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Mechanisms for pneumococcal meningitis and a new vaccine platform to raise a serotype-independent protection in the host

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Cover: Fluorescent microscopy picture of BV2 cells in red and intracellular pneumococci in green, TIGR4 pneumococci on a blood-agar plate and an IVIS image of a mouse subjected to experimental pneumococcal meningitis, the signal density of bioluminescent bacteria is shown on a RGB profile plot.

Mechanisms for pneumococcal meningitis and a new vaccine platform to raise a serotype-independent protection in the host

THESIS FOR DOCTORAL DEGREE (Ph.D)

By

Sigrun Thorsdottir

The thesis will be defended in public at lecture hall “Rockefeller” Nobels väg 11, Karolinska Institutet Campus Solna, Friday, 21.10.2022 at 09:30 am

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POPULAR SCIENCE SUMMARY OF THE THESIS

The notorious reputation of the pneumococcus can be traced back far in time. In 1918, Sir William Osler termed the bacteria as 'captain of the men of death', his famous words still ringing true to this date. Pneumococcal infections still account for a large portion of deaths and post-disease complications in the world, despite widespread access to antibiotics and vaccines. The pneumococcus is a common cause of pneumonia, from where its name derives from, especially in places where people live in close proximity to each other. The bacteria can also invade the bloodstream from where they can gain access to other organs, such as the brain, to cause meningitis. The largest burden of meningitis is in the so-called meningitis belt in sub-Saharan Africa. Up to half of pneumococcal meningitis patients succumb to the infection, and at least a third of survivors live with irreversible brain injury as a long-term consequence. The limited success of antibiotics and current vaccines to eradicate the disease, is related to the high capacity of the pneumococcus to modify itself, adapting to the environment. Further research is therefore needed to discover new alternative treatment strategies that outsmart this great escape artist.

Some crucial steps in the development of pneumococcal meningitis were investigated in this thesis. Firstly, we studied how the bacteria penetrate the brain from the blood circulation, via hijacking of host barrier functions, and found that blockade of bacterial entry protects the brain from invasion and damage. Secondly, the cross-talk of the bacteria with neuronal cells was examined, and was found to be mediated directly and indirectly by two pneumococcal virulence factors. Blockade of the interaction was also found to avert neuronal cell damage. Thirdly, we investigated the impact of bacterial presence in the brain on the brain's own cleaning system, and discovered that brain drainage is blocked during pneumococcal meningitis, leading to accumulation of waste products, contributing to worsened outcome. Lastly, we suggest a new way to prevent pneumococcal infections by delivering a vaccine containing particles present in all pneumococcal strains, i.e. vesicles produced by the bacteria themselves.

The potential value of mapping out the multi-faceted ways the pneumococcus causes disease, is to gain knowledge of how to more efficiently combat the disease. Hence, a step-by-step scrutinization of the invasion or damage inducing mechanisms of the bacteria can provide important cues of where in the disease progression therapeutic intervention is likely to benefit. Prevention of disease development, by an efficient vaccine approach, remains the ultimate goal, ridding the world of this devastating disease altogether.

ABSTRACT

Streptococcus pneumoniae is a highly relevant pathogenic bacterium, responsible for a large fraction of deaths and disease morbidity in the world. The pneumococcus remains the leading cause of life-threatening pneumonia, septicemia and meningitis beyond neonatal age, despite global implementation of vaccination programs. Due to its extraordinary adaptability, *S. pneumoniae* has developed evasion strategies against most therapeutic interventions. In addition to escaping vaccine conferred immunity, antibiotic resistance trends are continuously on the rise. The pneumococcal polysaccharide capsule is an important virulence factor with around 100 distinct capsular serotypes identified so far, that vary in invasiveness. Among other major virulence factors of the pneumococcus are the cytotoxin pneumolysin, pneumococcal pili, and adhesin factors PspA and PspC.

Vaccine-induced pressure drives capsular switching, and acquisition of resistance genes is promoted by antimicrobial pressure, complicating treatment strategies. The clinical management of pneumococcal meningitis is particularly troublesome, which is reflected in persistently high rates of permanent neurological sequalae among survivors. Therefore, there is an urgent need to scrutinize the pathogenesis of invasive pneumococcal disease (IPD), to identify new adjunctive therapeutic or prophylactic targets, and improve clinical outcomes. The work presented in the thesis aims to contribute to an improved understanding of key virulence mechanisms in the development of pneumococcal meningitis. These include bacterial invasion of the brain, bacterial interactions with fundamental cellular components of the brain, and bacteria-induced disruption of the brain's fluid dynamics. Moreover, we propose a new vaccine platform to prevent pneumococcal colonization and infection in a serotype-independent manner.

Pneumococcal invasion of the brain through the blood-brain barrier, and the potential therapeutic effect of blocking the endothelial cell host receptors PECAM-1 and pIgR, was investigated in paper I. In combination with antibiotics, antibody blockade successfully prevented bacterial invasion of the brain, and protected the brain from damage, in a murine bacteremia-derived meningitis model. The feasibility to modulate host responses as adjunctive therapy was demonstrated. Bacteria-host communication between the pneumococcus and human neuronal cells was shown to occur directly and indirectly in paper II. Neuronal cell injury was induced by pneumolysin and pilus-I interactions with cytoskeletal β-actin. Inhibition of the interaction, using a β-actin antibody, partially protected against cellular damage. The pneumococcal-induced pathophysiology of the brain's waste clearance system, the glymphatic system, and consequent neurofunctional damage, was characterized in paper III. A rat meningitis model, where bacteria were intracisternally administered together with a tracer dye, was employed, to study the accumulation of fluid and bacterial components in the brains CSF compartment. The findings of the study attest to the benefit of using lumbar drainage to alleviate intracranial pressure as adjunctive therapy in bacterial meningitis. Finally, in paper IV, pneumococcal vesicles were evaluated for their capacity to induce cross-protection against several pneumococcal serotypes, in a mouse immunization model. We found that the vesicles gave an excellent homologous and heterologous protection. The conserved lipoproteins MalX and PrsA were found to be the major components in the vesicles that conferred heterologous cross-protection. We suggest that vesicles represent promising novel vaccine targets to protect against pneumococcal disease.

LIST OF SCIENTIFIC PAPERS

I. Federico Iovino, **Sigrun Thorsdottir**, Birgitta Henriques-Normark
Receptor Blockade: A Novel Approach to Protect the Brain From Pneumococcal Invasion.
J Infect Dis. 2018 Jul 2;218(3):476.

II. Mahebali Tabusi, **Sigrun Thorsdottir**, Maria Lysandrou, Ana Rita Narciso, Melania Minoia, Chinmaya Venugopal Srambickal, Jerker Widengren, Birgitta Henriques-Normark, Federico Iovino
Neuronal death in pneumococcal meningitis is triggered by pneumolysin and RrgA interactions with β-actin.
PLoS Pathog. 2021 Mar 24;17(3):e1009432

III. Jaqueline S. Generoso*, **Sigrun Thorsdottir***, Allan Collodel, Diogo Dominguini, Roberta R. E. Santo, Fabricia Petronilho, Tatiana Barichello*, Federico Iovino*.
Dysfunctional glymphatic system with disrupted aquaporin-4 expression pattern on astrocytes causes bacterial product accumulation in the CSF during pneumococcal meningitis.
mBio, 2022 Aug 29 29:e0188622

IV. Ana Rita Narciso*, Federico Iovino*, **Sigrun Thorsdottir**, Peter Mellroth¤, Mario Codemo, Christian Spoerry, Francesco Righetti, Sandra Muschiol, Staffan Normark, Priyanka Nannapaneni, Birgitta Henriques-Normark
Membrane particles evoke a serotype-independent cross-protection against pneumococcal infection that is dependent on the conserved lipoproteins MalX and PrsA.
Proc Natl Acad Sci U S A. 2022 Jun 7;119(23):e2122386119

* = Equal contribution
¤ = Did not participate in the final version of the manuscript

SCIENTIFIC PAPERS NOT INCLUDED IN THE THESIS

- **Sigrun Thorsdottir**, Birgitta Henriques-Normark, Federico Iovino
  The Role of Microglia in Bacterial Meningitis: Inflammatory Response, Experimental Models and New Neuroprotective Therapeutic Strategies. Front Microbiol. 2019 Mar 25;10:576.

