in developed countries, and which may impact the activity of antimicrobial agents in meningitis (10, 11). The pharmacokinetic and pharmacodynamic properties of both vancomycin and daptomycin have been described in previous studies, and have been shown to vary considerably between animal models. Based on the pharmacokinetic characteristics in our mouse meningitis model, our best approximation of human pharmacokinetic parameters was achieved by giving daptomycin once and vancomycin twice, four hours apart. Vancomycin was rapidly cleared from the serum of the mice in our study. Thus, at the 4 hour time point, all treatment groups had received a single dose of antimicrobial. At the 8 hour time point, the vancomycin treated group had received two doses of vancomycin, and all mice had received a second dose of dexamethasone. Although pharmacokinetic approximation is clearly a limitation of animal studies, careful conclusions about the efficacy of both drugs in our model can still be made. The use of bioluminescent *S. pneumoniae* and the Lumazone imaging system allow for real-time, non-invasive determination of bacterial activity in CSF as a measure of infection and treatment thereof, thus limiting the need for multiple invasive CSF withdrawals per animal, and reducing the number of required animals per treatment group.

However, the bioluminescent detection limit corresponded to 4 log10 cfu/ml, almost 2 log10 higher than the detection limit using quantitative cultures (2.3 log10 cfu/ ml), making luminescent imaging most useful during the initial phase of the infection. Although bioluminescent data was not obtained on all animals in this study, animals with the complete spectrum of bacterial CSF concentrations were imaged. Because bioluminescence measured the activity of bacteria rather than actual bacterial killing, no definite conclusions can be inferred regarding the bactericidal versus bacteriostatic effects of the antimicrobial treatment regimens. Nevertheless, in vivo photonic imaging remains a relevant method for studying the pathogenesis and pathophysiology of bacterial meningitis. Clinical trials of daptomycin for the treatment of Grampositive bacteremia, endocarditis, and complicated skin and skin-structure infections have yielded favorable results (2, 3, 12). A case report of successful treatment of methicillin-resistant *S. aureus* meningitis with daptomycin has been reported (13). In vitro and animal model studies have shown diminished inflammatory response to infections treated with daptomycin compared with comparators (11, 14). The lack of noted differences in inflammation among treatment

groups that had statistically significantly lower mean bacterial titers (i.e., daptomycin and vancomycin groups compared to no antibiotic groups) may be related to the relatively early time point of collection of brains, and/or to the concomitant administration of dexamethasone. Histopathologic evaluation of brains at later time points may be required to appreciate treatment group differences in inflammation. Further animal studies are necessary to investigate the effects of daptomycin/ dexamethasone treatment on outcome parameters such as CSF inflammation, brain tissue damage and residual neurological deficit, in the context of dexamethasone treatment.

CONCLUSION

Daptomycin or vancomycin, when given in combination with dexamethasone, is active in the treatment of experimental pneumococcal meningitis. The observed bactericidal activity in this study is consistent with previous studies and provides support for future evaluation of daptomycin as an alternative to vancomycin in the treatment of bacterial meningitis.

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CHAPTER 5

GENETIC VARIATION IN INFLAMMASOME GENES IS ASSOCIATED WITH OUTCOME IN BACTERIAL MENINGITIS

Geldhoff M[*], Mook-Kanamori BB[*], Brouwer MC, Valls Seron M, Baas F, van der Ende A, van de Beek D.

[*]contributed equally in the writing of this paper.

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ABSTRACT

Bacterial meningitis is a severe and deadly disease, most commonly caused by *Streptococcus pneumoniae*. Disease outcome has been related to severity of the inflammatory response in the subarachnoid space. Inflammasomes are intracellular signaling complexes contributing to this inflammatory response. The role of genetic variation in inflammasome genes in bacterial meningitis is largely unknown. In a prospective nationwide cohort of patients with pneumococcal meningitis, we performed a genetic association study and found that single-nucleotide polymorphisms in the inflammasome genes CARD8 (rs2043211) and NLRP1 (rs11621270) are associated with poor disease outcome. Levels of the inflammasome associated cytokines interleukin (IL)-1β and IL-18 in cerebrospinal fluid also correlated with clinical outcome, but were not associated with the CARD8 and NLRP1 polymorphisms. Our results implicate an important role of genetic variation in inflammasome genes in the regulation of inflammatory response and clinical outcome in patients with bacterial meningitis.

INTRODUCTION

Bacterial meningitis is associated with high mortality, even in developed countries despite the implementation of childhood vaccination programs and effective antimicrobial agents (1, 2). The most common causative agent is *Streptococcus pneumoniae*, with case fatality rates ranging from 16 to 37% (1-3), and neurological sequelae, including hearing loss (4), focal neurological deficits, and cognitive impairment, occurring in 30–52 % of surviving patients (5, 6). Host genetic variation has been shown to influence susceptibility and outcome of pneumococcal and meningococcal infections (7, 8). The inflammasomes are intracellular signaling complexes belonging to the Nod-like receptors (NLRs) (9-11). To date, four major inflammasome complexes have been described, of which the Nod-like receptor protein 3 (NLRP3) inflammasome has been investigated most extensively (9, 10). The inflammasomes can be activated by several endogenous as well as exogenous danger signals, including ATP, changes in K^+ concentration, oxygen radicals, and uric acid released through cell injury in inflammation (12, 13). Bacterial components with inflammasome-activating properties include bacterial DNA and bacterial toxins. The primary result of inflammasome activation is the binding and activation of caspase-1 (12, 13). While several inflammasomes are capable of directly converting the inactive procaspase- 1 into the active form (e.g., NLRP1 and NLRC4), some (e.g., NLRP3) require the binding of an adaptor protein ASC (adaptor apoptosis-associated speck-like protein). The caspase recruitment domain (CARD) on either NLRP itself or ASC then binds to a CARD domain on the inactive caspase-1, which subsequently can be activated. Active caspase-1 contributes to the conversion of the inactive pro-interleukin-1 beta (pro-IL-1β) and pro-IL-18 into the respective active and secreted cytokines (9-11). One of the key regulators of caspase activity has been shown to be caspase-associated recruitment domain-8 (CARD8), which has been demonstrated to bind the CARD domain of caspase-1 and negatively regulate IL-1β and IL-18 production (14). Several findings in patients and animal models suggest a pivotal role for inflammasomes in the pathophysiology of bacterial meningitis (15). Firstly, in adults with bacterial meningitis, cerebrospinal fluid (CSF) levels of caspase-1 were elevated compared to noninfected patients (16). Furthermore, in children with pneumococcal meningitis, IL-1β concentrations in the CSF were elevated, a finding

that also has been observed in various animal models (17-19). While the role of IL-1β in the pathogenesis of pneumococcal meningitis has not been elucidated yet, various clinical effects have been attributed to caspase-1, IL-1β, and IL-18-mediated processes (15, 16, 18, 20). Finally, recent pneumococcal meningitis animal studies showed that lack of the inflammasome components ASC or NLRP3 decreased scores of clinical and histological disease severity in murine pneumococcal meningitis (20). We performed a prospective nationwide genetic association study in patients with community-acquired bacterial meningitis to investigate the role of common variants in genes encoding inflammasome components NLRP1, NLRP3, NLRC4, AIM2, PYCARD (ASC), as well as regulator protein CARD8 on clinical outcome. Subsequently, we determined the principle products of inflammasome activation, IL-1β and IL-18, in the CSF of patients with bacterial meningitis and looked for associations with clinical outcome and the genetic polymorphisms.

METHODS

In a nationwide prospective cohort study, we included bacterial meningitis patients older than 16 years of age with positive CSF cultures who were identified by The Netherlands Reference Laboratory for Bacterial Meningitis (NRLBM) from March 2006 to June 2009. The NRLBM provided the names of the hospitals where patients with bacterial meningitis had been admitted 2–6 days previously. The treating physician was contacted, and informed consent was obtained from all participating patients or their legally authorized representatives. Controls for exposure/susceptibility were patients' partners or their nonrelated proxies living in the same dwelling. Data on age, sex, and ethnicity of controls were collected. Secured online case-record forms were used to collect data on patient history, symptoms and signs on admission, treatment, complications, and outcome. Outcome was graded at discharge according to the Glasgow Outcome Scale, a well-validated instrument with good interobserver agreement (21). A score of 1 on this scale indicates death, a score of 2 a vegetative state, a score of 3 severe disability, a score of 4 moderate disability, and a score of 5 mild or no disability. A favorable outcome was defined as a score of 5, and poor outcome as a score of 1–4. The research ethics committee of the Academic Medical Center approved the study.

GENOTYPING

We selected nonsynonymous single-nucleotide polymorphisms (SNPs) in coding regions of genes involved in the inflammasome activation (NLRP1, NLRP3, NLRC4, PYCARD, AIM2, and CARD8) for which a commercial genotyping assay was available and the reported minor allele frequency (MAF) was >5 %. Selected SNPs in NLRP1 (rs12150220, rs11651270, and rs2301582), NLRP3 (rs10754558 and rs35829419), PYCARD (rs11648861), AIM2 (rs2276405), and CARD8 (rs2043211) were genotyped using TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City, CA, USA) in a LightCycler480 (Roche, Basel, Switzerland) using the TaqMan Genotyping Master Mix (Applied Biosystems), at the Department of Genome Analysis in the Academic Medical Center, Amsterdam, The Netherlands. We additionally genotyped one uncommon SNP in NLRP3 (rs35829479; reported MAF 2.5 %) as it was previously described to interact with the rs2043211 in CARD8 in inflammatory disease (22). No assays for common nonsynonymous SNPs in the coding regions of NLRC4 were commercially available. Laboratory personnel were

blinded to clinical information. IL-1β and IL-18 measurements in CSF samples of patients with bacterial meningitis We measured IL-1β and IL-18 in the CSF of patients with bacterial meningitis included in the cohort and 19 control patients with Luminex® technology using a Milliplex assay (Millipore, Billerica, MA, USA). CSF from the first diagnostic tap was collected, centrifuged, and supernatant was aliquoted and stored at −80 °C until analysis. Control CSF was obtained from patients evaluated for acute headache, without signs of meningitis and normal CSF findings. In these patients, a subarachnoid hemorrhage was excluded as cause of their headache by CSF examination. Leftover CSF was collected and centrifuged, and the supernatant was stored at −80 °C until analysis.

STATISTICS

The Mann–Whitney U test was used to identify differences in baseline characteristics among groups with respect to continuous variables, and dichotomous variables were compared with use of the $\chi 2$ test. These statistical tests were two-tailed, and $P<0.05$ was regarded as significant. Differences in genotype frequencies were analyzed with the $\chi 2$ or Fishers' exact tests by use of the programs SPSS 19. The main analysis was limited to common SNPs (i.e., minor allele frequencies >5 %). For the functional SNP rs2043211 in CARD8, $P<0.05$ was used to indicate significance. For the other four common SNPs, we performed the analysis both with ($P<0.0125$) and without ($P<0.05$) correction for multiple testing. A further analysis was performed to determine the effect of having either a variant allele for rs35829479 (NLRP3) or rs2043211 (CARD8) on outcome, as this combination was previously described to be associated with inflammatory disease, using $P<0.05$ to indicate significance. We calculated whether the genotype frequencies in the control groups concurred with the Hardy–Weinberg equilibrium (HWE) by use of a $\chi 2$ and exact test with one degree of freedom. SNPs deviating from the HWE were excluded. The genotype frequencies of patients with a favorable outcome were compared with those with poor outcome as defined by the Glasgow Outcome Scale. Survival data were plotted for the different genotypes using a Kaplan–Meier curve and analyzed using a log rank test. We corrected for possible confounders (age, sex, immunocompromise, and prehospital antibiotic treatment) by performing a multivariate logistic regression analysis including identified polymorphisms and potential confounders. Furthermore, we performed a test of formal interaction of gender and CARD8 rs2043211genotype to assess if a gender specific association of this SNP influenced outcome (23).

RESULTS

A total of 801 Dutch patients with bacterial meningitis were included as described previously (24). In this study, the distribution of causative organisms was *S. pneumoniae* in 576 episodes (72 %), Neisseria meningitidis in 92 (12 %), Listeria monocytogenes in 41 (5 %), and other bacteria in 92 (12 %) episodes. The case fatality rate was 18 %, and 38 % of patients had poor clinical functional outcome as defined as scores of 1–4 on the Glasgow Outcome Scale. DNA was available for 531 (66 %) patients and 376 controls. Clinical characteristics of this patient population are provided in Table 1. Genotyping success rate was >95 % for all assays. Three SNPs that were uncommon (PYCARD rs11648861) or monomorphic (NLRP3 rs35829419, and AIM2 rs2276405) were excluded from the analysis. The genotype frequency

concurred with the Hardy–Weinberg equilibrium in the control population for all SNPs. We identified rs2043211 in CARD8 to be associated with poor outcome of

Characteristic	Value/total	Characteristic	Value/total
Age (years)	55±17	Indexes of CSF inflammation[b]	
Male sex	262 (49 %)	Opening pressure (mmH$_2$O)	34±11
Pretreatment with antibiotics	63/527 (12 %)	WBC (/mm^3)	6778±13319
Predisposing conditions	227 (43 %)	WBC < 1,000/mm^3	142/496 (27 %)
Otitis or sinusitis	191 (36 %)	Protein (g/l)	4.3±3.0
Pneumonia	77 (15 %)	CSF blood: glucose ratio	0.14±0.19
Immunocompromise	124 (23 %)	Positive blood cultures	346/463 (75 %)
Symptoms and signs on presentation		Complications	
Headache	411/479 (85 %)	Systemic complications	166 (31 %)
Neck stiffness	398/510 (78 %)	Neurological complications	327 (62 %)
Systolic blood pressure (mmHg)	146±29	Glasgow Outcome Scale	
Heart rate (bpm)	99±21	1—Death	40/528 (8 %)
Body temperature (°C)	38.7±1.3	2—Vegetative state	1/528 (0.2 %)
Score on Glasgow Coma Scale[a]	11±3	3—Severe disability	21/528 (4 %)
<8 indicating coma	70/527 (13 %)	4—Moderate disability	78/528 (15 %)
Focal neurological deficits	141/528 (27 %)	5—Good recovery	388/528 (73 %)

[a]Glasgow coma scale score was evaluated in 527 patients

[b]CSF pressure was evaluated in 123 patients, CSF WBC in 496, CSF protein in 505, and CSF blood to glucose ratio in 498

TABLE 1. Clinical characteristics of 531 patients with community acquired bacterial meningitis [data are number/number evaluated (percentage) or mean ±SD]

Gene	SNP	Good outcome (GOS 5)[a]					Poor outcome (GOS 1–4)					Model	*P* value
	ID	A	B	AA	AB	BB	A	B	AA	AB	BB		
CARD8	rs2043211	525	237	183	159	39	169	107	57	55	26	Additive	0.04
NLRP1	rs12150220	433	327	127	179	74	173	103	57	59	22	Recessive	0.21
NLRP1	rs11651270	406	356	105	196	80	165	109	51	63	23	Recessive	0.12
NLRP1	rs2301582	473	299	143	187	56	183	97	58	67	15	Recessive	0.14
NLRP3	rs10754558	433	331	120	193	69	161	119	49	63	28	Recessive	0.65
NLRP3	rs35829419	701	35	334	33	1	227	7	110	7	0	Dominant	0.43

[a]Glasgow Outcome Scale Score

TABLE 2. Genotyping results of six genetic polymorphisms in inflammasome genes in 531 bacterial meningitis patients of which 388 patients with a good outcome and 143 with poor outcome

bacterial meningitis using an additive model (p=0.040; Table 2). Patients with the T/T genotype had the highest risk for poor outcome [odds ratio (OR), 2.09; 95 % confidence interval (CI), 1.17–3.71; p=0.009). In a multivariate analysis limited to white patients, rs2403211 was an independent risk factor for unfavorable outcome after correction for age, sex, causative bacteria, immunodeficiency, and pretreatment with antibiotics (OR, 2.10; 95 % CI, 1.04–4.21; p=0.038). The effect of rs2043211 was stronger in the subgroup of white patients with pneumococcal meningitis (Figure 1; OR for T/T genotype, 2.19; 95 % CI, 1.15–4.18; p=0.018). This effect on outcome seemed to be driven both by occurrence of systemic (OR T/T genotype, 2.48; 95 % CI, 1.29–4.7; p=0.016) and neurological complications (OR T/T genotype, 3.03; 95 % CI, 1.34–6.85; p=0.022). When testing the equality of the genotype versus outcome odds ratios in men and women, we could not demonstrate a statistically significant interaction between genotype and gender. Patients with either a variant allele for CARD8 rs2043211 or NLRP3 rs35829419, which was previously described to cause a deficient phenotype, were not at increased risk for death, unfavorable outcome, or complications. Rs11651270 (Met1154Val) in NLRP1 was associated with death in

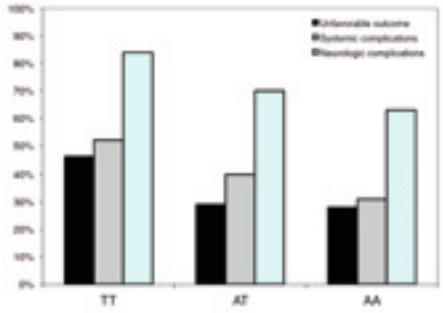

FIGURE 1. Rate of mortality, systemic, and neurological complications by CARD8 rs2043211 genotype in pneumococcal meningitis patients. CARD8 rs2043211 was associated with poor outcome an additive model (P=0.040). Patients with the T/T genotype had the highest risk for poor outcome [odds ratio (OR), 2.09; 95 % confidence interval (CI), 1.17–3.71). This effect on outcome seemed to be driven both by occurrence of systemic (OR T/T genotype, 2.48; 95% CI, 1.29–4.7; p=0.016) and neurological complications (p=0.022; OR T/T genotype, 3.03; 95 % CI, 1.34–6.85)

pneumococcal meningitis patients using a recessive model (14 % TT genotype vs. 6 % CC/CT genotype; OR, 1.97; 95%CI, 1.01–3.85, p=0.047; log rank survival analysis p=0.04, Figure 2). After correction for age, sex, immunodeficiency, and pretreatment with antibiotics, the effect of rs11651270 on mortality remained significant (OR, 2.32; 95%CI, 1.12–4.78; p=0.023). Using a Bonferroni correction, the effect of rs11651270 on death was no longer significant. Other SNPs in NLRP1 and NLRP3 were not associated with outcome or death. CSF was obtained from 289 patients with

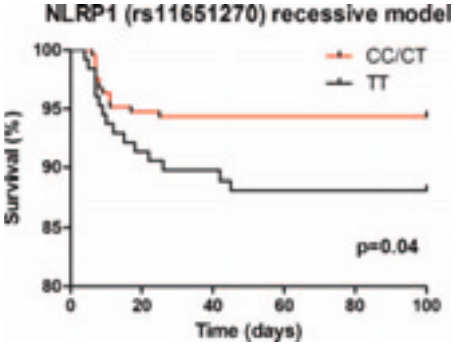

FIGURE 2. Kaplan–Meier survival curve in patients with pneumococcal meningitis according to rs11651270 genotype

bacterial meningitis and 19 control patients. Levels of IL-1β and IL-18 were elevated in the CSF of patients with bacterial meningitis as compared to controls [median, 1.31 ng/ml (IQR, 0.19–4.40) vs. 0.004 ng/ml (IQR, 0.002–0.006), p<0.001, and 10.76 ng/ml (IQR, 4.00– 25.02) vs. 0.71 ng/ml (IQR, 0.40–0.89), p<0.001 respectively]. High IL-1β levels were associated with occurrence of systemic complications [Figure 3; median, 1.94 ng/ml (IQR, 0.30–5.26) vs. 0.93 ng/ml (IQR, 0.15–3.11), p=0.003). There was a trend between high IL-1β levels and neurological complications [median, 1.62 ng/ml (IQR, 0.28–5.04) vs. 0.43 ng/ml (IQR, 0.08–4.73), p=0.10], as well as unfavorable outcome [median, 1.53 ng/ml (IQR, 0.28–5.19) vs. 1.03 ng/ml (IQR, 0.17–3.63), p=0.08]. High IL-18 levels were also associated with systemic complications [Figure 3; median, 15.13 ng/ml (IQR, 6.36–26.89) vs. 8.84 ng/ml (IQR, 3.09–19.91), p=0.004] and poor outcome [median, 14.48 ng/ml (IQR, 5.26–26.59) vs. 9.43 ng/ml (IQR, 3.37–22.68), p=0.039]. In the subgroup of patients with pneumococcal meningitis (n0207), associations with systemic complications remained significant. CSF levels of IL-1β and IL-18 between patients were not associated with rs2043211 and rs11621270 genotypes, also after a correction for total

CSF protein levels was applied.

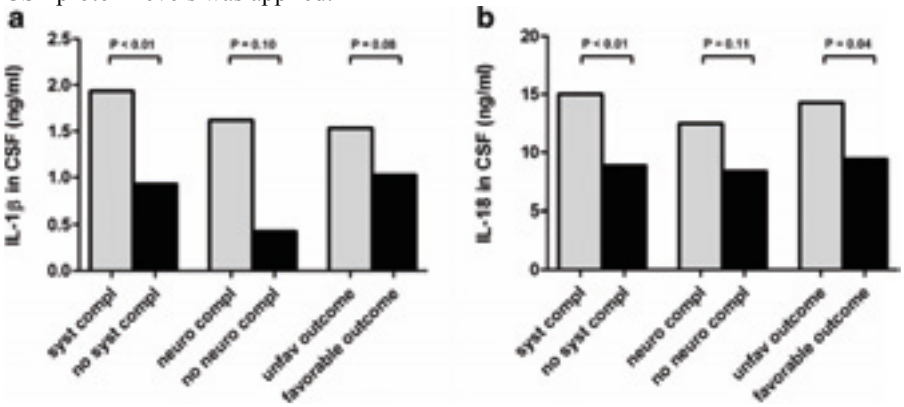

FIGURE 3. Median levels of IL-1β and IL-18 in CSF of patients with bacterial meningitis. a Elevated IL-1β was associated with systemic complications, and there was trend towards more neurological complications. b High IL-18 levels were associated with both systemic complications and unfavorable outcome

DISCUSSION

Our results implicate an important role of the inflammasomes in bacterial meningitis. We found that SNPs in the inflammasome genes CARD8 (rs2043211) and NLRP1 (rs11621270) are associated with death and poor disease outcome. IL-1β and IL-18 levels in CSF of patients with bacterial meningitis correlated with development of systemic complications and poor prognosis.

We identified the rs2043211 polymorphism in CARD8 to contribute to outcome of bacterial meningitis by influencing the risk of systemic complications. The A allele of rs2043211 generates a premature stop codon (Cys10X) and leads to a severely truncated CARD8 protein, which has been associated with various inflammatory diseases such as inflammatory bowel disease, Crohn's disease, and rheumatoid arthritis (22, 23, 25-27). Several functions have been attributed to CARD8. First is the inhibition of pathways to nuclear factor kappa B (NF-κB) activation (28, 29). In vitro studies have demonstrated that CARD8 interferes with NF-κB activation by established NF-κB activators, possibly through direct interaction between CARD8 and IkB kinase complex (28). Second, CARD8 also has antiapoptotic properties through the inhibition of caspases, including caspase-1, caspase-8, and caspase-9 (CARD8 is also known as TUNCAN, tumor-upregulated CARD containing antagonist of caspase-9) (30). Through direct interaction with caspase-1, CARD8 can negatively regulate caspase-1 dependent IL-1β generation in vitro (14). Lastly, CARD8 forms a physical component of the multiprotein complex of the NLRP3 inflammasome (31, 32). However, in vitro knockdown studies have shown that CARD8 may not be a requirement for the activation of the NLRP3 inflammasome in response to viral infection (33).
The truncated form of CARD8, therefore, has the potential to disrupt cytokine regulation at several key stages and could lead to higher levels of NF-κB mediated proinflammatory (pro)cytokines, incomplete NLRP3 assembly, and a limited caspase-1 activation, resulting in limited secretion of activated IL-1β and IL-18. Conversely, the normal form of CARD8 (T/T genotype) may lead to NF-κB inhibition and lower

proinflammatory (pro)cytokines. Despite proper NLRP3 assembly with CARD8, this could also lead to limited secretion of IL-1β and IL-18. Indeed, we do not see a difference in CSF levels of IL-1β and IL-18 between CARD8 genotypes. The suppressed non-caspase-dependent inflammation may, however, be insufficient to battle bacterial infection, resulting in the observed increased risk of systemic and neurological complications in patients with bacterial meningitis.

Our findings support the hypothesis that genetic variation in the inflammasome genes can influence the threshold for activation of the inflammatory response, presenting a double-edged sword: A more readily activated system will predispose to chronic inflammation (rheumatoid arthritis and inflammatory bowel disease), while a normally controlled system may result in suboptimal activation and less control of severe infection (bacterial meningitis).

We identified rs11651270 SNP in NLRP1 to influence mortality in bacterial meningitis, although the effect was no longer significant after correction for multiple testing. The exact function of NLRP1 remains unclear, though its relevance is underlined by associations between SNPs in NLRP1 and autoimmune diseases such as vitiligo, autoimmune Addison's disease, type 1 diabetes, and Alzheimer's disease (12, 34, 35). To our knowledge, this is the first report of rs11651270 to be associated the outcome of infectious disease. As the effect of rs11651270 was not significant after correction for multiple testing, this result should be regarded as explorative and needs validation in other populations before a firm conclusion can be drawn.

NLRP1 is activated by two known factors: anthrax lethal toxin derived from spore-forming bacterium Bacillus anthracis, and muramyl dipeptide and myramyl dipeptide, a peptidoglycan constituent of both Gram-positive and Gram-negative bacteria(36). Unlike NLRP3, NLRP1 has its own CARD domain and does not require ASC or CARD8 to activate caspase-1 (although the presence of ASC substantially increases caspase activation) (13). However, as the rs11651270 polymorphism does not seem to influence levels of IL-1β or IL-18 in the CSF of our patients, a caspase-dependent mechanism does not seem likely.

NLRP1 and CARD8 share a "function-to-find domain" (FIIND), which is a highly conserved domain only present in these two proteins. FIIND has an intraproteolytic function, of which the relevance is incompletely understood (37). Interestingly, the aforementioned NLRP1 SNP lies in, and the CARD8 SNP is situated before the respective FIIND regions (37). Although the influence of rs11651270 on the function of FIIND is unknown, one can hypothesize that a disruption of the FIIND domain could affect NLRP1 function and thereby influence clinical outcome following bacterial meningitis. IL-1β and IL-18 levels in CSF were found to correlate with outcome, but were not associated with the polymorphisms in NLRP1 or CARD8. A possible explanation for this discrepancy could be that IL-1β and IL-18 are also being produced in an inflammasome-independent manner. This was previously shown for IL-1β, which can be produced by neutrophil derived serine proteases or pathogen-derived proteases (38). Therefore, a small potential decrease in cytokine production due to NLRP1 and CARD8 polymorphisms may not be measurable in the total amount of IL-1β and IL-18 produced. Another explanation may be that the impact of these polymorphisms on secreted active IL-1β and IL-18 levels is limited, and NLRP1 and CARD8 may be involved in alternative inflammatory roles (38). Further functional studies of rs2043211 and rs11621270 are needed determine the influence on these SNPs on the immune response after stimulation with *S. pneumoniae*.

Our study has several limitations. First, our findings regarding the NLRP1 and CARD8 SNPs should be replicated in independent case–control study to validate our

observations. However, currently, no such studies are available for us to confirm our findings. Second, in this study, we show an association between the polymorphisms rs2043211 and rs11621270, and poor outcome and death, but we did not demonstrate changes in protein functionality or a causal relationship with outcome. Once the associations have been confirmed, further mechanistic studies of the functionality of these SNPs during infection should be performed.

In conclusion, our results implicate an important role of genetic variation in inflammasome genes in bacterial meningitis. Interference with inflammasome activation may therefore be a promising target for adjunctive therapy in bacterial meningitis.

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CHAPTER 6

INFLAMMASOME ACTIVATION MEDIATES INFLAMMATION AND OUTCOME IN HUMANS AND MICE WITH PNEUMOCOCCAL MENINGITIS

Geldhoff M*, Mook-Kanamori BB*, Brouwer MC, Troost D, Leemans JC, Flavell RA, van der Ende A, van der Poll T, van de Beek D.

*contributed equally in the writing of this paper.

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ABSTRACT

Background: Inflammasomes are multi-protein intracellular signaling complexes that have recently been hypothesized to play a role in the regulation of the inflammation response. We studied associations between inflammasome-associated cytokines IL-1β and IL-18 in cerebrospinal fluid (CSF) of patients with bacterial meningitis and clinical outcome, and pneumococcal serotype. In a murine model of pneumococcal meningitis we examined the pathophysiological roles of two inflammasome proteins, NLRP3 (Nod-like receptor protein-3) and adaptor protein ASC (apoptosis-associated speck-like protein).

Methods: In a nationwide prospective cohort study, CSF cytokine levels were measured and related to clinical outcome and pneumococcal serotype. In a murine model of pneumococcal meningitis using *Streptococcus pneumoniae* serotype 3, we examined bacterial titers, cytokine profiles and brain histology at 6 and 30 hours after inoculation in wild-type (WT), Asc and Nlrp3 deficient mice.

Results: In patients with bacterial meningitis, CSF levels of inflammasome associated cytokines IL-1β and IL-18 were related to complications, and unfavorable disease outcome. CSF levels of IL-1β were associated with pneumococcal serotype ($p<0.001$). In our animal model, Asc and Nlrp3 deficient mice had decreased systemic inflammatory responses and bacterial outgrowth as compared to WT mice. Differences between Asc−/− and WT mice appeared sooner after bacterial inoculation and were more widespread (lower pro-inflammatory cytokine levels in both blood and brain homogenate) than in Nlrp3-/-mice. Nlrp3 deficiency was associated with an increase of cerebral neutrophil infiltration and cerebral hemorrhages when compared to WT controls.

Conclusions: Our results implicate an important role for inflammasome proteins NLRP3 and ASC in the regulation of the systemic inflammatory response and the development of cerebral damage during pneumococcal meningitis, which may dependent on the pneumococcal serotype.

BACKGROUND

Bacterial meningitis is a life threatening infectious disease of the central nervous system that affects between 2.6 and 6.0 people per 100 000 per year in Europe and may be up to ten times higher in developing countries. The most common causative organism of community acquired bacterial meningitis in adults is *Streptococcus pneumoniae*, which is responsible for two-thirds of cases in Europe and United States (1). Pneumococcal meningitis has a case fatality rate of 16%-37% and of the survivors 30-52% suffer from neurological sequelae (2, 3). There remains a need for better (adjuvant) therapies, for which further understanding of underlying pathophysiology is necessary (4). Recently, several studies have examined the role of inflammasomes in bacterial meningitis. Inflammasomes are intracellular multiprotein complexes, belonging to the family of Nod-like receptors (NLRs) (5-7), and are triggered by exposure to microbial and endogenous danger signals such as ATP, changes in K^+ concentration, oxygen radicals and uric acid released through cell injury in inflammation. Upon activation, NLRP3 binds to procaspase via adaptor apoptosis-associated speck-like protein (ASC), which is shared by several inflammasome types. Procaspase-1 is converted to activated caspase-1, which subsequently converts interleukins 1beta (IL-1β) and IL-18 into their active secreted forms (5-7). Recently however, caspase-independent proinflammatory activity of

NLRP3 has also been described (8, 9). To date, four inflammasomes have been characterized, of which NLRP3 has been the most extensively researched. Further examination of the role of inflammasomes in pneumococcal meningitis is of interest for several reasons: First, inflammasomes are the well established activators of caspase-1, which has been shown to be elevated in the cerebral spinal fluid (CSF) of patients with pneumococcal meningitis compared to non-infected controls (10, 11). Moreover, mice deficient of caspase-1 displayed less severe inflammation, decreased brain water content and improved clinical score in a pneumococcal meningitis model (10, 12). Second, IL-1β, which is activated by caspase-1, has been shown to be elevated in the CSF of children with pneumococcal meningitis and correlates with disease severity (11), a finding that also has been observed in various animal models (13-15). Lastly, several murine models have demonstrated the importance of NLRP3 in the pathophysiology of invasive pneumococcal disease (16, 17). Most notably, a recent study showed that NLRP3 mediates brain damage in an experimental meningitis model using a serotype 2 *S. pneumoniae* (18). In this study we measured the CSF levels of inflammasome related cytokines IL-1β and IL-18 in a prospective nationwide cohort of community acquired bacterial meningitis and correlated these to clinical data and pneumococcal serotype. We then investigated the role of inflammasome gene NLRP3 and adapter protein ASC in a murine model of meningitis using serotype 3 *S. pneumoniae*, a common serotype in pneumococcal meningitis (19).

METHODS

Patients cohort

In a nationwide prospective cohort study we included bacterial meningitis patients older than 16 years of age with positive CSF cultures who were identified by The Netherlands Reference Laboratory for Bacterial Meningitis (NRLBM) from March 2006 to June 2009. The NRLBM provided the names of the hospitals where patients with bacterial meningitis had been admitted 2–6 days previously. The treating physician was contacted, and informed consent was obtained from all participating patients or their legally authorized representatives. Outcome was graded at discharge according to the Glasgow Outcome Scale, a well-validated instrument with good interobserver agreement (20). A score of one on this scale indicates death; a score of two a vegetative state; a score of three severe disability; a score of four moderate disability; and a score of five mild or no disability. A favorable outcome was defined as a score of five, and unfavorable outcome as a score of one to four. The study was approved by the medical ethical (review) committee of the Academic Medical Center of Amsterdam.

IL-1β and IL-18 measurements in CSF samples of patients with bacterial meningitis

We measured IL-1β and IL-18 in the CSF of 289 patients with bacterial meningitis included in the cohort and 19 controls with luminex¨ technology using a Milliplex assay (Millipore, Billerica, MA, USA). CSF from the first diagnostic tap was collected, centrifuged and supernatant was aliquoted and stored at −80°C until analysis. Controls were patients evaluated for acute headache, without signs of meningitis and normal CSF findings. In these patients a subarachnoid hemorrhage was excluded as cause of their headache by CSF examination. Leftover CSF was collected, centrifuged and supernatant was stored at −80°C until analysis.

Mouse model and tissue preparation

A well characterized and previously described murine model of pneumococcal meningitis was used in this study (21). Nlrp3$^{-/-}$ mice with C57BL/6 background (kind gift of Richard Flavell, Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, CT, USA) and Asc$^{-/-}$ mice with C57BL/6 background (kind gift of Fayyaz Sutterwala, University of Iowa, Iowa City, IA, USA), and specific pathogen-free C57BL/6 mice (Charles River, Wilmington, MN, USA) were weighed, clinically examined, and scored clinically. Inoculations were performed in several rounds, all with male mice aged 8–12 weeks. In each inoculation round knockout mice and an equal number of wild-type mice were inoculated with the same bacterial inoculum to control for variations between inocula. Experiments were approved by the Institutional Animal Care and Use Committee of the Academic Medical Center, Amsterdam, The Netherlands. Serotype 3 *S. pneumoniae* (ATCC 6303; American Type Culture Collection, Rockville, MD, USA) was grown to mid log phase in 4 hours at 37°C in Todd-Hewitt broth supplemented with yeast (Difco, Detroit, MI). Pneumococci were harvested by centrifugation at 4000 rpm for 10 min, and washed twice with sterile isotonic saline. Bacteria were diluted to a final concentration of $1x10^6$ CFU/ml and serial ten-fold dilutions were plated on sheep blood agar plates for quantification. Mice were inoculated in the cisterna magna under isoflurane anesthesia with 10 µl saline containing $1x10^4$ CFU (range $0.6 \times 10^4 – 1.2 \times 10^4$ CFU) of *S. pneumoniae* or sterile saline alone. Twelve mice per group (WT, Nlrp3$^{-/-}$ and Asc$^{-/-}$) were inoculated with *S. pneumoniae* and six mice per group (WT, Nlrp3$^{-/-}$ and Asc$^{-/-}$) with sterile saline. After intracisternal inoculation mice were assessed for neurologic damage as a result of the puncture, which was not present in any of the mice. At 6 or 30 hours post infection mice were anesthetized by intraperitoneal injection of ketamine (Eurovet Animal Health, Bladel, the Netherlands) and medetomidine (Pfizer Animal Health, Capelle aan den Ijssel, the Netherlands) followed by cardiac puncture for blood collection and perfusion of organs with sterile isotonic saline via the left ventricle. CSF was collected by puncture of the cisterna magna, and brains, lungs and spleen were harvested. The right hemisphere was suspended in 10% buffered formalin and embedded in paraffin for histopathology. The left hemisphere and spleen were taken up in 20% weight per volume sterile saline and were disrupted with a tissue homogenizer. Serial ten-fold dilutions of blood, CSF, brain homogenate and spleen homogenate were plated on sheep-blood agar plates and bacteria were allowed to grow overnight at 37°C. Heparin blood was centrifuged at 4000 rpm for 5 min. at 4°C. Tissue homogenates were lysed by adding 1:1 two times concentrated lysis buffer (150 mM NaCl, 15 mM Tris, 1 mM $MgCl(H_2O)_6$, 1 mM $CaCl_2$ $(H_2O)_2$, 1% Triton, AEBSF 4 µ g/ml, EDTA-NA2 50 µg/ml, pepstatin 10 ng/ml, leupeptin 10 ng/ml, pH 7.4), incubating on ice for 30 min. and centrifuged at 4000 rpm for 5 min. at 4°C. Plasma and lysed tissue supernatant were removed and stored at − 20°C for further analysis.