- Audur Anna Aradottir Pind, Magdalena Dubik, **Sigrun Thorsdottir**, Andreas Meinke, Ali M Harandi, Jan Holmgren, Giuseppe Del Giudice, Ingileif Jonsdottir, Stefania P Bjarnarson
  Adjuvants Enhance the Induction of Germinal Center and Antibody Secreting Cells in Spleen and Their Persistence in Bone Marrow of Neonatal Mice. Front Immunol. 2019 Sep 26;10:2214.

- Audur Anna Aradottir Pind, **Sigrun Thorsdottir**, Gudbjorg Julia Magnusdottir, Andreas Meinke, Guiseppe Del Giudice, Ingileif Jonsdottir, Stefania P. Bjarnarson
  A comparative study of adjuvants' effects on neonatal plasma cell survival niche in bone marrow and persistence of humoral immune responses.
  Front. Immunol. 2022 Aug 22;10:3389

CONTENTS

LIST OF ABBREVIATIONS

IPD	Invasive pneumococcal disease
CAP	Community-acquired pneumonia
CSF	Cerebrospinal fluid
Hib	*Heamophilus influenzae*
PCVs	Pneumococcal conjugate vaccines
PCW	Pneumococcal cell wall
TA	Teichoic acid
NanA	Neuraminidase A
Ply	Pneumolysin
Pav	Pneumococcal adherence and virulence factor
Eno	Enolase
CbpA	Choline binding protein A
PspC	Pneumococcal surface protein C
LytA	Autolysin
TGF-β	Transforming growth factor β
Treg	Regulatory T-cell
IL	Interleukin
MRC-1	Mannose receptor C-type lectin 1
Ig	Immunoglobulin
ChoP	Pneumococcal phosphocholine
PAF	Platelet-activating factor
pIgR	Polymeric immunoglobulin receptor
TLR	Toll-like receptor
CRP	C-reactive protein
LR	Laminin receptor
CNS	Central Nervous System
BBB	Blood-brain barrier
PECAM-1	Platelet endothelial cell adhesion molecule 1
PRRs	Pattern recognition receptors
GFAP	Glial fibrillary acidic protein

MHC	Major histocompatibility complex
ICP	Intracranial pressure
MMPs	Matrix metalloproteases
H2O2	Hydrogen peroxide
ROS	Reactive oxygen species
AIF	Apoptosis-inducing factor
ATM	Ataxia telangiectasia mutated
PPSV23	23-valent polysaccharide vaccine
PspA	Pneumococcal surface protein A
PsaA	Pneumococcal surface adhesin A
Pht	Polyhistidine triad
NETs	Neutrophil extracellular traps
EVs	Extracellular vesicles
MPs	Membrane particles
G-CSF	Granulocyte colony-stimulating factor
EBA	Evans blue-labelled albumin
IP	Immunoprecipitation
ORF	Open reading frame
PCR	Polymerase chain reaction
NES	Neuroepithelial stem
iPS	Induced pluripotent stem

1 INTRODUCTION

1.1 THE PNEUMOCOCCUS

1.1.1 Current outlook of global pneumococcal disease

Streptococcus pneumoniae, also known as the pneumococcus, is an important human pathogen, which still represents a major Public Health threat on a global scale [1]. The largest challenge in managing pneumococcal infections is the natural ability of the pneumococcus to transform, and thereby acquire resistance to antibiotics or evade targeted vaccines [2]. Although *S. pneumoniae* mainly stays as a commensal within the nasopharynx of children and sometimes in adults, it can become invasive and cause diseases such as pneumonia, septicemia and meningitis, i.e. invasive pneumococcal disease (IPD) [3].

To date, *S. pneumoniae* remains the leading etiological cause of community-acquired pneumonia (CAP) in the world, as well as being major causative agents of life-threatening septicemia and meningitis [4, 5]. Otitis media and sinusitis are examples of milder pneumococcal clinical manifestations. Pneumococcal diseases predominantly affect children under the age of 2 and the elderly above 65 years of age [6]. Other important risk factors include chronic diseases such as diabetes, HIV infection and other conditions weakening the immune system, smoking and alcoholism [7].

The burden of IPD varies greatly depending on geographical region, the incidence rates are higher in low-income countries compared to high-income countries [8, 9]. Disease outcomes, mortality and morbidity caused by pneumococcal infections, are largely affected by availability of health care, since early diagnosis and treatment is crucial [10]. The recommended treatment regimen includes intravenous administration of broad-spectrum antibiotics without delay, after sampling from the site of infection, pleural fluid, blood or cerebrospinal fluid (CSF) by lumbar puncture in case of suspected meningitis [7]. A targeted antibiotic may be selected depending on the detected bacterial strain and antibiotic sensitivity profile, and in many countries, glucocorticoids are used as adjunctive therapy to dampen inflammation [11].

The prevalence of *S. pneumoniae* resistant to antibiotics and the rise of non-vaccine type strains has increased over the recent years, complicating the therapeutic setting and prevention, threatening patient outcomes around the world [12-14].

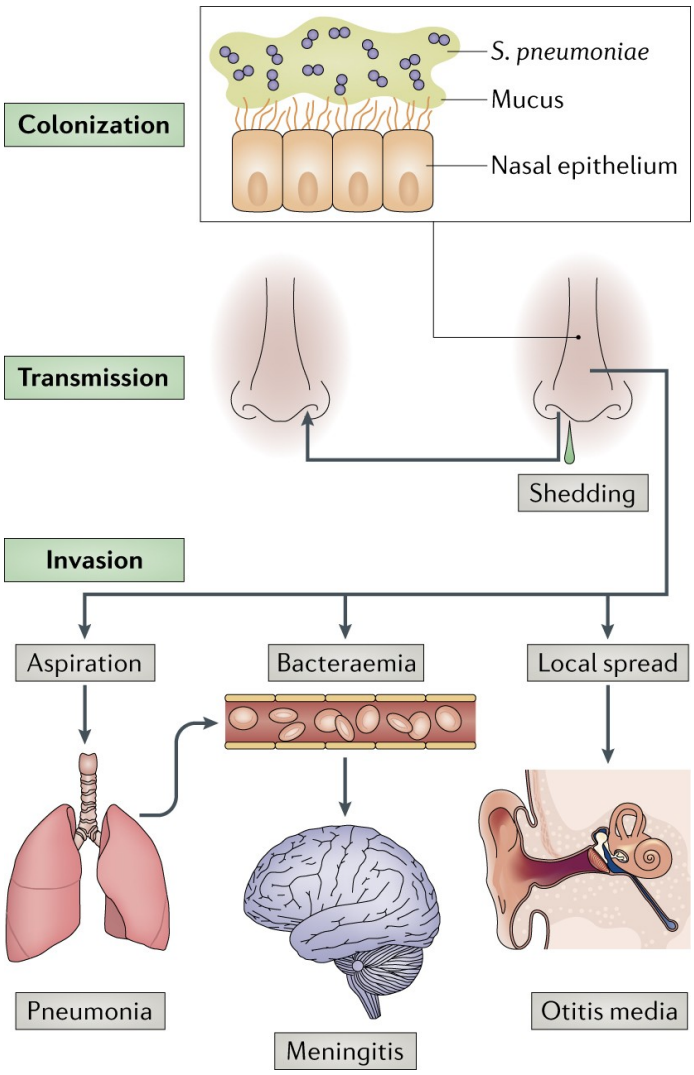


Figure 1. Schematic depiction of the life-cycle of *streptococcus pneumoniae,* spread and progression of pneumococcal disease, Reprinted from [2], with permission from Springer Nature.

1.1.2 Epidemiology of IPD

Community-acquired pneumonia (CAP) is an acute inflammation of the lower-respiratory tract caused by bacterial invasion and multiplication in the lungs, acquired outside of a hospital setting. CAP contributes to a large fraction of mortality and morbidity worldwide, in 2016 it accounted for ~650.000 deaths in children under the age of 5, approximately 1 million deaths in seniors over the age of 70, and 2.4 million in all age groups [4]. *Streptococcus pneumoniae* is the leading etiological cause of CAP globally, causing more deaths than all other respiratory pathogens combined [4]. Moderate reduction in the mortality rates of pneumococcal pneumonia in children under 5 years has been observed in areas with high pneumococcal vaccination coverage [4].