RT-PCR

Total RNA was isolated from murine brain homogenates with the Nucleospin® RNA II Purification kit (Clontech Laboratories, Mountain View, CA, USA; Bioke, Leiden, the Netherlands). Isolated RNA was converted to cDNA using oligo(dT) primer (Promega, Leiden, the Netherlands), Moloney murine leukemia virus reverse transcriptase (Invitrogen, Breda, the Netherlands), RT-buffer (Promega, Leiden, the Netherlands), deoxynucleotide triphosphate mix (Invitrogen, Breda, the Netherlands),

dithiothreitol (Duchefa Farma, Haarlem, the Netherlands) and RNAse inhibitor (Invitrogen, Breda, the Netherlands). After incubation for 10 min at 23□C, RT was carried out for 60 min at 42□C, followed by RT inactivation for 3 min at 95□C. Reverse transcription-PCR□s (RT-PCRs) were performed with LightCycler SYBR green I master mix (Roche, Mijdrecht, the Netherlands) and measured in a LightCycler 480 (Roche) apparatus under the following conditions: 5 min 95□C hot start, followed by 40 cycles of amplification (95□C for 15 sec - 60□C for 5 sec - 72□C for 20 sec). For quantification, standard curves were constructed on serial dilutions of a sample with known high cDNA content. Data were analyzed using LightCycler software. Gene expression is presented as a ratio of the expression of the housekeeping gene Glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

The primers used for RT-PCR were as follows: mouse GAPDH, 5'-CTCATGACCACAGTCCATGC-3' (forw) and 5'-CACATTGGGGGTAGGAACAC-3' (rev); mouse Nlrp3, 5'-CCACAGTGTAACTTGCAGAAGC-3' (forw) and 5'-GGTGTGTGAAGTTCTGGTTGG-3' (rev); mouse Asc, 5'-GGTGTGTGAAGTTCTGGTTGG-3' (forw) and 5'-GGTGTGTGAAGTTCTGGTTGG-3' (rev).

Cytokine measurements in murine tissue

IL-1β, IL-6, KC, TNF-α, IL-18, and MIP-2 were measured in plasma and brain homogenates with luminex¨ technology using a mouse cytokine and chemokine Bioplex kit (Bio-Rad Laboratories, Veenendaal, The Netherlands). Luminex assays were analysed on a Luminex 200 with Bio-Plex Manager software 5.0. Samples were 4 times diluted. Mouse myeloperoxidase (MPO) was measured by ELISA (Hycult Biotechnology, Uden, The Netherlands). Mouse albumin was measured by ELISA (GenWay Biotech, San Diego, CA).

Murine histopathology

Five µm paraffin brain sections were cut in a coronal plane from the olfactory bulb to the beginning of the cerebellum, and sections at intervals of 1400 µm or intervals of 700 µm throughout the hippocampal region were selected. Sections were mounted on slides and stained with hematoxylin and eosin. To assess differences in brain damage, coronal cut brain sections of WT and $Asc^{-/-}$ and $Nlrp3^{-/-}$ mice were scored for intracerebral hemorrhages, subpial hemorrhages, cerebral infarctions, and for neutrophil influx on a five point scale: 0) normal histopathology; 1) few inflammatory cells in the meninges but no perivascular cuffing; 2) moderate number of inflammatory cells in the meningitis and cuffing of some of the vessels; 3) extensive number of inflammatory cells in the meninges, prominent perivascular cuffs with mild infiltration of the neutrophil; 4) extensive number of inflammatory cells in the meninges, prominent perivascular cuffs, the presence of many inflammatory cells in the neutrophil and intraparenchymal pocket formation. Sections of mice 30 hours after induction of pneumococcal meningitis (n=8 per group) were scored. Sections were scored by two independent observers blinded for the experimental groups (interobserver kappa 0.75).

Statistics

The Mann□Whitney U test was used to identify differences in baseline characteristics, bacterial outgrowth, cytokine levels and histopathological scores among groups with respect to continuous variables. Dichotomous variables were compared using the $\chi 2$

test. Correlation analyses were performed with the Spearman's rank correlation coefficient. For all analyses a P-value < 0.05 was considered significant.

Results

CSF IL-1β and IL-18 levels in patients with bacterial meningitis are associated with complications and unfavorable disease outcome A total of 801 Dutch patients with bacterial meningitis were included as described previously (22). In this study the distribution of causative organisms was: 576 episodes (72%) *S. pneumoniae*, 92 (12%) Neisseria meningitidis, 41 (5%), Listeria monocytogenes, and other bacteria in 92 (12%) episodes. The case fatality rate was 18%, and 38% of patients had poor clinical functional outcome as defined as a score of 1-4 on the Glasgow Outcome Scale. CSF was available in 289 of the episodes with bacterial meningitis (36%), and 211 of 576 with pneumococcal meningitis (35%). Levels of IL-1β and IL-18 were elevated in the CSF of patients with meningitis as compared to controls. Higher IL-1β levels were associated with occurrence of systemic complications (median 0.91 ng/ml [IQR 0.15-3.00] versus 2.02 ng/ml [IQR 0.33- 5.26], p=0.001) and neurologic complications (median 0.81 ng/ml [IQR 0.15-3.36] versus 1.60 ng/ml [IQR 0.29-4.73], p=0.020). IL-1β levels were higher in patients with an unfavorable outcome although this difference was not statistically significant (median 1.04 ng/ml [IQR 0.17- 3.65] versus 1.53 ng/ml [IQR 0.27-5.16], p=0.11). High IL-18 levels were also associated with systemic complications (median 8.50 ng/ml [IQR 3.07-20.71] versus 15.13 ng/ml [IQR 6.35-27.32], p=0.006) and unfavorable outcome (median 9.27 ng/ml [IQR 3.36-22.83] versus 14.65 ng/ml [IQR 5.53-26.97], p=0.037). In the subgroup of patients with pneumococcal meningitis (n=211) associations with systemic complications remained significant.

CSF IL-1β and IL-18 levels in patients with bacterial meningitis are associated with pneumococcal serotype

Pneumococcal serotyping was performed in 509 pneumococcal strains (88%) and the most common serotypes were 3, 23 and 7 (Table III; serotype distribution has been partly published previously (23). CSF levels of IL-1β were related to pneumococcal serotype (Kruskal-Wallis 1 way ANOVA, p<0.001; Figure 1).

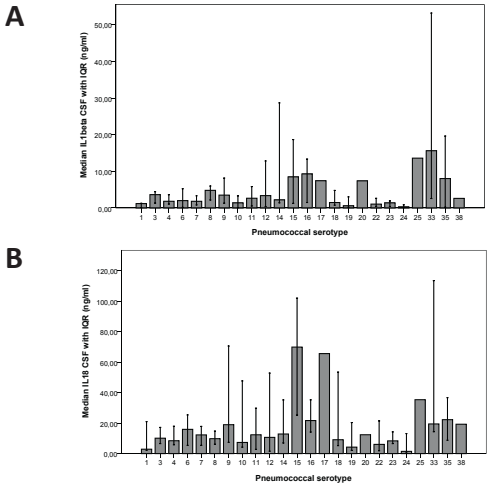

Figure 1 Median levels of IL-1β (A) and IL-18 (B) with interquartile range by pneumococcal serotype. CSF levels of IL-1β and IL-18 were related to pneumococcal serotype (Kruskal-Wallis 1 way ANOVA, p<0.001).

ASC and NLRP3 expression in Asc and Nlrp3 knockout mice

To confirm that the inflammasome components ASC and NLRP3 were expressed or

knocked out in our meningitis mouse model, we examined mouse brain homogenates from WT mice infected with *S. pneumoniae* serotype 3. At 6 and 30 h after infection ASC and NLRP3 expression in brain homogenates appeared upregulated as compared to saline inoculated mice (Figure 2). Nlrp3$^{-/-}$ mice showed expression of ASC and no NLRP3, and conversely Asc$^{-/-}$ mice showed expression of NLRP3 and no ASC.

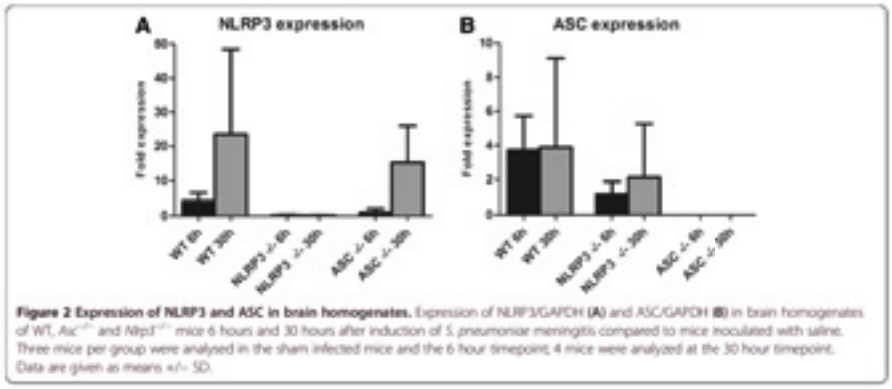

Figure 2 Expression of NLRP3 and ASC in brain homogenates. Expression of NLRP3/GAPDH (**A**) and ASC/GAPDH (**B**) in brain homogenates of WT, Asc$^{-/-}$ and Nlrp3$^{-/-}$ mice 6 hours and 30 hours after induction of *S. pneumoniae* meningitis compared to mice inoculated with saline. Three mice per group were analysed in the sham infected mice and the 6 hour timepoint; 4 mice were analyzed at the 30 hour timepoint. Data are given as means +/- SD.

Decreased systemic bacterial loads in Asc and Nlrp3 knockout mice

Nlrp3$^{-/-}$ mice showed more bacterial outgrowth in CSF at 6 h compared to WT mice (median 9.2 x 10^5 CFU/ml versus 2.2 x 10^5 CFU/ml, p=0.046 Figure 3A). However, Nlrp3$^{-/-}$ had less bacterial outgrowth in blood and spleen at 30 h, as compared to WT mice (blood, 5.7 x 10^3 CFU/ml versus 3.2 x 10^4 CFU/ml, p=0.017; spleen, 8.0 x 10^3 CFU/ml versus 4.7 x 10^5 CFU/ml, p=0.012 Figure 3B). No differences in bacterial outgrowth in brain homogenates were observed. Asc$^{-/-}$ mice showed less bacterial outgrowth at 6 h in blood and lung compared to WT mice (blood, 2.0 x 10^3 CFU/ml versus 6.6 x 10^3 CFU/ml, p=0.017; lung, 2.0 x 10^3 CFU/ml versus 1.1 x 10^5 CFU/ml, p=0.043 Figure 3C). At 30 h Asc$^{-/-}$ mice showed less bacterial outgrowth in blood (3.9 x 10^4 CFU/ml versus 1.4 x 10^5 CFU/ml, p=0.039 Figure 3D) and spleen (3.1 x 10^4 CFU/ml versus 6.7 x 10^4 CFU/ml, p=0.016). No differences in bacterial outgrowth in CSF or brain homogenates in Asc$^{-/-}$ mice were observed.

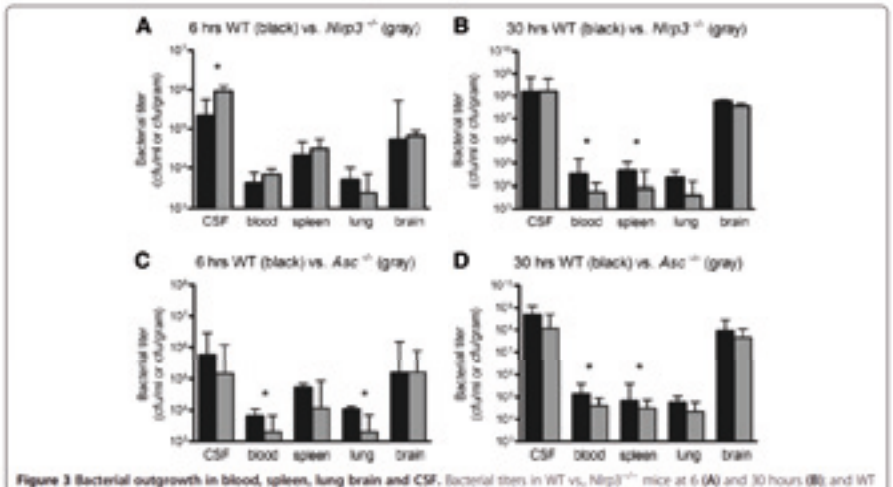

Figure 3 Bacterial outgrowth in blood, spleen, lung brain and CSF. Bacterial titers in WT vs. Nlrp3$^{-/-}$ mice at 6 (**A**) and 30 hours (**B**); and WT vs. and Asc$^{-/-}$ mice at 6 (**C**) and 30 hours (**D**) after induction of pneumococcal meningitis. Twelve mice per group were analyzed. Data are given as medians and 75th quartile. * P < 0.05.

Decreased systemic inflammatory response in both Nlrp3 and Asc knockout mice at 6 and 30 hrs

Nlrp3$^{-/-}$ mice showed decreased plasma levels of MIP-2 (median 12 pg/ml [IQR 5□20] versus 55 pg/ml [IQR 5□77], p=0.037) and IL-6 (52 pg/ml [IQR 24□90] versus 191 pg/ml [70□306], p=0.019) at 30 h as compared to WT mice (Figure 4A). No significant cytokine differences were found at 6 hours. Asc$^{-/-}$ mice showed decreased plasma levels of MIP-2 (12 pg/ml [IQR 5□27] versus 55 pg/ml [IQR 35□80] pg/ml, p=0.01), IL-6 (37 pg/ml [IQR 24□108] versus 202 pg/ml [IQR 46□448], p=0.034) and IFN-γ (3 pg/ml [3] versus 16 pg/ml [3-24], p=0.005) at 30 h, as compared to WT mice. At 6 h only KC levels were lower (105 pg/ml [78□202] versus 186 pg/ml [151□388], p=0.005). Notably, plasma levels of IL-1β and IL-18 were similar in Nlrp3$^{-/-}$, Asc$^{-/-}$ and WT mice (Figure 4C). For the other measured cytokines no significant difference was found at 6 or 30 hours.

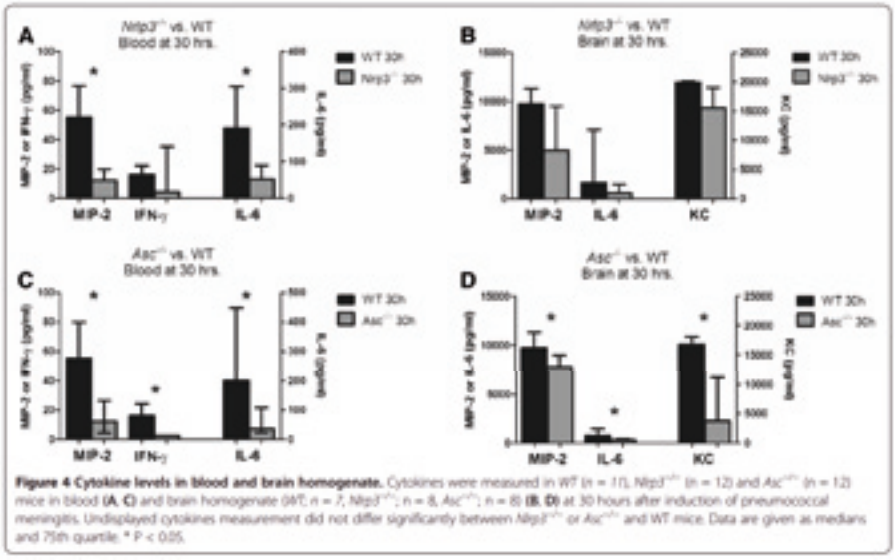

Figure 4 Cytokine levels in blood and brain homogenate. Cytokines were measured in WT (n = 11), Nlrp3$^{-/-}$ (n = 12) and Asc$^{-/-}$ (n = 12) mice in blood (**A, C**) and brain homogenate (WT, n = 7; Nlrp3$^{-/-}$, n = 8; Asc$^{-/-}$, n = 8) (**B, D**) at 30 hours after induction of pneumococcal meningitis. Undisplayed cytokines measurement did not differ significantly between Nlrp3$^{-/-}$ or Asc$^{-/-}$ and WT mice. Data are given as medians and 75th quartile. * P < 0.05.

Decreased brain cytokine and chemokine levels in Asc$^{-/-}$ but not Nlrp3$^{-/-}$ mice

Nlrp3$^{-/-}$ mice showed no differences in cytokine responses in the brain compared to WT mice, and brain albumin levels were also similar between WT and Nlrp3$^{-/-}$ mice (data not shown). Asc$^{-/-}$ mice displayed lower levels of KC (median, 3.81 ng/ml [IQR 1.57-11.24] versus 16.75 ng/ml [IQR 11.49-18.12], p=0.040), MIP-2 (7.74 ng/ml [IQR 1.46-8.91] vs. 9.74 ng/ml [IQR 8.20-11.3], p=0.049) and IL-6 (0.30 ng/ml [(24)IQR 0.10-0.45] versus 0.76 ng/ml [IQR 0.61-1.45], p=0.049) than WT mice in brain homogenates at 30 h (Figure 4D). Consistent with this finding, brain albumin concentrations were decreased in Asc$^{-/-}$ mice compared to WT mice at 30 h (p=0.026), indicating attenuated blood brain barrier disruption in Asc$^{-/-}$ mice compared to WT mice. No differences in brain IL-1β or IL-18 levels were measured between WT and Nlrp3$^{-/-}$ or Asc$^{-/-}$ mice.

Enhanced brain damage in Nlrp3$^{-/-}$ but not in Asc$^{-/-}$ mice

Neutrophil infiltrate in the brain was more pronounced in Nlrp3$^{-/-}$ mice at 30 h after

inoculation as compared to WT mice (median score 1.5 versus 2.4, p=0.018; Figure 5G). Nlrp3$^{-/-}$ mice also showed an elevated number of intracerebral and subpial hemorrhages and as compared to WT mice (median 12.5 versus 1.0 per slide, p=0.02; Figure 5H). Asc$^{-/-}$ mice showed no difference in neutrophil influx score and intracerebral hemorrhages compared to WT mice. Brain MPO levels were similar in both knockouts and wildtype mice (data not shown).

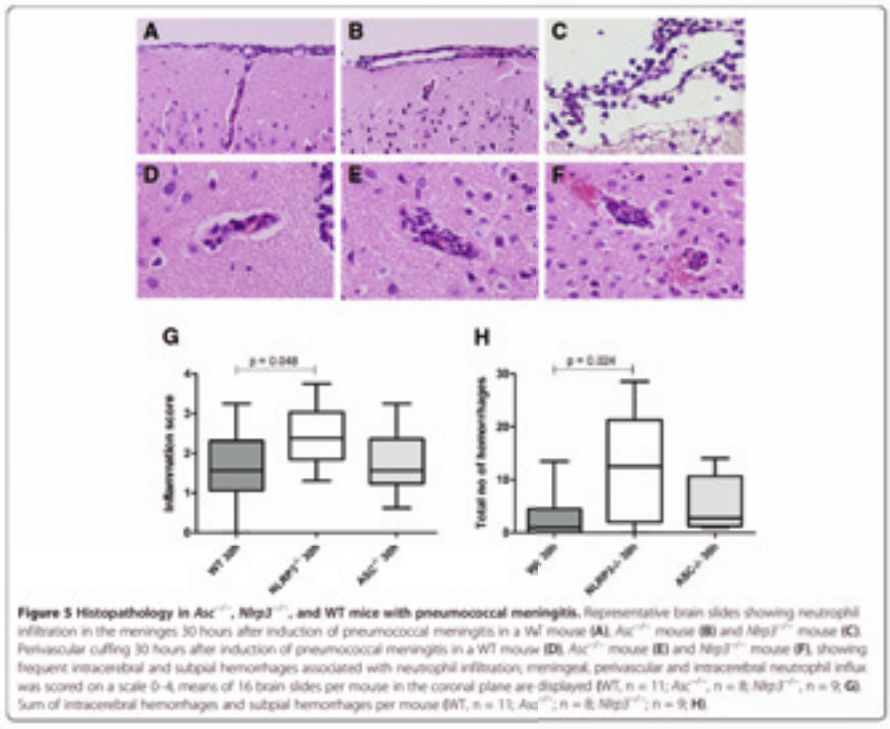

Figure 5 Histopathology in *Asc*$^{-/-}$, *Nlrp3*$^{-/-}$, and WT mice with pneumococcal meningitis. Representative brain slides showing neutrophil infiltration in the meninges 30 hours after induction of pneumococcal meningitis in a WT mouse **(A)**, *Asc*$^{-/-}$ mouse **(B)** and *Nlrp3*$^{-/-}$ mouse **(C)**. Perivascular cuffing 30 hours after induction of pneumococcal meningitis in a WT mouse **(D)**, *Asc*$^{-/-}$ mouse **(E)** and *Nlrp3*$^{-/-}$ mouse **(F)**, showing frequent intracerebral and subpial hemorrhages associated with neutrophil infiltration; meningeal, perivascular and intracerebral neutrophil influx was scored on a scale 0–4, means of 16 brain slides per mouse in the coronal plane are displayed (WT, n = 11; *Asc*$^{-/-}$, n = 8; *Nlrp3*$^{-/-}$, n = 9; **G**). Sum of intracerebral hemorrhages and subpial hemorrhages per mouse (WT, n = 11; Asc$^{-/-}$; n = 8; Nlrp3$^{-/-}$; n = 9; **H**).

DISCUSSION

This study implicates an important role for inflammasomes in regulation of systemic inflammation and development of cerebral damage during pneumococcal meningitis. In our patient cohort, inflammasome associated cytokines IL-1β and IL-18 levels in CSF of patients with bacterial meningitis correlated with development of systemic complications and unfavorable prognosis; and in the subgroup of patients with pneumococcal meningitis, IL-1β and IL-18 correlated with systemic complications only. In our murine model of pneumococcal meningitis, deficiency of inflammasome components ASC and NLRP3 led to decreased systemic inflammatory responses and bacterial outgrowth in the systemic compartment as compared with WT mice. Conversely, Nlrp3 deficiency led to enhanced central nervous system inflammation and increased brain damage. Differences between Asc$^{-/-}$ and WT mice occurred sooner after intrathecal inoculation with *S. pneumoniae* (lower bacterial titers and KC serum levels at 6 h) and were more widespread (lower pro-inflammatory cytokine levels in both the systemic compartment (blood) and central nervous system compartment (brain homogenate) than in the Nlrp3$^{-/-}$ mice.
In our murine model, NLRP3 was protective for brain damage, as Nlrp3$^{-/-}$ mice had

enhanced cerebral neutrophil influx and an increased number of cerebral hemorrhages. NLRP3 has been investigated with regard to pneumococcal infections in both lung infection models and a meningitis model (17, 18, 25), but findings are not unanimous. In a lung-infection model, $Nlrp3^{-/-}$ mice have higher bacterial titers and a higher mortality than WT controls. In a murine model of pneumococcal meningitis, better clinical outcome and decreased brain inflammation in $Nlrp3^{-/-}$ (and $Asc^{-/-}$) mice was found as compared to WT controls (18). Blocking of IL-1β and IL-18 in this meningitis model, led to a decrease in disease severity and which prompted the suggestion that the NLRP and ASC dependent changes are solely IL-1 and IL-18 related (18). Our findings that IL-1β and IL-18 levels were not significantly altered in $Nlrp3^{-/-}$ or $Asc^{-/-}$ mice, must be interpreted with caution as no assays are available that can discriminate between the pro- and active forms of murine IL-1β and IL-18. Previous studies showed increased IL-1β levels in brain homogenates of WT mice with pneumococcal meningitis at 30 hours compared to sham (21). We did not perform experiments blocking IL-1β, IL-18 or Caspase-1 in our model to further elucidate this mechanism.

Discrepancies in brain damage in $Nlrp3^{-/-}$ mice between our study and the previous study in experimental pneumococcal meningitis may be explained by the different pneumococcal serotypes used to establish meningitis between both models (18). We inoculated mice with a *S. pneumoniae* serotype 3 strain as opposed to the serotype 2 strain used in the previous study. Furthermore, we used a lower intrathecal dose of *S. pneumoniae* than the previous study (10^4 CFU vs. 10^5 /10^6 CFU). *S. pneumoniae* serotype 2 is less heavily encapsulated and less virulent than serotype 3 and needs high doses to induce infection (24). An in vitro study showed that high bacterial loads of *S. pneumoniae* serotype 2 are needed before IL-1β concentration in cell culture supernatants are elevated (26).

The variation of immune response between different serotypes of *S. pneumoniae* has been demonstrated by several groups (24, 27). In our patient cohort, we observed that the most common pneumococcal serotypes were 3, 23, and 7, and that CSF levels of IL-1β was serotype related. This observation may be due to serotype specific properties of the pneumococcal capsule. Alternatively, pneumolysin, a pore-forming toxin which is known to interact directly with the innate immune system (through, for instance complement or binding of Toll Like Receptor-4), is secreted in varying amounts depending on bacterial serotype. Pneumolysin has been reported to have both inflammasome inhibiting and activating properties (16, 17, 28), which may be caused by the recently described effects of pneumolysin polymorphisms on innate immune system recognition (17). We chose *S. pneumoniae* serotype 3 for our animal studies, as it is one of the most commonly encountered serotypes among patients with pneumococcal meningitis (19).

The more pronounced phenotype of the $Asc^{-/-}$ mice as compared to the $Nlrp3^{-/-}$ mice with pneumococcal meningitis can be explained by other, NLRP3 inflammasome independent, functions of ASC. The (functional) relationship between ASC, NLRP3, and caspase-1 activation during pneumococcal infection was recently described in murine pneumonia model (25), in which *S. pneumoniae* infection led to caspase-1 activation and IL-1β /IL-18 maturation through the activation of both the NLRP3 and the AIM2 (absent in melanoma) inflammasomes, in a process which was completely absent in the ASC deficient mice. Furthermore, ASC is capable of binding and facilitating the function of several other inflammasomes (such as NLRC4 and IFI16), though the relevance of this during pneumococcal infection is not evident (29). Lastly, independently of the inflammasomes, ASC has been shown to potent regulator

of a large number of inflammatory and cell-death related genes (30). The observation that NLRP3 deficient mice but not ASC deficient mice, expressed more brain damage suggests a protective mechanism in which NLRP3 may act independently of ASC and of the NLRP3 inflammasome. Such "inflammasome-independent" role of NLRP3 in tissue injury has been described in a mouse model of renal ischemia-reperfusion injury (8, 9), though a mechanism remains unclear.

CONCLUSION

In conclusion, although a definite mechanism remains elusive, our results provide additional evidence for an important role of inflammasomes (specifically the NLRP3 and ASC proteins and inflammasome associated cytokines IL-1β and IL-18) in the regulation of an inflammatory response and brain damage during pneumococcal meningitis. Further human and animal studies are necessary to clarify the pathophysiological mechanism, as well as explore the possibility of interference of inflammasome activation as a potential adjunctive therapy in the treatment of pneumococcal meningitis.

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CHAPTER 7

THROMBIN ACTIVATABLE FIBRINOLYSIS INHIBITOR INFLUENCES DISEASE SEVERITY IN HUMANS AND MICE WITH PNEUMOCOCCAL MENINGITIS

Mook-Kanamori BB, Valls Serón M, Geldhoff M, Havik SR, van der Ende A, Baas F, van der Poll T, Meijers JCM, Morgan BP, Brouwer MC, van de Beek D.

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ABSTRACT

Background: Mortality and morbidity in bacterial meningitis results from the pro-inflammatory response and dysregulation of coagulation and fibrinolysis. Thrombin-activatable fibrinolysis inhibitor (TAFI) is activated (TAFIa) by free thrombin or in complex with thrombomodulin, plays an anti-fibrinolytic role during fibrin clot degradation, but also has an anti-inflammatory role by inactivating proinflammatory mediators, such as complement activation products.
Objective: To assess the role of TAFI in pneumococcal meningitis.
Methods: We performed a prospective nationwide genetic association study in patients with bacterial meningitis, determined TAFI and complement levels in CSF, and assessed the function of TAFI in a pneumococcal meningitis mouse model using Cpb2 (TAFI) knockout mice.
Results: polymorphisms (reference sequence: rs1926447 and rs3742264) in the CPB2 gene, coding for TAFI, were related with development of systemic complications in patients with pneumococcal meningitis. Higher protein levels of TAFI in CSF were significantly associated with CSF complement levels (C3a, iC3b and C5b-9) and with more systemic complications in patients with bacterial meningitis. The risk allele of rs1926447 (TT) was associated with higher levels of TAFI in CSF. In the murine model, consistent with the human data, Cpb2-deficient mice had decreased disease severity reflected by lower mortality, attenuated cytokine levels and bacterial outgrowth in the systemic compartment during disease, without differences in the brain compartment, as compared with wild-type mice.
Conclusions: These findings suggest that TAFI plays an important role during pneumococcal meningitis, which is likely to be mediated through inhibition of the complement system, and influences the occurrence of systemic complications and inflammation.

INTRODUCTION

Despite advances in effective antibiotics and adjuvant drug treatment, bacterial meningitis still has a high mortality rate, ranging from 16 to 37%(1). The strongest risk factors for an unfavorable outcome are those that are indicative of systemic compromise, a low level of consciousness, and infection with *Streptococcus pneumoniae*(1). Of the patients who survive the infection, approximately half suffer from long-term neurological sequelae, such as hearing loss, focal neurological deficits and cognitive impairment(1-3).

Neurological damage in bacterial meningitis is only partially caused by direct harmful effects of bacterial invasion, but mainly results from the massive pro-inflammatory response and dysregulation of coagulation and fibrinolysis both in the central nervous system compartment and in the systemic compartment(4). Coagulation and inflammation are closely related and interact in severe infection(4). Patients with bacterial meningitis are at risk for cerebrovascular complications, mainly cerebral infarctions (25%) and to a lesser extent cerebral hemorrhages (2-3%)(5, 6). Studies suggest that dysregulation of coagulation and fibrinolysis is influenced by host genetic variation(7).

An important inhibitor of fibrinolysis during infection is thrombin-activatable fibrinolysis inhibitor (TAFI), which is activated following exposure to thrombin, either free or in complex with thrombomodulin, plasmin, trypsin and neutrophil elastase(8, 9). Active TAFI (TAFIa) modulates fibrinolysis by cleaving off the C-terminal lysine residues of partially degraded fibrin, thereby inhibiting its degradation by plasmin, and inflammation through inactivation of pro-inflammatory mediators such as bradykinin, thrombin-cleaved osteopontin and anaphylatoxins C5a and C3a(10). The importance of TAFI during infection has been demonstrated in various clinical studies(11-15), and animal models(16), but its role in bacterial meningitis is unclear. A genetic association study in meningococcal disease patient showed that genetic variation in the carboxypeptidase B2 gene (*CPB2*), coding for TAFI, was associated with a 10-fold increase in risk of diffuse intravascular coagulation(14).

We hypothesized TAFI is an important modulator of diseases severity and outcome in bacterial meningitis which is influenced by host genetic variation. We conducted a prospective nationwide genetic association study in patients with bacterial meningitis to assess the effects of common genetic variants (single nucleotide polymorphisms SNPs) of *CPB2* on disease presentation and outcome. We measured TAFI levels in cerebrospinal fluid (CSF) and related this to both the SNPs and clinical outcome. Finally, we further characterized the role of TAFI in pneumococcal meningitis using an animal model.

METHODS

Nationwide bacterial meningitis cohort

In a nation-wide cohort study we prospectively included adults with community acquired bacterial meningitis (17). Eligible patients were aged >16 years, had bacterial meningitis confirmed by CSF culture, and were identified by The Netherlands Reference Laboratory for Bacterial Meningitis (NRLBM) between March 2006 and October 2011. The NRLBM receives bacterial isolates from approximately 85% of bacterial meningitis patients in the Netherlands and provided

the names of the hospitals where these patients were admitted in the previous 2–6 days. Treating physicians were contacted and written informed consent was obtained from all participating patients or their legally authorized representatives. Patients with hospital-acquired bacterial meningitis and negative CSF cultures were excluded. Secured online case-record forms were used to collect clinical data. Systemic complications were defined as circulatory shock (diastolic blood pressure <60mmHg), respiratory failure or the need for mechanical ventilation. Outcome was graded at discharge according to the Glasgow Outcome Scale (GOS), a well validated instrument with good inter-observer agreement(18). Scores ranged from 1 (indicating death) to 5 (indicating mild or no disability). A favorable outcome was defined as a score of 5, and an unfavorable outcome was defined as a score of 1-4(1). Previously, other genetic association studies have been performed using (part of) this dataset, including genetic polymorphisms in complement and inflammasome genes, *SERPINE1* and *GLCCI1*(17, 19-21).

Genotyping

DNA was isolated from EDTA-blood using the Gentra Puregene Isolation Kit (Qiagen, Hilden, Germany). DNA was obtained from patients and controls: patients' partners or nonrelated proxies living in the same dwelling, having similar exposure to bacteria through nasopharyngeal colonization, and were matched for age, ethnicity, and sex. The rs3742264 (G505A encoding Ala147Thr) and rs1926447 (C1040T encoding Thr325Ile) SNPs in *CPB2* were genotyped by the Genetics Core Facility and the Department of Experimental Vascular Medicine in the Academic Medical Center, Amsterdam, using TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City, California, USA) in a LightCycler480 (Roche, Basel, Switzerland). Laboratory personnel were blinded to clinical information.

CSF measurements

Diagnostic lumbar puncture CSF was available in 371 of the 992 patients (37%). For the detection of TAFI in CSF a sandwich ELISA was used as described elsewhere except that CSF was diluted 50 times(22). CSF complement component C3 levels were determined using a C3 Luminex xMAP technology with a Milliplex MAP kit obtained from Millipore Corp. (St. Charles, Missouri, USA). CSF complement component C3a, iC3b, C5a and terminal complement complex (TCC; sC5b-9) levels were determined using the Microvue C3a, iC3b, C5a and sC5b-9 Quidel ELISA kits (San Diego, California, USA) according to manufacturer's instructions. CSF obtained from patients in whom a subarachnoid hemorrhage was excluded by CSF examination and had normal CSF composition served as negative controls.

Mouse model of pneumococcal meningitis

A well-characterized and previously described mouse model of pneumococcal meningitis was used in this study(23). Eight to 12 week old C57BL/6 female mice which received sterilized food and water *ad libitum* were weighed, clinically examined and scored (0-41 points)(23) prior to inoculation as described previously. Mice were inoculated in the cisterna magna under isoflurane anesthesia with $1x10^4$ CFU of *S. pneumoniae* serotype 3 (ATCC 6303; American Type Culture Collection, Rockville, MD, USA) or saline. After intracisternal inoculation all mice were directly assessed for neurologic damage, and if present they were excluded from further analysis. All mice were clinically scored at predetermined time-intervals following inoculation. Mice were sacrificed at predefined time-points or after reaching an illness

severity score of 15 by intraperitoneal injection of ketamine (190 mg/kg, Eurovet Animal Health, Bladel, the Netherlands) and dexmedetomidine (0.3 mg/kg, Pfizer Animal Health, Capelle a/d IJssel, the Netherlands). The experiments were approved by the Institutional Animal Care and Use Committee of the Academic Medical Center.

First a survival study was performed using *Cpb2*$^{-/-}$ mice with a C57BL/6 background (n=12)(24), and wild-type (wt) C57BL/6J (n=12, Charles River, Wilmington, Massachusetts, USA). Clinical scoring was performed at 4-6 hour interval following inoculation until a clinical score of 15 was reached. Second, in a treatment model, 36 *Cpb2*$^{-/-}$ mice and 36 wt mice were each divided into 3 groups of 12 mice: the first group was inoculated with NaCl (negative controls) and sacrificed at 68 hours, the second group with *S. pneumoniae* and sacrificed at 20 hours, and the third group with *S. pneumoniae*, treated 20 hours after inoculation with ceftriaxone 100 mg/kg i.p. and sacrificed at 68 hours. Clinical scoring was performed t=0, 6, 24 hours and thereafter twice daily until a clinical score of 15 of the end of the experiment was reached. To analyze whether mice suffered primarily from neurologic or systemic symptoms, we performed a separate analysis dividing neurologic and systemic scoring parameters of disease severity. Researchers were not blinded to the genetic status of the mice. Mice were euthanized using dexmedetomidine/ketamine, after which vena cava puncture was performed for blood collection on sodium citrate 3.2% (1:10 v/v) and organs were perfused with sterile isotonic saline via the left ventricle. CSF was collected by cisterna magna puncture and stored at -20□C. Brains, lungs, liver and spleen were harvested. The right cerebral hemisphere and liver were snap-frozen in liquid nitrogen. The left hemisphere, lung and spleen were resuspended in 4 volumes sterile saline and homogenized. Serial 10-fold dilutions in sterile saline were plated onto sheep-blood agar plates and incubated overnight at 37□C. CFUs were counted after 16 hours. Plasma and brain homogenate concentrations of IL-1β, IL-6, TNF-α, IL-10, KC and MIP-2 were measured using Luminex¨ technology and a mouse cytokine and chemokine Bioplex kit (Bio-Rad Laboratories, Veenendaal, The Netherlands). D-dimers (Diagnostica Stago, Asnieres-sur-Seine, France) and Thrombin-antithrombin complexes (TATc; *Siemens* Healthcare *Diagnostics,* Erlangen, Germany) were measured in plasma by ELISA. The soluble terminal complement complex sC5b-9 was measured in brain and plasma by ELISA (USCN life sciences, Wuhan, China). TAFI activity was measured as previously described(25).