Around 1.2 million cases of bacterial meningitis are estimated to occur yearly around the world [15]. The most heavily affected continent is Africa, in particular the so-called “meningitis belt”

which extends from Senegal to Ethiopia [15]. Several bacteria cause bacterial meningitis, the most common include *Neisseria meningitis*, *Haemophilus influenzae* type b (Hib) and *S. pneumoniae* [16]. Currently, the pneumococcus is the most frequent cause of bacterial meningitis in children beyond neonatal age, accounting for 23% of cases in Europe and 41% cases in Africa [17]. In adults, *S. pneumoniae* accounts for 10%-75% of the bacterial meningitis cases worldwide [17]. Before the introduction of pneumococcal conjugate vaccines (PCVs) just over 20 years ago, Hib was the most common cause of childhood meningitis, then implementation of PCVs vaccines has changed the epidemiology substantially [18]. Over the course of the last two decades, the incidence rates of bacterial meningitis in the Western countries, such as Sweden, Finland, the Netherlands, and the U.S., have declined by 3-4% each year to 0.7-0.9 per 100.000 people [16, 19]. The incidence rates remain significantly higher in sub-Saharan Africa at 10-40 per 100.000 persons yearly [16].

The case fatality of bacterial meningitis depends on several factors such as the patient's age, causative pathogen, and country income status. Pneumococcal meningitis fatality rates range from 20%-37% in high-income countries, and up to 51% in lower income countries [20]. High prevalence of neurological sequelae including: focal neurological deficits, hearing loss, impairment of motor-and cognitive functions, epilepsy and psychiatric disorders have been reported, affecting at least one third of surviving patients [21-23].

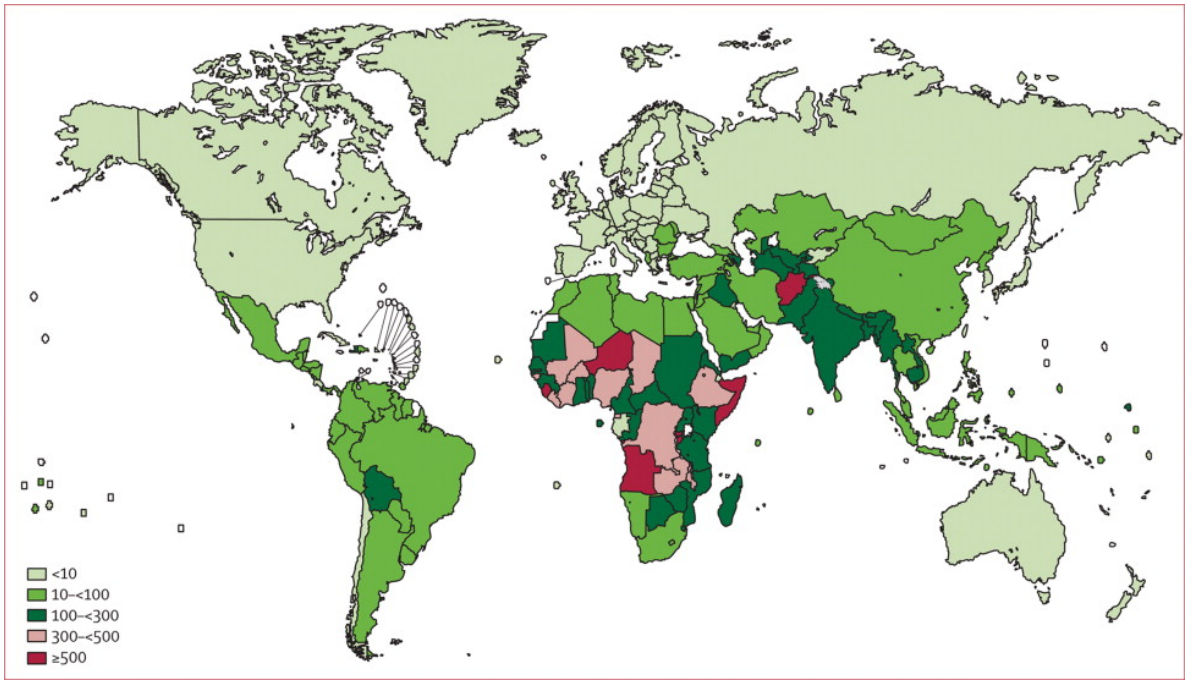


Figure 2. Global mortality rates in children below 5 years of age from pneumococcal diseases. Rates estimates are shown per 100.000 children younger than 5 years. Reprinted from [24] with permission from Elsevier.

1.1.3 Colonization of the upper respiratory tract mucosa

The main reservoir for *S. pneumoniae* is the human nasopharynx, where it can reside asymptomatically [25, 26]. Common carriers include children at day care center and adults living in close proximity to each other, and transmission occurs through respiratory droplets [5]. The pneumococcus has evolved several strategies to overcome physical barriers and modulate the immune system to colonize its host. The pneumococcal cell wall (PCW) consists of a thick layer of peptidoglycan and teichoic acid (TA) decorated with phosphocholine residues and is surrounded by a polysaccharide capsule which is considered the major virulence factor. The capsule is highly diverse in polysaccharide composition and structure with around 100 distinct serotypes identified varying in invasiveness [27]. Its most important role is to protect the bacteria from phagocytosis by the immune system [28, 29]. Due to its usually negative charge, the capsule can prevent mucus entrapment by facilitating colonization and is further aided by pneumococcal exoglycosidases like neuraminidase A (NanA) which alter mucosal viscosity by deglycosylation [23].

A functional pneumolysin (Ply), a pore-forming toxin released during bacterial autolysis, is present in nearly all invasive pneumococcal isolates. Its pore-forming activity can induce lysis of host cells and it is also known to promote adherence by inhibiting epithelial cell ciliary beating [23, 30]. Pneumococcal adhesion to extracellular matrix is facilitated by pneumococcal adherence and virulence factor (Pav) A, PavB and enolase (Eno) binding to fibronectin and plasminogen [31, 32], and the pneumococcal pilus-1 enables adherence to epithelium and endothelium through binding of the pilin protein RrgA to host-receptors [33, 34]. Choline-binding protein A (CbpA) is another important surface adhesin, which plays a major role in colonization [35]. Pneumococcal surface protein C (PspC) inhibits complement deposition through factor H binding [36]. So-called pneumocins, bacteriocins produced by the pneumococcus, are employed to outcompete other colonizing microorganisms [31]. Similarly, it has been proposed that non-competent pneumococcal cells can be outcompeted by induction of self-lysis or fratricide via autolysin (LytA), an enzyme present on all peptidoglycan containing bacteria [37]. Another suggested function of LytA is promoting the release of Ply and other virulence factors [30].

Escaping recognition and degradation by the immune system is also necessary to enable colonization. In addition to capsule mediated protection, *S. pneumoniae* has been suggested to induce immune tolerance through modulation of Transforming growth factor (TGF-β) signaling and promotion of regulatory T (Treg) cell responses over T helper type 17 (Th17) response [38, 39]. Although Ply has been shown to drive pro-inflammatory responses in host-

cells, interaction with the mannose receptor C-type lectin 1 (MRC-1) was recently shown to prompt anti-inflammatory responses driven by Interleukin (IL)-10 and TGF-β, promoting colonization [40]. *S. pneumoniae* expresses and secretes zinc metalloproteases which prevent initiation of inflammation through cleavage of immunoglobulin (Ig) A1, the most abundant antibody in the mucous membranes in the airway [41]. Numerous pneumococcal motifs are therefore required to obtain this first step of the pneumococcal life cycle, the carriage state, which is a prerequisite to cause invasive disease [32].

1.1.4 Pathogenesis and crosstalk with the host

The mechanisms by which the pneumococcus migrates from the nasopharynx into other organs such as the lungs or the meninges via the bloodstream, involves a wide range of virulence factors and host-interaction pathways. Firstly, the pneumococcus can translocate across the epithelium and endothelium by utilizing receptor recycling pathways. The pneumococcal phosphocholine (ChoP) can bind to Platelet-activating factor receptor (PAFR) and PspC to the Polymeric immunoglobulin receptor (pIgR), translocating bacteria from apical to basal membranes [42-44]. Lung transmission can be promoted by viral co-infections since epithelial integrity is compromised and nutrient availability increases feeding bacterial growth [45]. Also, to invade deeper into the tissue, pneumococci may use hyaluronan lyase to breakdown the extracellular matrix.