To determine the number of cerebral hemorrhages, the right cerebral hemisphere was snap-frozen and cut coronally into 10 µm-thick sections. Twelve serial sections were photographed at 3 mm intervals starting anteriorly of the lateral ventricles. Hemorrhagic areas were counted by two independent investigators (κ=0.8) and differences in scoring were resolved through discussion.

Statistics □genetic analysis

The Mann-Whitney U test was used to identify differences in baseline characteristics among groups with respect to continuous variables, and dichotomous variables were compared with use of the χ2 test. Differences in genotype frequencies were analyzed with the χ^2 or Fishers□exact tests by use of SPSS19, using a P-value of <0.05 to indicate significance. We calculated whether the genotype frequencies in the control groups concurred with the HWE by use of a χ^2 and exact test with 1 degree of freedom with a P-value of less than 0.05 to indicate significance. We did not perform

a power calculation prior to the design of this study on TAFI polymorphisms as the cohort study was designed as a genetic association study without prior definition of individual polymorphisms. With the current cohort size of pneumococcal meningitis patients (700) and a minor allele frequency of 0.3 of both studied SNP we would be able to detect a difference in unfavorable outcome rate with an odds ratio of 1.65 or higher using a p-value of 0.05 and power of 80%. The *CPB2* genotype frequencies of patients with a favorable outcome were compared with those with an unfavorable outcome. Furthermore, a preplanned analysis on the effect on systemic and neurological complications was performed. Subgroup analyses were defined by ethnicity (white), causative organism (*S. pneumoniae*), and a combination of these factors. When univariate analysis showed significant differences per genotype, multivariate analysis was performed correcting for age and score on the Glasgow Coma Scale on admission to correct for confounders.

Statistics – animal experiments

In the murine model, the survival study was analyzed using a Gehan-Breslow-Wilcoxon test, and cytokine levels and bacterial titers were compared using a Mann-Whitney U test. Clinical scores were compared using a linear mixed effects model with fixed effects for strain, time and their interaction, random intercept and slope effects and a heterogeneous autoregression 1 as covariance structure. Histopathological scores were compared using a Student's *t*-test. For all analyses a P-value of < 0.05 was considered to be significant.

RESULTS

Nationwide prospective cohort study of adults with community-acquired bacterial meningitis

992 patients with community acquired CSF culture proven bacterial meningitis were included (Figure 1). The causative bacterium was *S. pneumoniae* in 716 patients (72%), *N. meningitidis* in 109 patients (11%), and other bacteria in 167 patients (17%). DNA was available in 647 (65%) of these patients, and CSF in 393 (40%). CSF was available in 307 (47%) patients of whom DNA was obtained. The patients from whom DNA was obtained were younger, presented with less severe disease and more

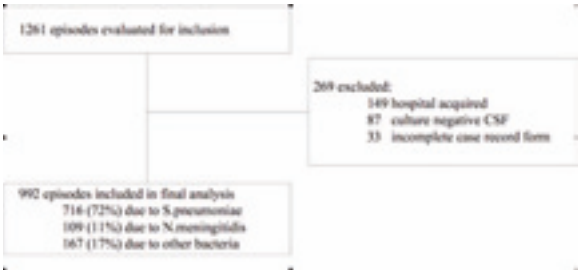


FIGURE 1. Prospective nationwide cohort study. Flowchart for inclusion of patients from the prospective nationwide cohort study of 992 episodes of bacterial meningitis.

often had a favorable outcome (suppl. Table 1). The average age at presentation was 55 yrs, and 49% of patients were male. Predisposing conditions for bacterial meningitis were present in 372 (57%) patients, of which 233 (36%) had otitis or sinusitis, and 152 (24%) were immunocompromised (Table 1). Complications occurred in 363 (56%) cases (including cerebral infarctions in 144 [22%] and

hemorrhages in 12 [2%]) and systemic complications in 201 (31%) patients. Systemic complications consisted of circulatory shock in 36 (6%) and respiratory failure in 183 (28%). The case fatality rate was 7% and 29% of patients had an unfavorable outcome.

Characteristic	Value/Total	Characteristic	Value/Total
Age (yr)	55 ±17	Indexes of CSF inflammation[c]	
Male sex	262 (49%)	Opening pressure (mmH_2O)	34 ±11
Pre-treatment with antibiotics	63/527 (12%)	WBC ($/mm^3$)	6778 ±13319
Predisposing conditions	227 (43%)	WBC < $1,000/mm^3$	142/496 (27%)
Otitis or sinusitis	191 (36%)	Protein (g/L)	4.3 ±3.0
Pneumonia	77 (15%)	CSF blood: glucose ratio	0.14 ±0.19
Immunocompromise	124 (23%)	Positive blood cultures	346/463 (75%)
Symptoms and signs on presentation		Complications	
Headache	411/479 (85%)	Systemic complications	166 (31%)
Neck stiffness	398/510 (78%)	Neurologic complications	327 (62%)
Systolic blood pressure (mmHg)	146 ±29	Glasgow outcome scale	
Heart rate (bpm)	99 ±21	1 □Death	40/528 (8%)
Body temperature (□C)	38.7 ±1.3	2 □Vegetative state	1/528 (0.2%)
Score on Glasgow coma scale[b]	11 ±3	3 □Severe disability	21/528 (4%)
< 8 indicating coma	70/527 (13%)	4 □Moderate disability	78/528 (15%)
Focal neurologic deficits	141/528 (27%)	5 □Good recovery	388/528 (73%)

[a]Data are number/number evaluated (percentage) or mean ±SD. [b]Glasgow coma scale score was evaluated in 527 patients. cCSF pressure was evaluated in 123 patients, CSF WBC in 496, CSF protein in 505 and CSF blood to glucose ratio in 498.

TABLE I. Clinical characteristics of 531 patients with community acquired bacterial meningitis.

Genetic association study on *CPB2* variants

Genotyping for rs1926447 and rs3742264 in *CPB2* was successful in 642 of 647 (99%) and 636 of 647 (98%) patients respectively and the genotype frequency of controls concurred with the Hardy-Weinberg equilibrium for both SNPs. We did not identify an association between rs1926447 and rs3742264 genotype and susceptibility for bacterial meningitis, unfavorable outcome or death (data not shown). However, a significant association of both rs1926447 and rs3742264 SNPs on the risk of developing systemic complications was identified in white patients (587 of 647 patients, 91%) with bacterial meningitis using an additive model for rs3742264 (P=0.015) and a recessive model for rs1926447 (P=0.021, Table 2). The risk of systemic complications in patients homozygous for the variant rs3742264 allele (AA) was 21%, compared to 38% in patients with a common allele (absolute risk difference 17%, OR 0.4, [95% CI 0.2-0.8], P=0.006). This effect was similar when limiting the analysis to white patients with pneumococcal meningitis (risk of systemic complications (21 vs. 41%, OR 0.40, [95% CI 0.20-0.79], P=0.008). The risk of systemic complications was higher in patients homozygous for the variant allele (TT) of the rs1926447 SNP (47% vs. 31%; OR 0.51, [95% CI 0.28-0.91], Table 2). In a multivariate analysis rs1926447 remained significantly associated with systemic complications after correction for age and score on the Glasgow Coma Scale (GCS) on admission (OR 0.44, 95% CI 0.21-0.93; P=0.032) in all patients. This effect was not significant when limiting the analysis to (white) pneumococcal meningitis patients. The effect of rs3742264 was no longer significant (OR 0.55, 95% CI 0.30-1.02; P=0.058) in a multivariate analysis correcting for age and GCS score in all patients.

TAFI and complement levels in CSF

TAFI and complement levels were determined for 283 patients (of the remaining 88 patients of which CSF was obtained, insufficient volume was available). CSF TAFI and complement levels were significantly higher in patients with bacterial meningitis than in negative controls (median TAFI level patients 2.05 μg/ml [IQR 0.85-3.73] vs controls 0.34 μg/ml [0.22-0.54], P<0.0001). Analysis of complement levels showed median C3a levels of 562 ng/ml, C3b 22.2 ng/ml, C5a 12.9 ng/ml and C5b-9 2207 ng/ml. Higher levels of TAFI in the CSF were significantly associated with more systemic complications in patients with bacterial meningitis (2.45 μg/ml [IQR 1.01–4.25] vs 1.95 μg/ml [IQR 0.85–3.39], P=0.03; Figure 2). When analyzed by type of systemic complications, TAFI levels were higher in patients with than those without respiratory failure (2.45μg/ml [IQR 1.02-4.33] vs 1.96 μg/ml [IQR 0.88-3.39], P=0.042) while there was no significant difference between patients with or without circulatory shock. The risk allele of rs1926447 (TT) was associated with higher levels of TAFI in the CSF (4.29 μg/ml [IQR 2.52–5.60] vs 2.04 μg/ml [IQR 0.96–3.44], P=0.002; Figure 2), whereas no influence on TAFI CSF levels was found for the rs3742264. CSF TAFI level was positively correlated to levels of C3a, iC3b and C5b-9 (correlation co-efficient C3a r=0.52, iC3b r=0.42, C5b-9 r=0.45, all P<0.001). These correlations were also present for patients with pneumococcal meningitis (C3a r=0.51, iC3b r=0.41, C5b-9 r=0.35; all P<0.001).

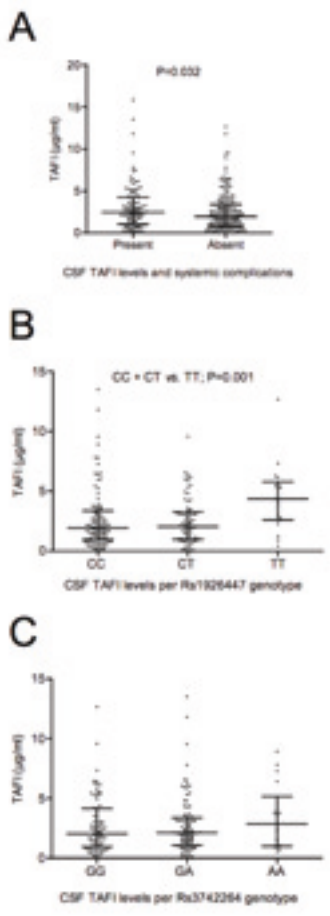


FIGURE 2. TAFI levels in CSF. CSF TAFI levels for all patients with and without systemic complications (A) and per TAFI genotype (B and C). Dots represent individual values, bars medians and whiskers interquartile range.

Functional analysis of TAFI in the mouse model of pneumococcal meningitis.
To examine the role of TAFI in pneumococcal meningitis, we first performed a survival experiment, comparing *Cpb2*$^{-/-}$ to wt mice, in which mice were inoculated with 10^4 CFUs of *S. pneumoniae* ATCC 6303. *Cpb2*$^{-/-}$ mice had a significantly longer survival than wt mice (P=0.03, Figure 3A).

Second, we designed a treatment model of pneumococcal meningitis, in which antibiotic treatment was given intraperitoneally at 20 or 24 hours after inoculation, to model the clinical disease course observed in pneumococcal meningitis patients. To model 30% mortality after 72 hours, we performed a dose finding study in which we varied the *S. pneumoniae* dose from 10^4 to 10^6 CFU (Supplemental Figure 1). With antibiotic treatment after 24 hours, mortality at 68 hours was 93% in mice inoculated with 10^4 CFU, and 100% in 10^6 CFU, a mortality of 33% at 68 hours was reached

using an inoculation of 10^4 CFU and antibiotic treatment after 20 hours. This regimen was used for experiments.

Third, we compared *Cpb2*$^{-/-}$ with wt mice at an early (t=20 h) time-point before treatment with a late time-point (t=68 h) in which mice were treated at 20 h. At 20 hours, bacterial outgrowth was lower in *Cpb2*$^{-/-}$ mice compared to wt in the systemic compartment (blood $2.8x10^4$ vs. $5.6x10^3$ CFU/ml, P=0.04, and spleen $1.1x10^5$ vs. $3.0x10^4$ CFU/ml, P=0.01; Figure 3E, F). After 68 hours bacterial outgrowth was significantly reduced in *Cpb2*$^{-/-}$ and wt mice compared to the 20h time-point (P<0.001 for all cultured organs) indicating effective antibiotic treatment (data not shown). Bacterial loads at 68h were similar between *Cpb2*$^{-/-}$ and wt mice. In the wt group, 8 of 11 (72%) survived the full-length of the experiment (68 h), as compared to 6 of 11 (55%) of the *Cpb2*$^{-/-}$ mice. Clinical severity did not differ significantly between the *Cpb2*$^{-/-}$ and wt mice (linear mixed effects model, P=0.26, Figure 3B). No differences in neurological and systemic parameters of disease severity included in the clinical score were observed between groups.

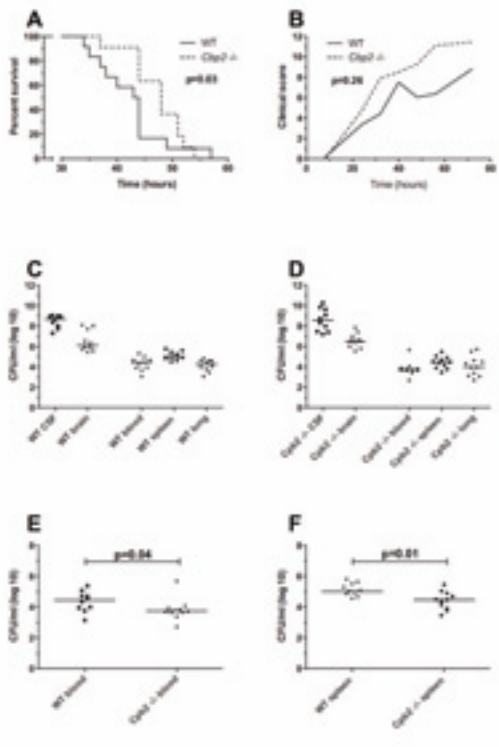
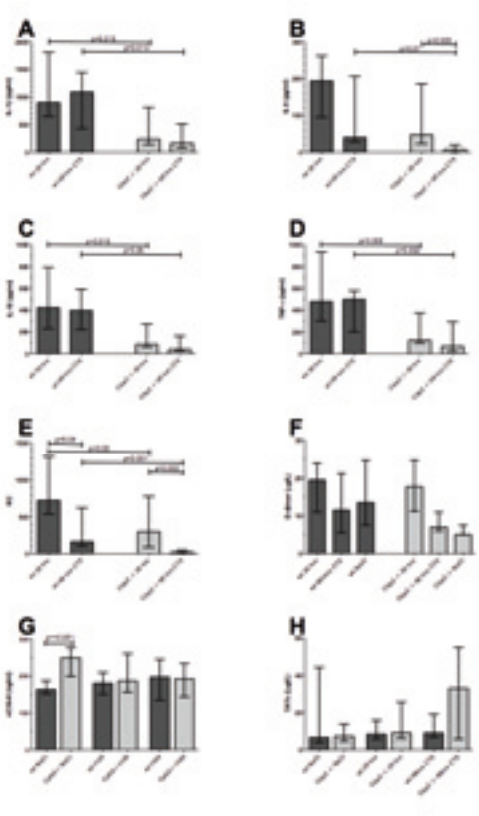


FIGURE 3. Kaplan-Meier survival curves of Cpb2-/- vs. wt mice with pneumococcal meningitis without antibiotic treatment (A); Clinical score of Cpb2-/- vs. wt mice inoculated with 104 CFU S. pneumoniae ATCC 6303 and treated with ceftriaxone (100 mg/kg i.p.) at 20 hours (B); CFUs of Cpb2-/- and wt mice inoculated with 104 CFU S. pneumoniae ATCC 6303 and sacrificed at 20 hours post infection (C-F, lines indicate medians).

Brain homogenate levels of IL-6 and KC were significantly higher in both infected *Cpb2*$^{-/-}$ and wt mice compared to negative controls at the 20 h time-point, whereas IL-1β and IL-10 were not. No differences were observed between wt and *Cpb2*$^{-/-}$ mice. Plasma levels of IL-6, IL-1β, TNF-α, IL-10 and KC were all elevated in *Cpb2*$^{-/-}$ and wt infected mice compared to negative controls (data not shown). In both the 20 h and 68 h time-point plasma levels of IL-6, IL-1β, TNF-α, IL-10 and KC were lower in *Cpb2*$^{-/-}$ than wt mice with the exception of IL-6 at 20 hours (Figure 4A-E).

To gain insight into the effect of TAFI on the fibrinolytic host response, D-dimer levels were measured in the plasma and brain homogenate. Most brain homogenate D-dimer levels were below the detection limit. Plasma D-dimer levels were elevated

at 20 hours after inoculation in both *Cpb2*$^{-/-}$ and wt mice compared to mice injected with saline (median 18 µg/l and 20 µg/l, respectively, Figure 4F). At 68 hours plasma D-dimer levels were similar in the *Cpb2* $^{-/-}$ and wt mice (median 7 µg/l and 11 µg/l). TATc levels in brain homogenate were below detection levels; plasma TATc levels in de wt and *Cpb2*$^{-/-}$ mice were similar at 20 and 6 hours after inoculation (median 8.4 µg/L and 9.18.4 µg/L; median 9.18.4 µg/L and 33.0 µg/L, respectively, Figure 4H). TAFI activity was elevated at 20 hours in wt infected mice compared to saline injected controls, and similar between the 20 and 68 hour time-points (median 74% and 109%; P=0.23). TAFI activity was undetectable in *Cpb2* $^{-/-}$ mice. Pathological analysis showed no difference in the total number and area of cerebral hemorrhages between *Cpb2* $^{-/-}$ and wt mice (data not shown).



A significant difference in plasma C5b-9 levels was observed between *Cpb2*$^{-/-}$ mice injected with saline compared to wt mice (162 ng/ml [IQR 152-187] vs. 249 ng/ml [199-277], P=0.0007). Complement levels in infected animals at the 20h and 68h time-points were similar (Figure 4G). There were no differences in brain sC5b-9 levels at baseline or during infection between wt and knockout mice (data not shown).

FIGURE 4. Plasma cytokines levels (A-E) as well as D-dimer, TATc and complement C5b-9 levels (F-H) in wt and Cpb2-/- mice inoculated with S. pneumoniae ATCC 6303, treated at 20 hours with ceftriaxone (CTX, 100 mg/kg i.p.), and sacrificed at 20 and 68 hours after infection. Data are shown as median and interquartile range (IQR).