Survival in the blood stream is heavily dependent on encapsulation, since the thick capsule protects the bacteria from opsonization and thus the pneumococcus cannot be eliminated by complement alone. Both the innate and the adaptive arm of the immune system are involved in host-mediated killing at the site of infection. Toll -like receptor (TLR) signaling, in particular TLR2 recognition of TA/lipoproteins, and consequent immune activation, are examples of innate immune system involvement [46]. Neutrophils and macrophages require specific signals, through opsonization with or without antibodies, to recognize and engulf the bacteria [47, 48]. Also, the adaptive immune response, in an immunocompetent host, will eventually produce anti-capsular antibodies which flag the bacteria for phagocytosis [49]. The concentration of C-reactive protein (CRP) in plasma increases during a systemic inflammation, and its role in pneumococcal infection is to activate the classical pathway of the complement system after specifically binding to ChoP [50]. Furthermore, Ply and PspC have been reported to aid the pneumococcus in escaping opsonophagocytosis [23].

Once the bacteria gain access to the bloodstream, they can disseminate multiple organs, such as the brain to cause meningitis. Less frequently, *S. pneumoniae* can translocate into

myocardial tissue by CbpA binding to the Laminin receptor (LR) and PAFR on the cardiac vasculature, causing Ply-mediated microlesions in the heart [51]. The pneumococcus is predominantly an extracellular pathogen, but in some instances, it may survive and even replicate intracellularly. The spleen is the organ that carries out pneumococcal clearance from the blood. Contradictory, the spleen may also serve as a reservoir feeding bacteremia, since *S. pneumoniae* has been shown to be able to replicate within splenic macrophages

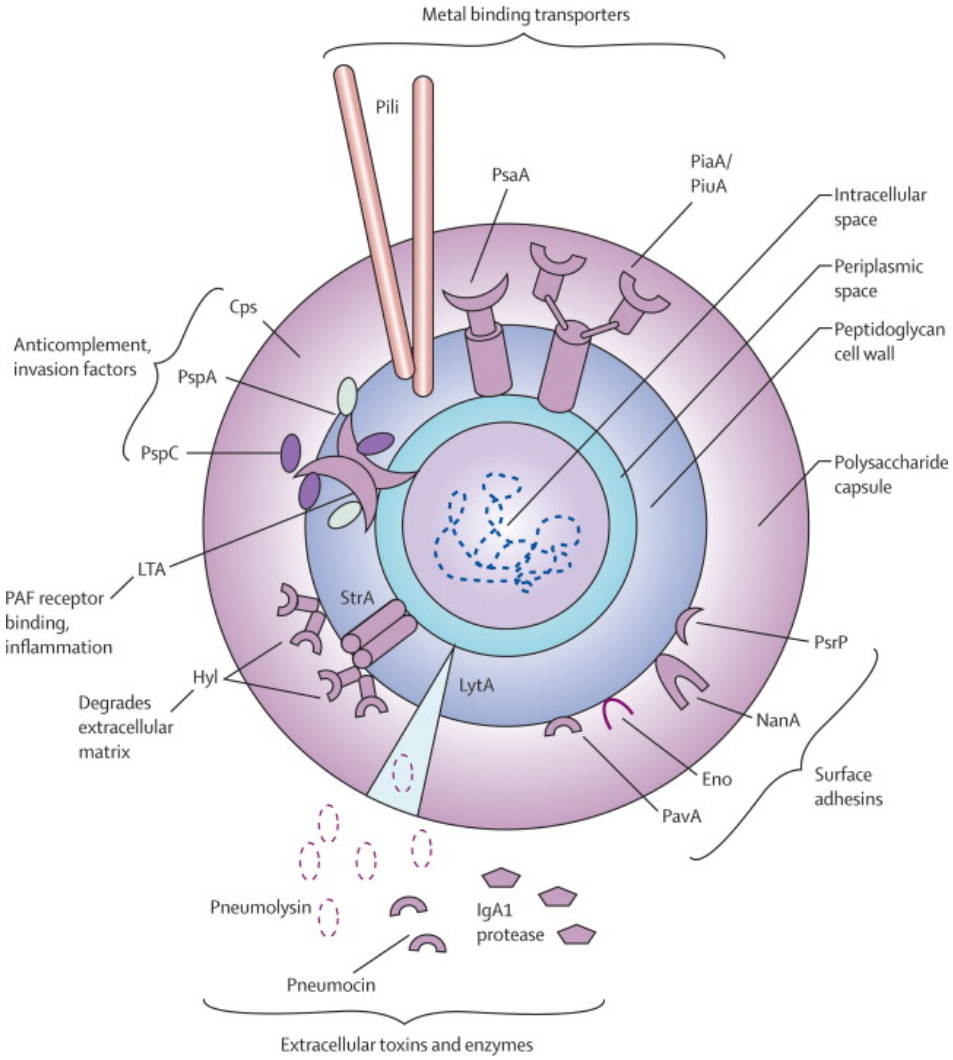


Figure 3. Graphical summary of the structure of a pneumococcal cell and virulence factors involved in colonization and pathogenesis. Reprinted from [52] with permission from Elsevier.

1.2 PNEUMOCOCCAL MENINGITIS

1.2.1 Bacterial invasion of the Central Nervous System

Bacterial meningitis is a severe bacterial infection of the Central Nervous System (CNS) characterized by an inflammation of the meninges that surround the brain and the spinal cord. Although improved healthcare and implementation of childhood vaccination programs have modified the epidemiology, the disease burden remains unsatisfactory with a high case-fatality, especially in low-income countries [5, 21]. The main causative agents in children and adults are *S. pneumoniae* and *N.* meningitidis which together account for 80% of all meningitis cases, and *Escherichia coli* K1 in neonates [5].

To enter the CNS, blood-borne bacteria need to cross the Blood-brain barrier (BBB), a physical and mechanical barrier made of semipermeable microvasculature, separating the brain from the circulating blood [43]. The physiological role of the BBB is to maintain biochemical balance of the brain, nutrition supply and filtering out harmful substances or pathogens. The BBB capillary walls are made out of tightly packed endothelial cells elaborately covering the whole brain by a large surface area, facilitating solute exchange in proximity to all brain cells [53].

Receptor mediated transcytosis was originally suggested as a pathway used by pneumococci to cross BBB and enter the brain [44, 54-56]. In a bacteremia-derived meningitis model, where mice were challenged intravenously with *S. pneumoniae*, bacterial adherence to the brain vascular endothelium in regions of cortex, septum, and choroid plexus, was observed preceding brain invasion [57]. Leukocyte recruitment was delayed until later stages of disease progression, but astrocyte and microglial activation was detected upon adhesion, hence suggesting spatiotemporally separated invasion [57]. The respiratory pathogens commonly causing childhood meningitis *S. pneumoniae*, *N. meningitidis* and Hib all interacted with the Laminin receptor, initiating contact with the BBB [55]. In the case of the pneumococcus the contact was mediated through PspC [55]. pIgR and Platelet endothelial cell adhesion molecule 1 (PECAM-1), one of the major adhesion molecules expressed by endothelial cells, have been suggested to act as BBB receptors for *S. pneumoniae* [42, 43, 58]. Pneumococci colocalized with both pIgR and PECAM-1 in super-resolution confocal microscopy of human postmortem brain biopsies from patients that died from meningitis [59]. Pneumococcal expression of the pilus adhesin RrgA was found to promote passage through the BBB, and RrgA has been identified to act as a major interacting factor for both receptors [59]. Thus, receptor-mediated

transcytosis, where bacteria hijack epithelial and endothelial cell transportation systems without disrupting the BBB, seems to be the principal route of pneumococcal brain invasion.

Another possible route is direct passage from the nasopharynx, along olfactory nerves into the brain [60]. Furthermore, skull fractures where the dura mater is torn, may open up an entry way for the bacteria to invade and cause meningitis [61].

1.2.2 Potentiation of Neuroinflammation

The neurovascular unit consists of several cell types in addition to BBB microvascular endothelial cells. These include, neurons, pericytes, myocytes, astrocytes, and microglia [62, 63]. Of those, astrocytes and microglia constitute the primary line of the innate immune system in the brain [64]. Once bacteria enter the brain after penetrating the BBB, bacterial products such as teichoic acids and lipoproteins, are recognized by pattern recognition receptors (PRRs) on microglia and astrocytes and the inflammatory cascade is initiated [65]. Examples of PRRs involved in responses to bacteria in the brain are scavenger receptors, mannose receptors and TLRs [66, 67]. Signaling through the TLR2/MyD88/NF-κB pathway is prominent in both cell types to fight gram-positive bacteria, like the pneumococcus [64].

Astrocytes and microglia together constitute the primary source of complement in the brain [68, 69]. Other important cues for microglia include damage signals from injured host cells that are recognized by purinergic receptors, tachykinin receptors, estrogen receptors and cannabinoid receptors [70-73]. Activated microglia draw in their long ramifications and obtain a larger ameboid shape [57, 74]. In this state, microglia become reactive and release various cytokines and chemokines and gain migratory, proliferative, and phagocytic properties, designed to fight a particular stimulus. Activated astrocytes contribute to the inflammatory response mainly by secreting pro-inflammatory mediators, cytokines and chemokines [75].

Like microglia, astrocytes are structurally plastic, meaning they undergo morphological changes upon activation. Reactive astrocytes display cytoskeletal hypertrophy and upregulate Glial fibrillary acidic protein (GFAP) along their processes [76]. Microglia reactive states are sometimes divided into M1-like “classically activated” and M2-like “alternatively activated” polarized phenotypes depending of effector functions, but such dichotomous is an oversimplification since they exist on a spectrum of functional phenotypes [77]. M1-like skewed microglial response, is a hallmark of neuroinflammation and thus microglia are fundamental players in the pathophysiology of bacterial meningitis. Reactive astrocytes can be subdivided into two reactive states called A1 and A2, respectively. The A1 state is regarded as harmful or neurotoxic through potentiating neuroinflammation, while the A2 state is associated

with neuroprotective functions [66]. It remains debatable whether astrocytes obtain the A1 phenotype directly or by indirect stimuli by activated microglia [78].

The presence of pro-inflammatory cytokines in the brain increases the permeability of the BBB, and leukocytes from the blood, neutrophils, and peripheral macrophages are recruited into the brain [23, 73, 79, 80]. Glia can further act as a bridge for the adaptive immune system, interacting with lymphocytes. Microglia display antigens of engulfed and processed pathogens on major histocompatibility complex (MHC) class II and thus act as antigen presenting cells. Astrocytes also drive the adaptive immune response, possibly contributing to T-cell activation, through expression of MHC class II and co-stimulatory molecules [81]. A well-orchestrated host-inflammatory response is crucial to eradicate bacteria from the brain and regain tissue homeostasis. Excessive or prolonged neuroinflammation, however, can cause severe damage to the host [82].

1.2.3 Mechanisms of CNS injury and neurological sequelae

A devastating consequence of meningitis is the occurrence of neurological sequelae including hearing loss, motor, and cognitive impairment, present in at least one third of surviving patients after the acute infection has been resolved [21]. The sequelae are caused by direct bacterial toxicity and host-inflammatory mediated responses [79]. Even though the resulting physical disability often improves over time, due to the vulnerability of the developing brain, cognitive loss may continue for life, particularly in pediatric cases [83, 84]. Long term neuronal sequelae occurs in up to 50% of patients following an acute pneumococcal meningitis episode, developing in the first 90 days following infection [22]. Deficits in sensory and motor functions, hearing loss and neurological impairment are highly prevalent.

As suggested by human histopathological data the damage can be caused by several factors; the increased intracranial pressure (ICP), edema, herniation, leukocyte infiltration, abscess formation and necrosis in cortical regions and hippocampal neuronal loss [85, 86]. Reactive oxygen and nitrogen species, and matrix metalloproteases (MMPs) produced by activated microglia, contribute to BBB breakdown and leakage [87]. The resulting increase in ICP and a hyperactivated and self-amplifying neuroinflammatory response finally leads to a vast cellular respiratory burst [65, 80].

The major pneumococcal toxin Ply, detected in the CSF of patients, can induce neurotoxicity by several modes of action. Persistently high levels of Ply in the CSF are associated with poor overall prognosis [88]. Upon binding to cholesterol in membranes of host cells, Ply forms pores, causing cytolysis. Deafness is partially caused by direct action of Ply, through induction

of cell death in cochlear hair cells [89]. Ply has also been found to inhibit ciliary beating by ependymal cells in the brains ventricular system, leading to CSF buildup and accumulation of waste products [90].

Neuronal apoptosis can occur as an indirect result of the exacerbated neuroinflammation and direct effects of the bacterial toxicity [79]. The injury often occurs in the hippocampus which in an area important area for neurogenesis and for memory and learning [85]. When pneumococci were injected directly into the subarachnoid space, hippocampal neurons were found to undergo two phases of apoptosis. The early phase is caspase dependent triggered by Ply and hydrogen peroxide (H_2O_2) leading to increase in reactive oxygen species (ROS) and calcium influx which in turn result in mitochondrial dysfunction and recruitment of apoptosis-inducing factor (AIF) into the cytosol. The second phase occurring ~24 h after pneumococcal challenge, is mediated by PCW or peptidoglycan stimulation of leukocytes. These leukocytes diffuse into the brain parenchyma and induce caspase dependent apoptosis in neurons via p53 and Ataxia telangiectasia mutated (ATM) checkpoint initiating the release of cytochrome C, activating the caspase cascade [91-94]. A third mechanism of neuronal damage, preceding both waves, has been suggested as intravascular administered PCW or capsule outside of the CNS compartment indirectly caused hippocampal apoptosis in mice. This effect was found the be partially reversed by IL-10, indicating the neuroinflammatory response as the damage causing factor [95, 96].

Cerebrovascular complications, including ischemic and hemorrhagic strokes are common events during and following pneumococcal meningitis [22]. The inflammation in the subarachnoid space, characterized by infiltration of neutrophils and lymphocytes into the subarachnoid arteries, contributes to the vasculitis, vasospasm, and thrombosis of vessels. Dysregulation of coagulation and fibrinolytic pathways may also play a role [23]. The consequent narrowing of the lumen causes hypoxia and gives rise to focal necrotic lesions. Also, disruption of CSF flow, due to high protein and leukocyte content, can lead to hydrocephaly where the increase in ICP leads to generalized hypoxic brain damage [97].

The recently discovered glial-lymphatic system, termed the glymphatic system, in neurological diseases has gained attention for its role in neurological diseases. The system consists of A) an influx route for CSF to enter into the brain interstitial system through arterial perivascular spaces facilitated by aquaporin-4 (AQP4) water channels on astrocytes and B) clearance mechanism for removal of solutes from venous perivascular and perineuronal spaces draining into meningeal and cervical lymphatic vessels [98]. Pneumococcal meningitis decreases the

glymphatic system function, increasing the neurotoxic waste debris in the brain which could be another important cause for brain sequelae.

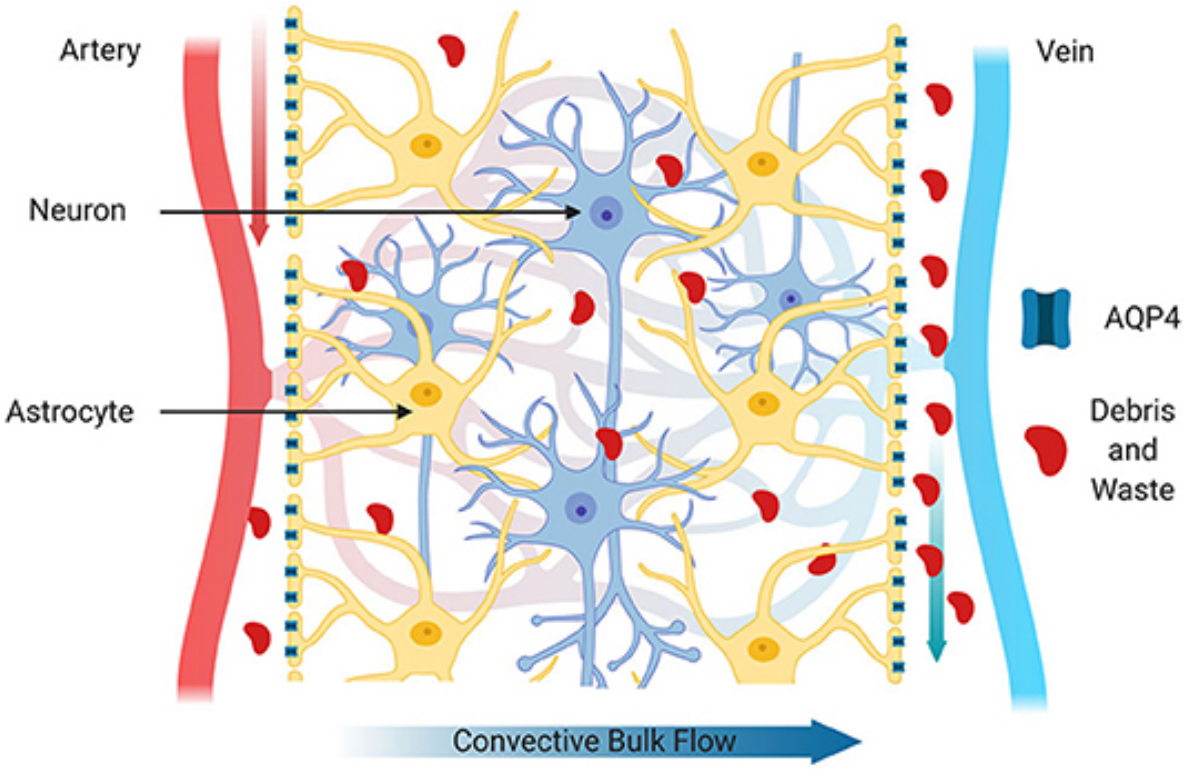


Figure 4. Basal structure and convective flow of the glymphatic system. Reprinted from [99]

Further, it remains unknown whether the neurological damage, typically resulting from sequalae in specific regions of the brain, occurs because of tropism of bacteria to those regions, or whole brain systematic effects of the presence of bacteria on the host itself.