DISCUSSION

In a nation-wide prospective cohort study we found that common *CPB2* polymorphisms rs1926447 and rs3742264 were associated with systemic complications in bacterial meningitis and pneumococcal meningitis, but did not influence outcome or mortality. The risk genotypes of both SNPs showed higher levels of TAFI in CSF, although the association was only significant for rs1926447. To our knowledge, this is the first time that TAFI protein has been reported to be measureable in CSF. Higher TAFI levels in CSF were associated with an increased risk of systemic complications and TAFI was positively correlated with complement components C3a, iC3b and C5b-9 in the CSF of the diagnostic lumbar puncture in patients with bacterial meningitis as well as in the subgroup of patients with

pneumococcal meningitis. There was no difference in neurological complications and outcome between TAFI genotypes or TAFI CSF levels.
Our results concur with previous studies in meningococcal disease showing that patients homozygous for the variant allele of *CPB2* rs1926447 (Thr325Ile) have an increased disease severity, reflected by higher rate of diffuse intravascular coagulation and mortality(14, 26). The TAFI Thr325Ile has been described to result in a TAFIa enzyme with a longer half-life compared to the Thr325 variant(27-29). We observed a similar effect of rs1926447 on CSF TAFI concentration. Rs3742264 (Ala147Thr) has also been associated with plasma TAFI levels, with higher levels in patients homozygous for the Thr147 variant allele(29, 30). Rs3742264 has not been previously described to affect disease severity in infectious disease before, but was identified as a risk factor for other diseases, such as venous thromboembolic disease and chronic heart disease(31-35). Previous studies hypothesized that inhibition of TAFI activation may aid in preventing diffuse intravascular coagulation(14).
Our results in the pneumococcal meningitis mouse model show delayed mortality in the *Cpb2*$^{-/-}$ mice compared to wt and attenuated cytokine levels and bacterial outgrowth in the systemic compartment early during disease without differences in brain cytokine levels, bacterial outgrowth and cerebral hemorrhages between *Cpb2*$^{-/-}$ and wt mice. Also higher level of baseline complement activation was observed in *Cpb2*$^{-/-}$ mice compared to wt mice, an effect no longer significant at later time-points. There were no differences in D-dimer and TATc levels between *Cpb2*$^{-/-}$ and wt mice at all time-points.

The lack of effect on fibrinolysis during infection has been previously observed in *Escherichia coli* sepsis in *Cpb2*$^{-/-}$ mice, which had similar plasma thrombin-antithrombin complexes, plasma D-dimer levels and fibrin depositions in lung and liver tissues compared to wt(16). *Cpb2* deficiency in this model also resulted in an altered immune response to sepsis, although bacterial outgrowth, levels of TNF-α and IL-6 were higher compared to wild type, whereas in the meningitis model reduced severity was observed(16).

Taken together, our results suggest that the effect of TAFI on disease severity in bacterial meningitis may be primarily due to baseline suppression of complement activity rather than reduced fibrinolysis. We hypothesize that during the initial phase of infection low or absent TAFI results in higher initial complement levels, allowing for a more readily activated innate immune system, as is illustrated by *Cpb2*$^{-/-}$ mice having better survival and humans with lower TAFI having fewer systemic complications. As the disease progresses, TAFI fails to inhibit complement, despite elevated TAFI levels, as shown by the similarity in complement activation in later stages of infection between *Cpb2*$^{-/-}$ and wild-type mice (Figure 4G). Our findings do not show any effect of TAFI on fibrinolysis parameters.

Our study has several limitations. Because DNA and CSF were not available for all patients, particularly not for those with more severe disease, a selection bias was introduced, possibly resulting in type II errors. However, this does not undermine our findings of the association between rs1926447 and rs3742264 and systemic complications. A similar bias is present in the murine cytokine, coagulation and histology data, where mice that died before the defined end-points were not included, possibly leading to an underestimation of the observed differences. Because of the multiple analytes studied in the mice model at multiple time-points there is a risk of

overtesting. Researchers were not blinded to genetic status of the mice, possibly resulting in a bias in assessing the clinical score. However, as the groups did not differ, this is unlikely to have influenced the results. A further limitation is that we did not collect plasma samples during the acute phase of disease. Therefore, plasma levels of TAFI and complement were unavailable, which may have elucidated the association between systemic complications, TAFI CSF levels and complement activation. Furthermore, biochemical properties of TAFI differ somewhat between mice and humans (8). TAFI in mice is an acute-phase protein, whereas in humans *in vitro* transcription is not enhanced by acute-phase mediators(36). Lastly, to further clarify the specific substrate of TAFI, the effect of complement receptor antagonists or substances that deplete fibrinogen should be assessed in future experiments.

In conclusion, we found that TAFI plays an important role during bacterial meningitis, influencing the occurrence of systemic complications and inflammation, without effect on outcome. Our experimental data show that the effect of TAFI on disease severity may be mainly driven by its anti-inflammatory capacity rather than its inhibition of fibrinolysis. As initial inhibition of the complement system appears beneficial, this may prove to be useful therapeutic approach in patients with bacterial meningitis, and needs to be further investigated.

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CHAPTER 8

CEREBROSPINAL FLUID COMPLEMENT ACTIVATION IN PATIENTS WITH PNEUMOCOCCAL AND MENINGOCOCCAL MENINGITIS

Mook-Kanamori BB, Brouwer MC, Geldhoff M, van der Ende A, van de Beek D

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ABSTRACT

Background: Recent research into the treatment of bacterial meningitis has examined the innate immune system, specifically the complement system, as a potential target for adjuvant therapy. However, the effects of blocking the complement system may be pathogen dependent.
Methods: We measured cerebrospinal fluid (CSF) levels of complement components C1q, C3a, iC3b, C5a, sC5b-9, CFH and MBL in 310 patients with pneumococcal and meningococcal meningitis from a prospective nationwide cohort study. The CSF complement component levels were successfully determined for between 289 (93%) and 307 (99%) patients, depending on available volumes of stored CSF.
Results: Complement factors C1q and MBL as well as common complement pathway factors C3a, iC3b, C5a, sC5b-9 and complement regulator CFH were all elevated in patients with bacterial meningitis as compared to the controls. CSF levels of complement components C5a and sC5b-9 were higher in patients with pneumococcal meningitis compared to those with meningococcal meningitis. After correction for age, immunocompromised state and level of consciousness, the CSF concentrations of C5a and sC5b-9 remained different between causative microorganisms (P=0.006 and P=0.016 respectively). In pneumococcal meningitis high C5a and C5b-9 levels are associated with the occurrence of systemic complications, unfavorable outcome and death, whereas an inverse relationship between C5b-9 levels and mortality is observed in meningococcal meningitis.
Conclusions: Our study shows striking variations in complement activation depending on the pathogen responsible for the bacterial meningitis. In pneumococcal meningitis, high CSF complement levels were a strong indicator of disease severity and mortality, however in meningococcal meningitis, an inverse relationship between sC5b-9 and mortality was observed.

INTRODUCTION

Bacterial meningitis remains an important cause of mortality and morbidity worldwide, despite the implementation of antibiotic therapy, adjunctive dexamethasone treatment and childhood vaccination strategies(1-3). The most common causative organisms are *Streptococcus pneumonia* and *Neisseria meningitidis*, which together account for 85% of all cases of bacterial meningitis in Europe and the United States(2). The rates of mortality caused by these bacterial infections are 26% and 9% respectively, and of those patients who survive, up to half suffer from neurological sequelae, including hearing loss, cognitive impairment and focal neurological deficits.
Recent studies have looked at the innate immune system, specifically the complement system, as a potential target for adjunctive therapy(4). Complement factor 5 (C5), a principle component of the common complement pathway, was investigated as a novel potential target in pneumococcal meningitis(5). Murine studies showed that mice with pneumococcal meningitis with adjuvant treatment with antibodies against C5 had no mortality, and significantly less histopathological damage and cerebral spinal fluid (CSF) inflammation as compared to mice treated with adjuvant dexamethasone or isotype antibodies(5). The subsequent question is whether adjuvant treatment with antibodies against C5 will also be beneficial for patients with meningococcal meningitis(6). Currently no reproducible murine meningococcal meningitis model is available. Genetic studies have shown that complement

deficiencies in the common complement pathway predispose to meningococcal meningitis(7), warranting caution in complete blocking complement as it may increase severity of meningococcal disease(6). Evaluation of complement activation patterns in pneumococcal and meningococcal infection may provide insight in the role of complement in bacterial meningitis due to these pathogens and whether complement inhibition has potential as an adjuvant treatment.
We investigated patterns of complement activation in CSF of bacterial meningitis patients included in a prospective nation-wide cohort study. We discuss the differences in complement activation between causative pathogens, the potential consequences for future treatments and directions for further study.

METHODS

We included bacterial meningitis patients older than 16 years of age with positive CSF cultures and who were identified by the Netherlands Reference Laboratory for Bacterial Meningitis (NRLBM) between March 2006 and June 2010.

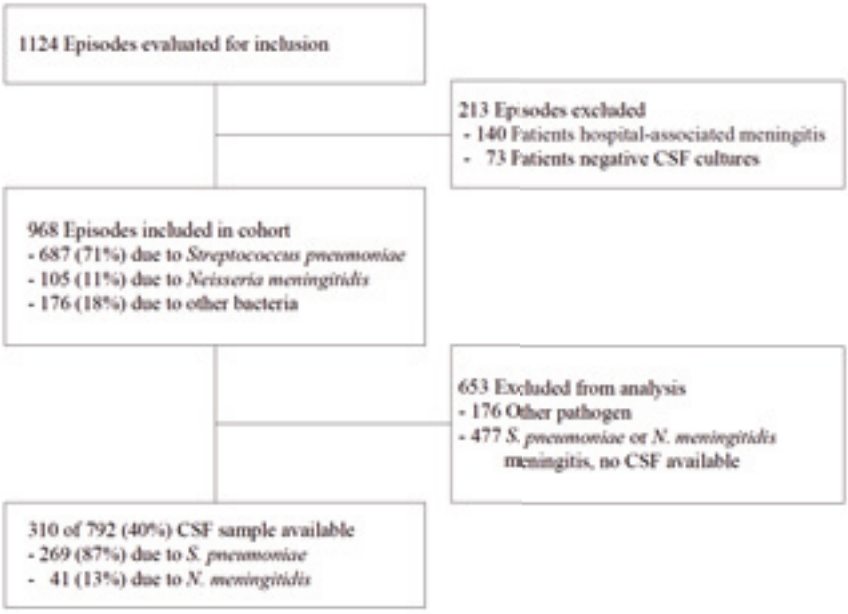

FIGURE 1 Selection of patients.

The NRLBM receives bacterial isolates from approximately 85% of bacterial meningitis patients in the Netherlands and provided the names of the hospitals where patients with bacterial meningitis had been admitted 2e6 days previously. The treating physician was contacted, and written informed consent was obtained from all participating patients or their legally authorized representatives. We did not include patients with hospital-acquired bacterial meningitis, those with a neurosurgical device (CSF drain) or negative CSF cultures. CSF samples from 18 patients without bacterial

meningitis, in whom a subarachnoidal hemorrhage was excluded by CSF examination, served as negative controls.
CSF of participating patients was obtained by lumbar puncture and stored at -80^{o}C. Complement levels were determined as follows: C3 using Luminex technology with Milliplex MAP kit (Millipore corp, St. Charles, MO, USA); C3a, iC3b, C5a, sC5b-9, CFH by ELISA from Microvue Quidel, (Quidel, San Diego, CA, USA); C1q and MBL by ELISA (Hycult biotech, Uden, the Netherlands). Secured online caserecord forms (CRFs) were used to collect patient data including medical history, symptoms and signs on admission, treatment, complications, and outcome. Neurological complications were defined as impairment of consciousness, seizures, or focal neurological abnormalities. Systemic complications were defined as cardiorespiratory failure or need for mechanical ventilation.
The clinical outcome was graded at discharge using the validated Glasgow Outcome Scale (GOS)(8), a 5 point scale in which a score of 1 indicates death, and a score of 5 indicates mild or no disability (the patient is able to return to work or school). We defined a favorable outcome as a GOS score of 5, and unfavorable outcome as a score of 1e4. The study was approved by the medical ethical review committee of the Academic Medical Centre, Amsterdam, the Netherlands.
Continuous data are presented as medians and interquartile ranges (IQR). Differences in complement levels between different patient groups were compared using a Mann-Whitney U-test for continuous variables and a Chi-square test or Fisher's exact test regarding dichotomous variables. The main analysis was performed using nonparametric tests (Mann-Whitney U). Logistic regression analysis was used to correct for possible confounders such as age, immunocompromised state and level of consciousness at time of presentation determined by the Glasgow Coma Scale (GCS) score. Strength of relationships between continuous variables was assessed by Spearman's correlation tests. All statistical tests were 2-tailed, and a p-value of <0.05 was considered to be significant. All analyses were executed using SPSS software, version 19.0.

RESULTS

Between March 2006 and April 2011, 1181 patients were identified (Figure 1); 213 patients were excluded (140 with hospital-acquired meningitis, 73 with negative CSF cultures). Of the remaining 968 patients with bacterial meningitis, 71% (n=687) was caused by *S. pneumoniae*, 11% (n=105) by *N. meningitidis*, and 18% (n=176) by other bacteria. Of the 792 patients with either meningococcal or pneumococcal meningitis, CSF was obtained in 310 (40%) patients. Patients with meningococcal meningitis from whom CSF was obtained were younger than those with pneumococcal meningitis (median 29 vs. 62 years, $P < 0.001$), presented with less severe disease, reflected by a higher score on the GCS (median 14 vs. 10, $P < 0.001$, Table 1) and had fewer predisposing factors (22% vs. 65%, $P < 0.001$).
The CSF complement component levels were successfully determined for between 289 (93%) and 307 (99%) of patients of whom CSF was obtained, depending on available volumes of stored CSF. Complement factors C1q and MBL as well as common complement pathway factors C3a, iC3b, C5a, sC5b-9 and complement regulator CFH were all elevated in patients with bacterial meningitis as compared to the controls (Table 2). CSF levels of complement components C5a and sC5b-9 were higher in patients with pneumococcal meningitis compared to those with meningococcal meningitis (Table 2). After correction for age, immunocompromised

TABLE 1 Baseline patient characteristics of included patients with pneumococcal or meningococcal meningitis with CSF avail- able for analysis (n Z 310).[a]

Clinical Characterisics	Pneumococcal meningitis (n=269)	Meningococcal meningitis (n=41)	P-value
Age (yrs)	61 (49-70)	29 (19-52)	<0.001
Male	128/269 (48)	21/41 (51)	0.66
Predisposing factors for meningitis	176/269 (65)	9/41 (22)	<0.001
Otitis or sinusitis	110/268 (41)	2/41 (5)	<0.001
Pneumonia	24/260 (9)	1/41 (2)	0.22
Immunocompromised state[b]	81/269 (30)	6/41 (15)	0.04
Symptoms and signs on admission			
Symptoms < 24 hrs	125/261 (48)	21/39 (54)	0.49
GCS	10 (8-13)	14 (11-15)	<0.001
Headache	189/231 (82)	31/37 (84)	0.77
Neck stiffness	195/266 (73)	33/41 (81)	0.61
Temp >= 38hrs	196/244 (80)	26/37 (70)	0.16
Rash	13/269 (5)	28/41 (68)	<0.01
Diast bloodpressure <60mmHg	23/266 (9)	7/41 (17)	0.09
Blood Chemistry Tests[c]			
ESR	46 (25-72)	28 (7-50)	0.004
CRP	209 (93-319)	230 (183-385)	0.07
Leucocytes	17 (12-23)	19 (16-24)	0.14
Blood culture positivity	192/237 (81%)	21/38 (55%)	<0.001
CSF values[d]			
WBC	3109 (605-9855)	12900 (4480-28856)	<0.001
Protein	4.0 (2.6-5.9)	4.6 (2.3-6.2)	0.89
CSF/blood glucose ratio	0.02 (0.00-0.23)	0.04 (0.00-0.36)	0.28
Outcome			
Death	35/268 (13)	2/41 (5)	0.20
Unfavorable outcome	106/269 (39)	6/41 (15)	0.002
Neurological complications	164/206 (80)	16/22 (73)	0.45
Systemic complications	116/263 (44)	6/41 (15)	<0.001

[a] Data are number/number evaluated (%) or median (interquartile range).
[b] Immunocompromise was defined by the use of immunosuppressive drugs, a history of splenectomy, or the presence of diabetes mellitus, alcoholism, as well as patients infected with the human immunodeficiency virus (HIV).
[c] erythrocyte sedimentation rate (ESR) was determined in 167 patients, C-reactive proteint (CRP) in 294 patients, and leukocyte count in 308 patients.
[d] CSF white blood cell count was determined in 300 patients, CSF proteins levels in 298 patients and the CSF/blood glucose ratio in 295 patients.

TABLE 2 CSF levels of C1q, MBL, CFH, C3, C3a, iC3b, C5a, and sC5b-9.

Complement factor	Control patients (n=18)	Patients with pneumococcal meningitis (n=269)	Patients with meningococcal meningitis (n= 41)
C1q	105 (73-139)	188 (115-301) **	226 (106-420) **
MBL	3.0 (2.0-3.9)	13 (5-46) **	16 (4-52) **
C3	3030 (259-5418)	1223 (357-3673)	1562 (400-3874)
C3a	6.0 (4.0-9.1)	568 (195-1200) **	481 (51-997) **
iC3b	3.6 (2.7-4.9)	23 (11-45) **	17 (3-36) **
C5a	0.0 (0-0.51)	17 (4-58) **,††	4 (2-6) **,††
C5b-9	155 (57-405)	2389 (566-5863) **,††	1805 (91-2963) **,††
CFH	1166 (928 – 1516)	11413 (7546-15931) **	12710 (4160-17990) **

Concentrations are expressed in ng/ml, values are medians with interquartile ranges.
*P <0.05; **P<0.01 as compared to controls. †P<0.05 ††P<0.01, comparison between patients with meningococcal and pneumococcal meningitis.

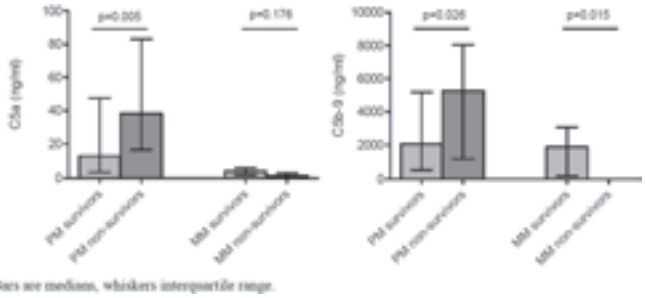

FIGURE 2 CSF levels of complement factors C5a and C5b-9 in deceased and surviving patients with pneumococcal and meningo- coccal meningitis.[a]

[a]Bars are medians, whiskers interquartile range.

state and level of consciousness (GCS score), the CSF concentrations of C5a and sC5b-9 remained different between causative microorganisms (P=0.006 and P=0.016 respectively).
In patients with pneumococcal meningitis, levels of C5a and sC5b-9 were higher in those patients who eventually died compared to survivors (C5a 38 vs. 14 ng/ml, P0.005; sC5b-9 5216 ng/ml vs. 2085 ng/ml, P=0.026, Figure 2). In the subgroup of patients with meningococcal meningitis, those who died had lower levels of C5b-9 than those patients who survived (0 vs. 1863 ng/ml, P=0.015, Figure 2).