1.3 PREVENTION AND TREATMENT

1.3.1 Vaccines

The polysaccharide capsule is the target of existing vaccines against *Streptococcus pneumoniae* infections; thus, they provide a serotype-specific protection. Most of the ~100 capsular serotypes can cause disease, but their invasiveness and prevalence differs markedly [27, 100].

The first pneumococcal polysaccharide vaccine was licensed in 1977, contained the 14 polysaccharides that caused 70%-80% of invasive disease in the U.S, parts of Europe and South Africa [101]. A second-generation formulation, the currently available 23-valent polysaccharide vaccine (PPSV23), targets the following serotypes; 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, lOA, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, and 33F. The additional serotypes were selected based on epidemiological data from Europa and North America [101], and the vaccine confers around 70% protection in immunocompetent adults [102]. One of the most vulnerable groups to acquire pneumococcal infections, children under the age of 5, only elicit transient antibody responses to the polyvalent purified polysaccharide vaccine, especially to the important pediatric serotypes 6, 14, 19F and 23F [103]. The poor immunogenicity of polysaccharides is due to lack of T-cell involvement when mounting the antibody response, hence they are considered as T-independent antigens [104]. These antigens also fail to induce immunologic memory, making them unfavorable to induce long-term protective immunity [105].

To overcome this, protein conjugate vaccines were developed, where the polysaccharide is conjugated to a carrier protein such as diphtheria toxoid or tetanus toxoid, which are T-cell dependent antigens [104]. First out was the heptavalent polysaccharide conjugate vaccine (PCV) 7 which targeted serotypes 4, 14, 18C, 19F, 23F, 6B and 9V, followed by PCV10 and PCV13 in 2010, with three (1, 5 and 7F) and six added serotypes (1, 3, 5, 19A, 6A and 7F), respectively. PCV13 is now the most widely used vaccine towards pneumococcal diseases and carriage in the world [106, 107]. Additionally, PCV15 and PCV20 have recently been licensed [108].

Compared to PPSV23, PCVs induce considerably higher and more long-lasting antibody titers of all isotypes, IgG, IgA and IgM via T-cell dependent B-cell activation [109]. The conjugate vaccines have proven effective in reducing pneumococcal disease by the serotypes included in the vaccines, as well as indirectly inducing herd immunity in non-vaccine recipients in some studies, but not in other studies [110, 111]. However, carriage rates have not decreased to the same extent [112]. Instead, non-vaccine types have expanded, occupying the empty niche

created by the absence of vaccine-type serotypes [113, 114]. In 2016 more than 70% of the IPD cases in Sweden were caused by non-vaccine types in the elderly, emphasizing the need for new vaccine approaches for this age group [111]. Capsular switching, partly driven by vaccine pressure, is thought to be an inherent mechanism of pneumococci, in which genetic recombination occurs in the capsule synthesis loci, via horizontal gene transfer from neighboring bacteria [115, 116]. The high production cost of the vaccines, which delays distribution to low-income countries, and the evident serotype replacement disease are the main shortcomings of the PCVs [31].

The naturally acquired immunity to pneumococcal infections provides important cues to identify promising protein antigen candidates. Although anti-capsular antibodies constitute the main part of the humoral immune response, titers of antibodies to other pneumococcal surface antigens, simultaneously increase in serum [117]. The protective efficacy of the naturally acquired protein antibodies has even been suggested to be superior to those conferred by capsular antibodies [118]. Surface availability, high immunogenicity and conservation among clinical disease-causing isolates are favorable characteristics of a pneumococcal protein antigen to be considered as a promising vaccine target. Examples of vaccine candidates fitting some of these criteria, include detoxified Ply, choline-binding proteins such as Pneumococcal surface protein A (PspA), Pilus-I, metal-binding proteins like pneumococcal surface adhesin A (PsaA), polyhistidine triad (Pht) proteins, NanA and ChoP [119-121].

Having various functions for virulence in the host, PspA is one of the most abundant surface proteins of *S. pneumoniae*. Immune evasion mechanisms include inhibition of complement deposition and escaping neutrophil extracellular traps (NETs) [122, 123]. Although PspA is present in all pneumococcal strains, it is highly diverse structurally and serologically, thus providing the bacteria with high competence to evade antibody responses [124, 125]. Previous studies have found protective effects against colonization or disease using recombinant PspA in vaccination models, often with added efficacy in combination with other choline-binding proteins or Ply [126, 127]. Anti-PspA antibodies reverse the characteristic immune evasion mechanisms of PspA, which include inhibition of complement deposition, escaping neutrophil extracellular traps (NETs), and binding of lactoferrin [120, 122, 123]. Decoding of the serological responses to recombinant PspA, identifying conserved epitopes that are pan-protective, could hold potential for a superior PspA-based vaccine [128, 129]. Another important consideration in designing such a vaccine, is potential homology with human proteins. In the case of PspA, some structural homology to human cardiac myosin is present in

coiled-coil regions. To avoid the risk of cross-reactivity, this region should be avoided [130, 131].

On the account of the increased availability of genomic data on *S. pneumoniae*, in the recent years, genome-based approaches have been the main tool to identify novel immunogens. For example, via whole genome sequencing, and protein screening, stratifying for surface-localized proteins, six novel pneumococcal surface antigens; Sp36, Sp46, Sp91, Sp101, Sp128, and Sp130 where identified, as potential vaccine targets [132]. The potential of the surface protein-based protein vaccines, however, might be limited to protect against colonization. In a recent study, five pneumococcal novel protein antigens that were tested for their immunogenicity in a colonization model failed to protect against colonization, in presence of the capsule. Even though antibodies were generated at high titers, the capsule blocked the antibody binding to the bacterial cells, thus having no inhibitory effect on colonization [133].

A universally cross-protective vaccine, which is safe and effective even in young children, is the ultimate goal. In addition to identifying the best mixture of antigens, the mode of delivery is another crucial component. Since *S. pneumoniae* natural route of infection occurs via mucous membranes, involvement of the mucosa-associated immune system could be the key to optimize vaccine responses. Instead of delivering vaccines by the classical intramuscular injection, delivery directly into the mouth or nose in drop or spray form, would mimic natural exposure to the pathogen more accurately [134, 135]. Furthermore, the selection of an appropriate adjuvant to deliver the antigen and co-stimulate the immune system, affects the longevity of the humoral response. This has been suggested to be particularly important in early life when the immune system is immature and antibody responses are generally transient [136].

Whole killed, non-capsulated pneumococcal cells (WCA) have been evaluated in a mucosal vaccination model. WCA was shown to facilitate protection against colonization and invasive disease to encapsulated bacteria, across serotypes [137, 138]. The antibody responses raised towards WCA were specific to a wide range of known and unknown pneumococcal components, and the protection mediated in a self-adjuvanticity manner i.e., in the absence of a classical adjuvant [134, 139]. Bacteria-derived particles such as the adjuvants LT-K63, mmCT, derived from heat-labile toxin from *E. coli* and cholera toxin, respectively, stimulate stronger and more long-lived immune responses, compared to aluminum hydroxide, by other immunization routes than the mucosal one [136]. *S. pneumoniae* has recently been found to produce membrane derived extracellular vesicles (EVs), like other gram-positive bacteria, enriched with bacterial protein content capable of inducing immunomodulatory effects [140, 141]. As a vaccine platform, pneumococcal EVs or membrane particles (MPs) could represent

great advantages as delivery systems carrying and displaying different types of cargo, such as lipoproteins in their native conformation [142]

Pneumococcal vaccine/ vaccine candidate	***Vaccine type***	***Status***	***Reference***
23-valent polysaccharide vaccine (PPSV23)	Polysaccharide only	In use	[101]
Polysaccharide conjugate vaccines PCV7, PCV10, PCV13	Conjugate	In use	[106, 107]
Polysaccharide conjugate vaccines PCV15 and PCV20	Conjugate	Recently licensed	[108]
PspA with Ply or PspC	Protein antigen	Clinical trials	[126, 127]
Pneumococcal Pilus	Protein antigen	Under pre-clinical evaluation	[143]
Trivalent protein vaccine, PsaA, StkP PcsB	Protein antigens and TLR stimulating adjuvant	Under pre-clinical evaluation	[144, 145]
Whole killed, non-capsulated pneumococcal cells	Pan-antigens	Under pre-clinical evaluation	[137, 138]

Table 1. Overview of currently used pneumococcal vaccines and some novel candidates

1.3.2 Antibiotics and antimicrobials

The highly adaptive nature of the pneumococcus, which allows the bacteria to evade vaccine strategies e.g. by capsular switching, has major implications for clinical interventions of IPD [115]. Trends of antimicrobial resistance have been reported, worldwide, especially in non-vaccine serotypes [146].

The emergence and spread of multi-drug resistant pneumococcal clones is particularly alarming [147]. Resistance to beta-lactam antibiotics, macrolides, fluoroquinolones and tetracyclines is on the rise, especially in areas with high antibiotic consumption within a geographical region [12, 148-150]. The antimicrobial pressure drives the bacteria to undergo transformation of genes that sensitize them to a certain antibiotic, like those encoding penicillin binding proteins in the case of beta-lactam resistance [151].

The mechanisms for acquiring resistance genes include homologous recombination where the exogenous DNA is supplied by other pneumococci or related streptococcal species, or by mobile elements via bacteriophages, plasmids and transposons [116]. There is an urgent need to reduce the speed of acquired antimicrobial resistance and spread of multi-resistant strains of *S. pneumoniae*. In order to combat this evolutionary escape-artist and maintain an effective treatment regimen, these aspects should be considered; promoting responsible antibiotic use, exploration of new adjunctive therapeutic targets and antibiotics, as well as re-visiting older drugs [12].

Antimicrobial peptides such as LL37, lysozyme and lactoferrin, which have exhibited activity against *S. pneumoniae*, could represent future alternatives or adjunctive to antibiotics [152, 153].

1.3.3 Treatment and management of pneumococcal meningitis

The current treatment regimen for pneumococcal meningitis consists of intravenous antibiotic administration sometimes in combination with an anti-inflammatory agent such as corticosteroids and glycerol [79]. The efficacy of corticosteroids as adjunctive therapy to prevent neurological sequalae has been debated, since the beneficial effects for patients have only been reported in high-income settings [154]. Multiple other strategies, which aim to skew the immune response, towards anti-inflammatory and neuroprotective functions, have been proposed. In this context, M1 polarized microglial cells and A1 polarized astrocytes, the major propagators of inflammation, represent attractive targets for pharmacological manipulation. Exogenous TGF-β and IL-10 agonists have been found to dampen neuroinflammation,

promoting M2 phenotypic shift of microglia, but with possible unfavorable side effects for hydrocephalus development [155, 156].

Modulation of TLR signaling, either by prophylactic receptor stimulation or inhibition of NF-κB pathway by tyrosine kinase inhibitors, have yielded interesting results. TLR stimulation resulted in increased phagocytosis of bacteria, and tyrosine kinase inhibitors, accordingly, limited cytokine production and release by microglia [157-159].

An important hallmark of advanced neuroinflammation in bacterial meningitis is BBB breakdown. MMP inhibitors and exogenous stimulation with the cytokine CD34, which indirectly upregulates tight junctions in microvascular endothelial cells, have been suggested to protect the integrity of the BBB in bacterial meningitis [84]. Interference with vast leukocyte infiltration into the brain, which contributes to BBB breakdown, was attempted by pharmacological inhibition of leukocyte rolling, but this had detrimental effects on increased bacterial load in the blood [160]. In contrast, Granulocyte colony-stimulating factor (G-CSF) therapy, which increases the number of neutrophils in the circulation, had positive effects in a meningitis model, promoting hippocampal neurogenesis and spatial learning [161].

Finally, antimicrobial delivery to the brain represents an additional challenge due to restricted penetration of larger molecules through the BBB. The cephalosporin ceftriaxone, which belongs to the family of β-lactams, is a common treatment of pneumococcal meningitis in a clinical setting. β-lactams are bactericidal, killing the bacteria through interference and penetration of the cell wall, inducing lysis and release of bacterial components into the physiological milieu. The suitability of using a bactericidal antibiotic to treat bacterial meningitis has been brought into question, since the released bacterial components i.e. cell wall components, Ply, TA, lipoproteins, may further enhance the already hyperactivated neuroinflammatory response [154]. The bacteriostatic antibiotics daptomycin and minocycline which instead of lysing the bacteria inhibit bacterial protein synthesis, have been proposed as alternatives to ceftriaxone. In experimental meningitis models, bacteriostatic antibiotics have displayed advantageous properties, leading to attenuated neuroinflammation and prevention of neurological sequalae [162-164]. Taken together, a growing knowledge of bacterial meningitis pathophysiology, and exploration of adjunctive therapy targets is needed to improve prognosis and disease outcome [165].

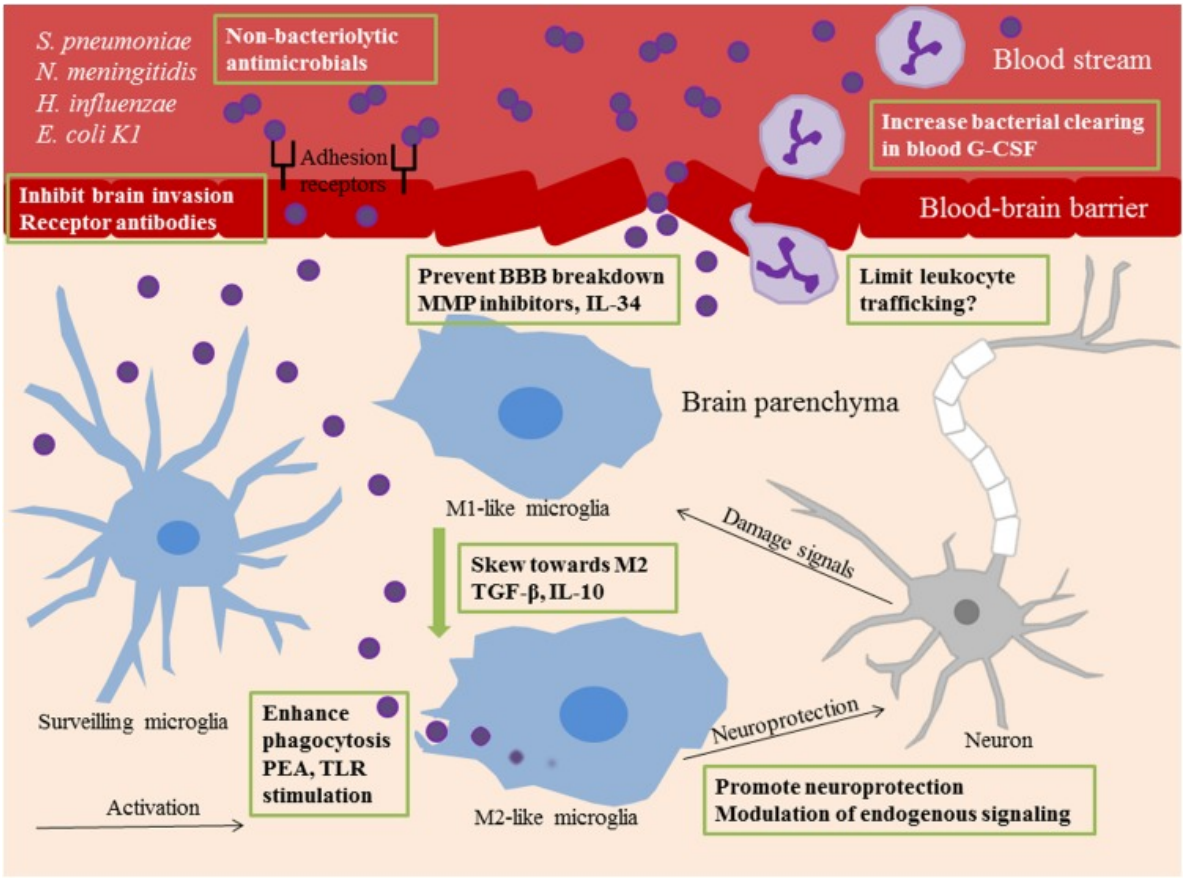


Figure 5. Summary of novel therapeutic approaches for bacterial meningitis. Reprinted from [165].