DISCUSSION

We found that CSF patterns of complement activation differ between patients with pneumococcal and meningococcal meningitis. In patients with pneumococcal meningitis, high CSF complement levels were a strong indicator of disease severity, systemic complications, unfavorable outcome and death. In patients with meningococcal disease, an inverse relationship between sC5b-9 and mortality was observed, indicating that insufficient complement activation may result in worse prognosis in these patients.
Elevated levels of CSF complement factors C3a, C3b and MBL in patients with pneumococcal meningitis has been previously reported and were shown to be related to host genetic factors. The novel findings in this study center around the

measurement of complement in all major complement pathways, and in both pneumococcal and meningococcal meningitis, allowing analysis of the differences between the two most common causative pathogens of bacterial meningitis.
The observed difference in complement response between patients with meningococcal and pneumococcal meningitis may be explained several ways. First, several baseline characteristics differed between patients who acquired the respective infections, of which the difference in age at time of presentation was most notable. Several complement mediated diseases are age-related, such as atypical hemolytic uremic syndrome which occurs at very young age and age-related macula degeneration, occurring at old age(9). However, there is no indication that the complement system activity varies with age. To correct for possible confounding factors such as age, level of consciousness at presentation, and immunocompromised status, we performed a multivariate analysis showing a significant difference between C5a and sC5b-9 levels in meningococcal and pneumococcal meningitis. Therefore, it is unlikely that baseline clinical characteristics or preexistent co-morbidity fully explain the observed differences in complement activation in patients with pneumococcal and meningococcal meningitis.
Furthermore, genetic difference between patients with meningococcal and pneumococcal meningitis may explain differences in complement activation. There is ample evidence that complement deficiencies predispose patients for both invasive meningococcal and pneumococcal disease(7, 10). In meningococcal disease, the importance of the common and terminal complement pathways has been illustrated by the high incidence of invasive meningococcal disease in patients with late complement component deficiencies (C5eC9) compared to control patients(7). Deficiency of the alternative pathway proteins properdin and complement factor D have also been shown to result in increased risk of meningococcal disease in case series(6, 10). A genome wide association study showed that complement factor H is associated with susceptibility for meningococcal disease(11). For pneumococcal disease, a meta analysis showed patients with invasive pneumococcal disease more often have the variant alleles of MBL2 polymorphisms than control patients(10), which was recently confirmed in pneumococcal meningitis patients(12). Patients deficient for complement factor 2 had an increased risk for both meningococcal and pneumococcal disease, and a recent genetic association study showed that complement factor 3 polymorphisms were associated with increased susceptibility to bacterial meningitis in general(13).
The pathophysiological response to *N. meningitidis* and *S. pneumoniae* infection differs considerably. Both pathogens reside in the nasopharynx of human hosts(4, 14). *N. meningitidis* has evolved to be resistant to complement mediated lysis through its polysaccharide capsule and the recruitment of negative complement regulators such as complement factor H (CFH, which blocks the alternative pathway) and C4- binding protein (C4BP, which blocks the C3 convertase-C4b2a) (14, 15). When complement activation does occur during meningococcal disease, the formation of the C5b-C9 membrane attack complex is vital for meningococcal clearance. Upon invasion, the pneumococcal polysaccharide capsule acts as a non-specific barrier, reducing complement deposition and subsequent phagocytosis(4). Surface proteins inhibit complement factors C1q (classical pathway) and subsequent C3 deposition, and like meningococcal surface proteins, also bind CFH and C4BP. Pneumolysin, a pore-forming toxin, is a potent activator of the classical complement pathway. Secreted pneumolysin limits opsonophagosytosis, possibly by depleting complement factors away from the bacterium(4). In contrast to meningococcal infection, the membrane

attack complex (C5b-9) does not play an important role in the clearance of pneumococci(4).
Because we did not perform serum complement measurements, we cannot determine whether the observed changes in complement levels reflect changes in the systemic compartment or are compartmentalized in the CSF. Blood cultures were positive in 55% and 81% of patients with meningococcal and pneumococcal meningitis respectively (Table 1), reflecting systemic involvement in both patient groups. As blood-brain disruption is a pathophysiological hallmark of bacterial meningitis, it seems likely that the CSF findings are at least in part, reflective of processes in the systemic compartment.
Our study has several limitations. First, CSF was available for under half of the patients included in our cohort study. Although baseline characteristics were similar between patients with or without CSF available for analysis, those from whom CSF was not obtained had a higher rate of mortality and may therefore have had different CSF complement values (Supplement Table 1). Furthermore, a relatively small number of CSF samples from patients with meningococcal meningitis was available (n=41), limiting the power to detect associations of complement levels with clinical and laboratory characteristics. Also, both time between presentation and lumbar puncture, and time between lumbar puncture to storage at -80^{o}C were not recorded. However, we think it is unlikely that methodological causes resulted in the observed differences. Despite these limitations, this is the largest series of CSF complement measurements in pneumococcal and meningococcal meningitis to date and provides insight into the underlying pathophysiological differences between these causative bacteria.
Our study shows striking variations in complement activation depending on the pathogen responsible for the bacterial meningitis. Although the value of treatment with complement inhibition (antibodies against C5 and C1q) has been demonstrated in pneumococcal meningitis and sepsis animal models, treatment of patients with bacterial meningitis is rarely initiated with knowledge of the underlying causative organism. Thus, potential treatment options should be investigated in a broader setting; experimental studies using multiple pneumococcal serotypes and a meningococcal meningitis model, as well as selective complement pathway blocking agents (for instance C5a) may provide more insight into the clinical value of the blocking of complement as an adjuvant therapy.

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CHAPTER 9

SUMMARY AND GENERAL DISCUSSION

This chapter reviews the main findings of this thesis, views the results in the context of current research in the field, and gives suggestions for further directions in which research may be of benefit.

Introduction

Bacterial meningitis remains a deadly disease. Currently, the most common causative agents are the *Streptococcus pneumoniae* and *Neisseria meningitidis*, which together are responsible for over 80% of the cases of bacterial meningitis in the developed world. Presently, despite advances in medical care, mortality from pneumococcal meningitis occurs in up to 30%, and neurological sequelae, including hearing loss focal neurological deficits and cognitive impairment, are estimated to occur in almost half of surviving patients(1).

Advances in curbing the impact of bacterial meningitis may be divided into two areas: prevention and treatment. Prevention has been and will continue to be primarily through vaccination strategies. History has shown the profound effect of vaccination programs: *Haemophilus influenzae* meningitis has been nearly eliminated; pneumococcal meningitis due to vaccine serotypes has decreased by 92%; and in regions implementing meningococcal C conjugate vaccines, substantial reductions in serogroup C disease have been recorded(2). As the epidemiology of causative bacteria changes, for example through serotype shifting following vaccination efforts or though the increasing problem of drug resistant bacteria, vaccination development and implementation strategies will have to adapt.

Bacterial meningitis was uniformly lethal until the introduction of meningococcal antisera in the 1920s, followed by the advent of penicillin in the 1940s(3). Since then, the rate of mortality remained largely stable, until the introduction of dexamethasone as an adjuvant treatment some 60 years later. Still, current rates of fatality and morbidity remain uncomfortably high, particularly in developing countries fuelling further efforts for treatment and vaccine development.

In the 21st century, the genetic basis of bacterial virulence factors and host genetic factors determining susceptibility and outcome as well as pathophysiological interactions taking place during the disease process are becoming increasingly clear. The future breakthroughs will likely come from combining the efforts from the microbiological to large population studies in so-called translational research.

The context of research of the 21st century:
Translational research:

At the foundation of our translational approach lies a large nationwide prospective cohort of patients with bacterial meningitis of which isolates of the causative bacteria, DNA of patients as well as matched controls and extensive clinical data are collected. The genome of bacterial isolates is being scrutinized to determine what proteins are responsible for in- or decreased virulence and outcome in patients. Whole genome sequencing is revealing factors such as ArgH in *Streptococcus pneumoniae* and CFHbp in *Neisseria meningitidis*(4, 5). Genome wide studies of patients with bacterial meningitis have yielded many genes and proteins associated with susceptibility, outcome, or the occurrence of complications(6). As an increasing

number of these variables are characterized, our understanding of the underlying mechanisms is increasing, and potential targets for vaccination or treatment are emerging.

How the murine model is contributing to our understanding of bacterial meningitis:

What factors make an animal model usable in bacterial meningitis? It must model the human disease course as closely as possible; it must be reproducible and easily usable; and it must be genetically modifiable. Prior efforts made use of a wide range of animals (mice, rats, rabbits) as well as many inoculation techniques (intranasal, intrathecal, intracerebral, intravenous). Limitations of these models included problems with reproducibility, validity and limited possibilities of host genetic modification. We therefore elected to create our own model.

In **Chapter 3**, we describe a murine model using adult C57BL/6 mice, a well characterized, widely used inbred strain of laboratory mouse, in which genetic modifications are readily made. Many transgenic lines already exist with modifications in genes known to be involved in the pathogenesis of bacterial meningitis. We used serotype 3 streptococcus pneumonia, one of the most frequently isolated serotypes in human setting(1), and in subsequent experiments other serotypes have been used successfully(4). After revisiting intranasal, intravenous, intraperitoneal and intracerebral inoculation methods, we chose intracisternal injection, and a dose of 10^4 CFU/mouse in which the mice became symptomatic after more than 40 hours, allowing for systemic inflammatory and histopathological features as seen in the human setting to develop and be studied. Outcome parameters included survival, clinical scoring, cultures including blood and CSF, brain histology (including microglial activation and hippocampal apoptosis) and cytokine assays. The findings mirror those in human studies, making this model a useful platform for future investigations.

The use of a single model, though well characterized, brings with it important limitations. None of the conclusions resulting from its use may be generalized. Mice strains vary in their response to a single pneumococcal strain; pneumococcal strains vary in their pathogenicity in a single mouse strain; treatment regimens are likely to have variable effects when investigated in different models, most importantly when extrapolated to the human setting.

Treatment studies:

Animal models have been used for many treatment studies as described in **Chapter 2**, sometimes leading to clinical trials and changes in standard care, exemplified by the addition of adjuvant dexamethasone treatment(7, 8). One interesting area of improvement of antimicrobial therapy lies in the use of non-bacteriolytic antibiotics, such as rifampicin, moxifloxacin and daptomycin(9). These agents should limit the amount of cell debris, which is thought to contribute to the overwhelming inflammatory response and poor outcome.

In **Chapter 4** we compare the use of daptomycin to vancomycin in the presence of adjuvant dexamethasone treatment. We use a serotype 3 *S. pneumoniae*. In vitro

studies showed comparable bactericidal activity of daptomycin and vancomycin, which was not altered by the addition of dexamethasone. Using pharmacokinetic studies and therapeutic monitoring we adjusted dosing regimens to best approximate human drug serum concentrations. Our data showed that daptomycin and vancomycin to be similar at clearing *S. pneumoniae* in CSF in the presence of dexamethasone, though the bactericidal effect of daptomycin occurred more rapidly.

The findings are consistent with previous reports using rabbit and infant rat models, though in these studies no adjuvant dexamethasone was administered(10-12). Following the publication of this manuscript two articles using rabbit models and two pneumococcal strains confirmed the bactericidal effect of daptomycin in the presence of dexamethasone (13, 14).

In spite of our findings and the favorable results of clinical trials in which daptomycin was used to treat Gram-positive bacteremia, endocarditis and complicated skin infections, we cannot presently conclude that daptomycin may be used in the treatment of patients with pneumococcal meningitis for several reasons: Firstly, as outlined in **Chapters 2**, the outcome of patients with bacterial meningitis is not determined by solely decreasing bacterial load or inflammation; few studies have addressed functional outcome after treatment with daptomycin. Second, the effects of daptomycin in pneumococcal meningitis have been investigated in a very limited subset of pneumococcal serotypes. Lastly, in experimental models, the effects of daptomycin when used in combination with dexamethasone are achieved at relatively high doses. At lower doses, rabbit models showed lower rates of bacterial clearance(14). These dosage regimens (after conversion of Animal Doses to Human Equivalent Doses) are higher than current guidelines for the treatment of complicated skin and soft tissue infections and right side infective endocarditis in the human setting. The obvious possibility of increased side effects and adverse events must therefore be considered.

Pathophysiology studies (1)

Studying pathophysiological pathways and mechanisms is possibly where the murine model can contribute most significantly. In this thesis studies are performed in two areas, inflammation and coagulation.

As described in **Chapter 2**, inflammation is initiated almost immediately following contact with *S. pneumoniae*. Once in the bloodstream, the complement system is activated and the pneumococci are recognized by antigen presenting cells (APCs) through the binding of pattern recognition receptor to specific bacterial proteins. Once activated, these APCs release various cytokines such as TNF-α, IL-1 and IL-6, which induce upregulation of adhesion molecules on the vascular epithelium, mediate leukocyte influx and further inflammation.

The detection of pneumococci by APCs occurs via pattern recognition receptors, such as Toll-like receptors (TLRs) 2, 4 and 9, and Nod-like receptors (NLRs). Inflammasomes are a group of NLRs, which when activated leads to activation of Caspase-1, which in turn activates IL-1β and IL-18. Though much research continues to be done on TLRs, little is known about the role of inflammasomes in bacterial meningitis.

In **Chapters 5 and 6** we examine the role of the inflammasome in bacterial meningitis. Why? Because levels of IL-1β has been shown to be elevated in the CSF of children with bacterial meningitis and correlates with CSF bacterial concentrations(15); because the deficiency of caspase-1 causes less severe inflammation and decreased brain edema in mice with pneumococcal meningitis(16); and because the inflammasome may be an important link between the detection of pneumococci and the modulation Caspase-1 and IL-1β levels.

Our most important findings are that single-nucleotide polymorphisms in two inflammasome genes (CARD8 and NLRP3) are associated with poor disease outcome in adult patients with bacterial meningitis. IL-1β and IL-18 levels in CSF are correlated with systemic complications and unfavorable outcome, and with only systemic complications in the subgroup of pneumococcal meningitis. The cytokine levels appear to be serotype dependent, prompting caution when interpreting experimental data using a single serotype pneumococcus. Knocking out key inflammasome proteins NLRP and ASC results led to decreased systemic inflammation and bacterial loads, whereas the Nlrp-/- mice displayed no decreased CNS inflammation and even an enhancement of brain damage.

Review of the literature shows why we must have caution in interpreting these results: murine studies of pneumococcal pneumonia suggest that NLRP may be beneficial, but emphasize the large variation in IL-1β production between pneumococcal serotypes(17), a finding that mirrors the observations in our clinical cohort. In contrast to our findings, a previous murine pneumococcal meningitis model found a better clinical outcome and brain inflammation in Nlrp-/- and Asc -/- mice, but used serotype 2 pneumococcus (which rarely is found as the cause of human pneumococcal meningitis).

Thus, though the inflammasome plays clearly a role in pneumococcal meningitis as a whole, its role and the role of IL-1β and IL-18 appear dependent on pneumococcal serotype.

Pathophysiology studies (2)

Another large area for improvement of understanding and potential therapeutic gain is the disruption of coagulation/fibrinolysis during bacterial meningitis. Ischemic stroke occurs in nearly 25% of patients, and intracerebral hemorrhages in 2-3%(18, 19). **Chapter 2** describes how the long held presumption that vasculitis is solely responsible for ischemic stroke may not be accurate, and that coagulopathy and dysregulation of the fibrinolytic pathways are likely to play an important role(20). The same inflammatory cytokines, IL-6, TNF-α and IL-1β to be involved in inflammation-induced coagulopathy as well as inhibition of fibrinolysis(21).

One of the proteins involved in both coagulation and inflammation is TAFI. It is activated by high levels of thrombin (when complexed with thrombomodulin), thus during a prothrombotic state, and it inhibits fibrinolysis by inhibiting the degradation of fibrin by plasmin. TAFI can also inactivate complement factors C3a and C5a (potent inflammatory mediators and chemoattractants).

In **Chapter 7** we describe that TAFI does play a role in bacterial meningitis. We find that 2 SNPs in the CPB2 gene (encoding for TAFI) are associated with the occurrence of systemic complications; that the risk genotypes of both alleles showed higher TAFI levels in CSF; and that higher TAFI protein levels in CSF are similarly associated with the occurrence of systemic complications. In the murine model TAFI deficiency led to attenuated inflammation and bacterial outgrowth only in the systemic compartment. Both in the human and murine setting, there was no evidence of TAFI being mediating CNS pathology. And just like in previous murine studies on sepsis induced liver damage(22), it seems that TAFIs effect on disease severity is mainly driven by its anti-inflammatory capacity rather than impacting fibrinolytic activity in the advanced stages of bacterial meningitis.

An important caveat is that TAFI's role may well lie in the baseline suppression of complement activity, as illustrated by the higher initial complement levels at baseline measurement in the TAFI knockout mice, and therefore be primarily important in the initial phases of infection. TAFI does not seem to affect complement (or fibrinolysis) at later stages of infection. I therefore suspect that TAFI itself is not a likely beneficial target for therapeutic intervention studies. Rather direct complement inhibition, such as demonstrated by Woehrl et al.(23), could prove more effective in curbing the clinical course of patients with pneumococcal meningitis.

With **Chapter 7** and the complement inhibition studies in mind, we further explore the patterns of complement activation in bacterial meningitis in **Chapter 8**. By measuring complement in CSF of over 300 patients with bacterial meningitis, we discover that complement activation varies considerably depending which causative pathogen is involved. Specifically, high levels of CSF complement activation are predictive of greater disease severity and mortality in patients with pneumococcal meningitis, whereas an inverse relationship is observed patient with meningococcal meningitis.

These findings raise interesting questions regarding the practical implementation of therapies that target the complement system. In clinical practice treatment is often initiated before a definite causative organism has been found, underlining the need for treatment effectiveness across pneumococcal and meningococcal serotypes. If possible, treatment studies aiming at complement inhibition will therefore need to be conducted in experimental models using several of the most common pathogens. Likewise, we must be cautious to generalize conclusions regarding complement inhibition when derived from models using a single pathogen.

Suggestions for future research

Bacterial meningitis presents us with an evolving therapeutic challenge. The introduction of conjugate vaccines and increasing emergence of drug resistant strains present us with a moving target. Therapeutic advances will lie in the optimization of antimicrobial treatment and the reduction of inflammation by adjuvant drugs.

For antimicrobial therapy, future regimens may include the use of non-bacteriolytic drugs such as daptomycin, rather than the current bacteriolytic agents, which cause the rapid release of proinflammatory bacterial components. Experimental models are

providing the foundation for clinical trials, which are urgently needed as drug resistance increases.

Future adjuvant therapy, aimed at reducing inflammation, may move beyond blanket anti-inflammatory strategies to an approach more tailored towards regional epidemiology of bacterial pathogens and host genetics. Research is needed to learn why certain populations (bacterial and host) benefit more/less from dexamethasone, and in certain situations other anti-inflammatory agents might be of value. Adjuvant targets aimed at modulation of ROS/RNS mediated damage, caspase inhibition or complement and coagulation cascades (**Chapter 2**), are being explored in animal models such as our own. So far no clinical trials using these new targets has been performed in patients with bacterial meningitis.

Lastly, genomic studies will continue to expose new bacterial virulence factors and host factors associated with susceptibility or clinical outcome. As illustrated in this thesis with inflammasomes and TAFI, animal models will provide the platform to study these factors, furthering our understanding of the underlying pathophysiology and generating potential targets for new adjuvant treatment and vaccine development.

I can't help but anticipate that major treatment advances are right around the corner. These are exciting times, and I am grateful that the work in this thesis has contributed in laying the groundwork for future discovery.

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CHAPTER 10

SUMMARY IN DUTCH
SAMENVATTING IN HET NEDERLANDS

In dit hoofdstuk worden de belangrijkste bevindingen van dit proefschrift besproken, alsmede hun plaats in de context van de huidige wetenschappelijke stand van zaken. Tevens worden mogelijke toekomstige onderzoeksrichtingen voorgesteld.

Inleiding

Bacteriële meningitis blijft een dodelijke infectieziekte, waarvan op dit moment in ontwikkelde landen 80% wordt veroorzaakt door *Streptococcus pneumoniae* en *Neisseria meningitidis* samen. Ondanks vorderingen in medische zorg is de mortaliteit rond de 30 procent. Van de patiënten die bacteriële meningitis overleven kampt bijna de helft met blijvende neurologische klachten waaronder gehoorverlies, focale neurologisch uitval en cognitieve problemen(1).

Vooruitgang in de strijd tegen bacteriële meningitis komt voort uit ontwikkelingen in zowel preventie als behandeling. Preventie bestaat met name uit ontwikkeling en implementatie van vaccinatieprogramma's, waarvan de effectiviteit historisch meerdere malen is geïllustreerd: *Haemophilus influenza* is thans vrijwel geëlimineerd als oorzaak van bacteriële meningitis; pneumokokken meningitis door vaccinatie-serotypes is met 92% gedaald; serotype C meningokokken komen minder vaak als verwekker voor in regio's waar gevaccineerd wordt(2). Door deze successen, maar ook door de opkomst van antibiotica-resistente bacteriën, verandert de epidemiologische achtergrond van bacteriële meningitis waardoor de ontwikkeling en implementatie van vaccinatie strategieën in de toekomst zal moeten blijven mee veranderen.

Tot de ontwikkeling van antisera in de jaren 20 van de vorige eeuw was het beloop van bacteriële meningitis vrijwel zeker fataal(3). In de jaren 40 werd met de komst van penicilline de mortaliteit verder gereduceerd tot een niveau die sindsdien globaal onveranderd is gebleven tot de introductie van dexamethason als adjuvante behandeling bijna 60 jaar later. Toch blijven tot op heden de mortaliteit en morbiditeit hoog, zeker in de minder ontwikkelde landen, hetgeen de noodzaak voor verder onderzoek naar zowel behandeling als vaccinatie strategieën blijft onderbouwen.

De 21^{e} eeuw heeft de mogelijkheid tot uitgebreid genetisch onderzoek van zowel bacteriële verwekker als patiënt met zich meegebracht, waardoor nieuwe inzichten zullen ontstaan t.a.v. de reden waarom bepaalde verwekkers bij bepaalde groepen patiënten resulteren in een beter of slechter ziektebeloop. Nieuwe inzichten zullen zeer waarschijnlijk voortvloeien uit integratie van het gehele spectrum van onderzoek (van bacteriële verwekker tot patiënt/populatie niveau) in een zogenoemde "translationele benadering".

Translationeel onderzoek:

De basis van onze translationele benadering wordt gevormd door een groot landelijk prospectief cohort van patiënten met bacteriële meningitis. Uit dat cohort is naast uitgebreide klinische informatie ook genetisch materiaal verkregen van zowel verwekkers, patiënten als naasten. Zodoende zijn bij analyse van bacterieel DNA reeds meerdere genen geïdentificeerd die het beloop van infectie blijken te beïnvloeden(4, 5). En zijn bij analyse van DNA van patiënten genen gevonden die van belang zijn voor de gevoeligheid voor infectie, klinisch beloop en de kans op

complicaties(6). Deze bevindingen dragen bij aan onze kennis van de onderliggende ziektemechanismen, waaruit vervolgens nieuwe behandeling- en vaccinatiestrategieën kunnen worden ontwikkeld.

De bijdrage van een muismodel aan het translationeel onderzoek:

Een diermodel wordt bij een translationele benadering voornamelijk gebruikt (1) ter uitbreiding van onze kennis van het onderliggende ziekteproces (bv. het onderzoeken van de verschillende genetische factoren bij verwekker en patiënt die het ziektebeloop kunnen beïnvloeden) en (2) ter evaluatie van mogelijke nieuwe therapeutische strategieën.

Om een diermodel effectief te gebruiken moet het aan een aantal voorwaarden voldoen: het moet de menselijke situatie zo goed als mogelijk weerspiegelen; het moet eenvoudig bruikbaar en reproduceerbaar zijn en er moet de mogelijkheid zijn voor het creëren van genetische veranderingen bij zowel verwekker als proefdier. Vooraf aan dit proefschrift zijn reeds meerdere diermodellen beschreven waarbij gebruik is gemaakt van diverse diersoorten en inoculatie-methoden. De beperkingen liggen vooral in de reproduceerbaarheid, juiste weerspiegeling van het klinisch beloop van de mens, of beperkte mogelijkheden tot genetische modificatie. Deze beperkingen heeft ons er toe gezet ons eigen diermodel te ontwikkelen.

In Hoofdstuk 3 wordt ons muismodel van pneumokokkenmeningitis beschreven waarbij gekozen wordt voor een muissoort die zeer algemeen gebruikt wordt en waarvan reeds zeer veel genetische gemodificeerde soorten beschikbaar zijn (ook met mutaties in genen waarvan bekend is dat deze een belangrijk rol spelen bij bacteriële meningitis). Verder maken wij initieel gebruik van serotype 3 *S. Pneumoniae*, een van de meest voorkomende serotypen bij de mens(1); later worden ook andere serotypes gebruikt(4). Als methode voor inoculatie wordt uiteindelijk gekozen voor intracisternale injectie waarbij de verwekker direct in de cisterna magna wordt geïnjecteerd. Het ziekteproces wordt door middel van klinische scoringslijsten, bloed- en liquoranalyse en histologie beschreven. De bevindingen weerspiegelen de menselijke situatie en derhalve wordt dit model gebruikt voor toekomstige onderzoek.

Hoewel het model voldoet aan onze eisen en wensen, heeft het gebruik van een enkel model uiteraard de belangrijke beperking dat geen algemene conclusies hieruit kunnen worden getrokken, zeker niet wanneer een stap naar de menselijke situatie wordt overwogen.

Behandelstudies:

Diermodellen zijn in het verleden veelvuldig gebruikt bij ontwikkeling en toetsing van nieuwe therapieën, soms met als gevolg een verandering in standaard behandeling, zoals toevoeging van adjuvante behandeling met dexamethason(7, 8). Een van de vele mogelijke strategieën qua verbetering van antibiotische behandeling (zoals beschreven in hoofdstuk 2) is het gebruik van zogenoemde niet-bacteriolytische antibiotica(9). Hierbij wordt beoogd de "debris" van bacteriële lysis, waarvan wordt gedacht dat deze bijdragen aan de overweldigende inflammatie (en daarmee slechtere uitkomst), te beperken.

In Hoofdstuk 4 wordt het effect van het niet-bacteriolytische antibioticum daptomycine vergeleken met het bekende vancomycine in de aanwezigheid van adjuvante dexamethasone bij de behandeling van serotype 3 *S. pneumoniae* meningitis. *In vitro* wordt geconstateerd dat het bactericidale effect van daptomycine en vancomycine in aanwezigheid van dexamethasone gelijkwaardig lijken. Verder blijkt de bacteriële klaring uit het liquor voor beide behandelingen *in vivo* ongeveer gelijk, hoewel dit effect door daptomycine sneller wordt bereikt.

Deze bevindingen komen overeen met voorgaande observaties in rat- en konijnmodellen, hoewel bij die studies geen adjuvante dexamethason werd gegeven(10-12). Sinds de publicatie van ons onderzoek zijn de resultaten van twee verdere onderzoeken gepubliceerd waarbij in konijnmodellen bij twee pneumokok serotypes het bactericidale effect van daptomycine in de aanwezigheid van dexamethason wordt aangetoond(13, 14).

Ondanks deze bevindingen kan om meerdere redenen op dit moment niet zonder meer worden gesteld dat daptomycine geschikt is voor de behandeling van patiënten met pneumokokkenmeningitis. Ten eerste wordt de uitkomst van patiënten met bacteriële meningitis niet uitsluitend bepaald door bacteriële klaring dan wel inflammatie. Slechts enkele studies hebben functionele uitkomst trachten te objectiveren na behandeling met daptomycine. Ten tweede zijn de effecten slechts bij een zeer beperkt aantal pneumokokkenserotypen onderzocht. Tenslotte worden bij de diermodellen relatief hoge doseringen daptomycine gegeven (ook na omrekenen naar humane equivalente hoeveelheden); bij lagere dosering bleek daptomycine minder effectief(14). Omdat deze doseringen hoger zijn dan geadviseerd voor indicaties waarvoor daptomycine reeds is geregistreerd (gecompliceerde huidinfecties en rechtszijdige infectieve endocarditis), moet rekening gehouden worden met meer bijwerkingen en complicaties t.g.v. de daptomycine zelf.

Pathofysiologiestudie (1)

In dit proefschrift werd het muismodel gebruikt om twee belangrijke aspecten van het onderliggend ziekteproces nader te onderzoeken, zijnde inflammatie en stolling.

Inflammatie ontstaat vrijwel gelijk na contact van *S. pneumoniae* met bloed. Het complement systeem wordt geactiveerd en de pneumokokken worden herkend door antigeen-presenterende cellen (APC) die diverse ontstekingsmediatoren produceren. Vaatwand ontsteking, verhoogde vaatwand doorgankelijkheid en verdere ontsteking zijn o.a. het gevolg.

De herkenning van de pneumokok door een APC gebeurt via specifieke receptoren waarvan de zogenaamde “inflammasome” er een is. Bij activatie van de inflammasome wordt caspase-1 geactiveerd, die vervolgens leidt tot activatie van cytokinen IL-1β en IL-18.

Over de rol van de inflammasome in bacteriële meningitis is weinig bekend. Wel is bekend dat IL-1β verhoogd is in liquor van kinderen met bacteriële meningitis(15), en dat in muizen met pneumokokken meningitis caspase-1 deficiëntie leidt tot minder inflammatie en hersenoedeem(16). Omdat de inflammasome een link zou kunnen zijn

tussen de detectie van de pneumokok en de modulatie van caspase-1 en IL-1β werd deze verder onderzocht.

Onze belangrijkste bevindingen waren dat variaties (single-nucleotide polymorphisms, SNPs) in twee inflammasome genen (CARD 8 en NLRP3) geassocieerd waren met een slechtere uitkomst bij patiënten met bacteriële meningitis. Tevens correleerden de IL-1β en IL-18 concentratie in liquor met het optreden van systemische complicaties en slechtere uitkomst in patiënten met bacteriële meningitis. De cytokine spiegels lijken overigens pneumokok serotype-afhankelijk. In ons muismodel werd geobserveerd dat het defici nties in NLRP en ASC, twee belangrijke inflammasome genen, leidt tot minder systemische inflammatie en bacteri le uitgroei; daarentegen hadden de NLRP defici nte muizen geen verminderde inflammatie van het centraal zenuwstelsel en mogelijk zelfs een toename van cerebrale schade.

Hoewel het duidelijk lijkt dat het inflammasome een rol speelt bij de pneumokokken meningitis is enige terughoudendheid nodig bij interpretatie van de bovenstaande bevindingen. In de literatuur worden namelijk zeer uiteenlopende effecten van NLRP en ASC beschreven afhankelijk van type infectiemodel (bv muis pneumokokkenpneumonie) of welk serotype pneumokok wordt gebruikt(17). Zo wordt recentelijk door een onderzoeksgroep bij NLRP en ASC defici ntie juist klinische verbetering en minder cerebrale inflammatie gezien, maar wordt hierbij gebruik gemaakt van *S. Pneumoniae* type 2, een relatief minder vaak voorkomende serotype(18).

Pathofysiologiestudie (2)

Een andere richting van potenti le therapeutische vooruitgang is het beperken van de ontregeling van stolling/fibrinolyse die bij pati nten met bacteri le meningitis lijkt op te treden. Ischemische herseninfarcten treden op bij bijna 25% van pati nten, hersenbloedingen bij 2-3%(19, 20). Dat vasculitis de enige oorzaak zou zijn hiervoor lijkt onwaarschijnlijk zoals in Hoofdstuk 2 beschreven.

TAFI is een eiwit die zowel bij stolling als inflammatie betrokken blijkt. Het kan na activatie door hoge concentraties thrombine (na complexvorming met thrombomoduline) fibrinolyse remmen door de remming van degradatie van fibrine door plasmine. Daarbij kan het ook complement factoren C3a en C5a (sterke inflammatie mediatoren) blokkeren.

In Hoofdstuk 7 tonen we dat TAFI een rol speelt in bacteri le meningitis en dat 2 SNP s in het gen wat voor TAFI codeert geassocieerd zijn met het optreden van systemische complicaties. De hoog-risico allelen van deze SNP s zijn geassocieerd met hogere TAFI spiegels in liquor; en hogere TAFI liquorspiegels zijn geassocieerd met meer systemische complicaties. In het muismodel werd bij TAFI-defici ntie alleen verminderd inflammatie in het systemisch compartiment gezien. Zowel bij muizen als in het pati nten cohort waren geen aanwijzingen voor TAFI s effect op liquor pathologie. Het lijkt derhalve dat het effect van TAFI vooral gericht is op remming van inflammatie en niet zozeer remming van fibrinolyse.

Een belangrijke overweging is dat de betrokkenheid van TAFI zeer mogelijk ligt bij de baseline remming van complement activiteit, zoals wordt ge llustreerd door

hogere initiële complement spiegels bij de TAFI deficiënte muizen. Het geobserveerde effect zou derhalve vooral het gevolg kunnen zijn van complementinhibitie tijdens de initiële fase van de infectie, zonder dat TAFI complement (of fibrinolyse) in latere stadia beïnvloedt. Ik verwacht derhalve niet dat TAFI blokkade als therapeutische interventie tot een positief klinisch effect zal leiden. Meer waarschijnlijk is dat directe complementinhibitie effectief zal zijn, zoals reeds door Woehrl et al. is beschreven(21).

Met complement inhibitie van Hoofdstuk 7 in gedachte is in Hoofdstuk 8 gekeken naar de patronen van complement activatie in bacteriële meningitis. Bij meer dan 300 patiënten zijn in liquor complementspiegels bepaald. Hierbij hebben we geconstateerd dat het activatiepatroon aanzienlijk varieert afhankelijk van welk bacteriële verwekker verantwoordelijk is. Specifiek wordt gezien dat complement activatie geassocieerd is met een ernstig ziektebeloop en mortaliteit bij patiënten met een pneumokokken meningitis; daarentegen wordt een omgekeerde relatie gezien bij patiënten met een meningokokken meningitis.

Deze bevindingen zijn vooral interessant voor de praktische implementatie van therapieën die het complement systeem zouden beïnvloeden. In de klinische praktijk wordt vaak behandeling geïnitieerd voordat een definitieve verwekker is aangetoond, waardoor een brede effectiviteit gewenst is. Gezien de bovenstaande bevindingen zullen studies waarbij complement remming wordt onderzocht moeten worden beoordeeld in modellen waarin effectiviteit tegen meerdere verwekkers en serotypes kan worden geëvalueerd.

Voorstellen voor toekomstig onderzoek:

Bacteriële meningitis blijkt een in de tijd veranderd ziektebeeld, waarbij veranderingen o.a. worden veroorzaakt door geografie, vaccinatieprogramma's en het opkomen van multiresistente verwekkers. De therapeutische vooruitgang zal naar alle waarschijnlijkheid komen uit optimalisering van antibiotische behandeling en beperking van inflammatie met adjuvante behandeling.

Toekomstige (adjuvante) behandeling zou mogelijk gerichter gegeven kunnen worden afhankelijk van verwekker en genetische achtergrond van de patiënt. Brede anti-inflammatoire behandeling is niet voor alle patiënten even gunstig en meer onderzoek is nodig om te achterhalen welke factoren (van zowel verwekker als patiënt) voorspellend zijn voor een goed therapeutisch effect. Overige behandeling (zoals ROS/RNS gemedieerde schade, caspaseremming, complement- en stollingscascadeinhibitie) worden nu in diermodellen onderzocht, zoals die in dit proefschrift beschreven. Er zijn tot op heden geen klinische trials die deze strategieën hebben onderzocht.

Tenslotte zullen genetische studies nieuwe bacteriële virulentie factoren blijven opleveren, evenals genetische factoren die geassocieerd zijn met gevoeligheid voor infectie en klinisch beloop. Het muismodel zal een platform blijven waarmee deze factoren kunnen worden onderzocht, hopelijk leidend tot een beter begrip van de onderliggende ziekteprocessen en ontwikkeling van nieuwe targets voor (adjuvante) behandeling en vaccinatie ontwikkeling.

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CURRICULUM VITAE

Barry Brendan Mook was born on January 29th 1974 in Hagen, Germany. He attended the Stedelijk Gymnasium Arnhem and graduated in 1992. In 1996 obtained a B.A. with a major in biology from Swarthmore College, Pennsylvania, USA. For 2 years he worked at Yale Medical School Department of Pathology as a research associate before returning to The Netherlands to start medical school in Nijmegen, in 1998. In 2004 he obtained his medical degree and, after working at the Rijnstate hospital in Arnhem for 1 year, he started his residency at the neurology department of the Academic Medical Center in Amsterdam (under Prof. Dr. J. Stam; Dr. J.H.T.M. Koelman; Prof Dr. R. Vermeulen). He spent one year of his training at the Medical Center Alkmaar (under Dr. R. ten Houten). In 2007 he joined the research group of Prof. Dr. D. van de Beek, and spent six months in the Mayo Clinic in Rochester, Minnesota, USA, before returning to the AMC to conduct the research described in this thesis. He completed his residency in 2012 and is now working as a clinical neurologist at the Haga hospital in the Hague, the Netherlands.

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