2 RESEARCH AIMS

The general aims of the thesis were to unravel bacterial and host factors important for bacterial meningitis pathogenesis, with a focus on the major contributor globally, *Streptococcus pneumoniae*. We set out to scrutinize several checkpoint mechanisms which underlie the progression of pneumococcal meningitis, and manifestation of neurological sequelae. We focused on bacterial invasion of the brain, bacteria-cell interactions in the CNS and interruption of fluid dynamics. Modulation of such host defenses could hold potential to advance therapeutic strategies and reduce the prevalence of long-term sequelae among meningitis survivors. Furthermore, we studied pneumococcal membrane particles as a novel serotype-independent vaccine candidate.

2.1 SPECIFIC AIMS

Paper I

To understand the mechanisms for pneumococcal entry from the blood stream through the BBB into the brain. This, by evaluating the BBB receptor pIgR and PECAM-1 blockade as a therapeutic approach to reduce bacterial invasion of the brain, potentiation of neuroinflammation and survival.

Paper II

To investigate the molecular mechanisms underlying pneumococcal interactions with neuronal cells leading to neuronal cell death.

Paper III

To assess the role of the glymphatic system in pneumococcal meningitis, its potential dysfunction leading to brain edema and neurotoxic waste build-up in the brain, in turn exacerbating neuroinflammation and causing neurofunctional impairment.

Paper IV

To evaluate pneumococcal membrane particles as a vaccine platform to induce a serotype-independent protection, and to identify the major antigens contained in these particles that are involved in the cross-protection to heterologous serotypes.

3 METHODOLOGICAL CONSIDERATIONS

This chapter provides an overview of the infectious agents, in vitro models and animal models used in the thesis, and the rationale behind the chosen methodology. The protocols are thoroughly described in the Material and Methods sections in the respective papers.

3.1 BACTERIA

In paper I we used the bioluminescent derivate of *S. pneumoniae* of serotype 4, TIGR4 strain, Xenogen 35 [54, 59]. This strain, which expresses *lux* genes, providing it with bioluminescent properties, was originally developed to monitor trafficking of bacteria in infection models by *in vivo* imaging. Indeed, we chose this strain for its compatibility with the IVIS imaging system, which allowed us to track brain infection in mice challenged i.v. with bacteria, by measuring the bioluminescence signal intensity. Some reservations regarding maintenance of virulence compared to the parent TIGR4 strain, have been raised, and thus it should not be used as an analog of TIGR4 [166]. Since only intercomparison between groups of mice subjected to Xen35 was performed in the study, the concern for our application is limited.

TIGR4 of serotype 4 [167], isogenic mutants lacking Ply (TIGR4Δ*ply*) [40], Pilus-I (TIGR4Δ*rrgA-srtD*) [33, 54] RrgA(TIGR4Δ*rrgA*) [54] were used in paper II, and the double mutant lacking both RrgA and Ply (T4Δ*rrgA-srtDΔply)* was constructed for the study. The TIGR4 strain is an invasive disease clinical isolate in which pilus-I was first characterized and found to be important for adherence, virulence and cross-talk to the immune system [168]. The prevalence of pilus-I expression in all pneumococcal isolates is estimated to ~ 30% [169]. The choice of using TIGR4 to study the characteristics and interaction pathways of piliated pneumococci to neuronal cells was therefore straightforward. Several knockout strains, constructed to study pilin interactions to other cell types, were readily available to us from the BHN strain collection.

S. pneumoniae of serotype 3 was the choice for paper III. In addition to being highly clinically relevant, causing invasive disease despite being including in PCV13, it is known to shed a lot of its capsule [170]. This was a favorable characteristic for the study since efficient capsule detection was a prerequisite to answer whether pneumococcal products were accumulating in different fluid compartments of the brain.

In paper IV we also used TIGR4 of serotype 4 [167], isogenic mutants lacking Ply (TIGR4Δply) [40], lacking capsule (T4R) [171], and PspA, PrsA, MalX knockout strains were constructed for the study. In the model with heterologous infection, serotype 1 (BHN733) and serotype 3 (BHN428) were used as challenging strains. EVs were isolated from liquid grown bacteria, and MPs were isolated by scraping plate-grown bacteria off blood-agar plates. The mutants used in paper II and in paper IV were all constructed utilizing the natural transformability of the pneumococcus. Open reading frames (ORFs) of the gene of interest were replaced with an antibiotic resistance gene or an antibiotic resistance gene,

by overlap Polymerase chain reaction (PCR), allowing for selection on blood-agar containing the antibiotic.

3.2 *IN VITRO* MODELS

When modeling pneumococcal infections to study host-pathogen interactions, one should aim for an experimental setting which most closely resembles the natural host, human [172] We selected the neuroblastoma cell line SH-SY5Y as a model for neuronal cells in paper II. The SH-SY5Y is a secondary cell line, derived from the parental SK-N-SH, which was established from a bone-marrow aspirate from a 4 year old female patient in 1970 [173]. This cell line is immortalized which represents advantages when large numbers of cells are needed, they divide quickly and may be passaged often, in comparison to non-cancerous cells. Further, the SH-SY5Y cell line can be differentiated into mature neuronal-like cells, with retinoic acid and brain-derived neurotrophic factor (BDNF) [174]. After the differentiation protocol, the cells do not divide anymore. Even though the differentiated SH-SY5Y cells provide a better model for physiological neuronal cells, there are still vast differences to consider. To account for some of these, we decided to also include human primary neurons in the study. Neuroepithelial stem (NES) cells derived from induced pluripotent stem (iPS) cells were induced and differentiated into mature neurons. The primary neurons represent greater similarities to neurons *in vivo*, but these are not optimal when larger quantities of cells are needed, thus calling for a combination of approaches. The NES cells can be differentiated into a mixed culture of neurons and glia, a large subset of neuronal cell types, and organoids [175].

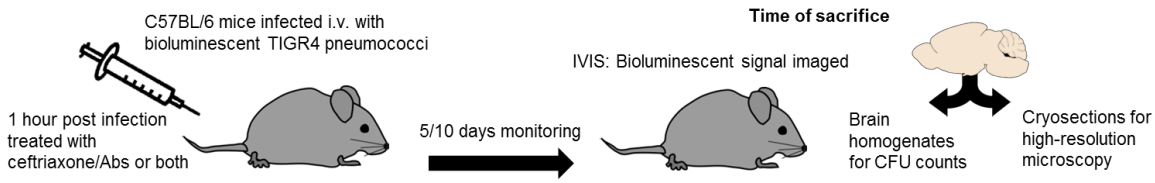
3.3 *EX VIVO* MODELS

The organization and architecture in relation to other cell types within the brain tissue is also highly relevant for the cellular functions, and likely for cellular responses to bacterial infection. To study functional areas of the brain, i.e., the hippocampus, cortex, cerebellum, and thalamus during bacterial infections, organotypic slice-cultures from postnatal rodents could be a useful tool for continued studies [176]. Biopsies from pneumococcal meningitis patients that died from the disease have been used in co-localization studies [59]. Although post-mortem tissue is cytoarchitecturally accurate, the experimental applications are limited, only representing fatal disease.

3.4 *IN VIVO* MODELS

The complex interplay between the pneumococcus and its human host, entails the need for experimental models with similar organ systems as humans. To study the pathogenesis, therapeutic or preventive interventions of pneumococcal infections, rodents and lagomorphs represent the most common *in vivo* models. Mice, rats, and rabbits are mammals and evolutionary close to humans, with an evolved CNS and an immune system. The animals are inoculated with bacteria by several routes: Intranasal, intravenous, or intrathecal route, depending on the organs of interest and study purpose [177, 178].

In paper I, we utilized the murine bacteremia-derived meningitis model, as follows.



The model was designed to mimic the natural route of bacterial invasion of the brain from the blood, through the BBB [55, 57]. Intrathecal inoculation, administration of bacteria directly into the spinal fluid, is another commonly used route to induce experimental meningitis [23]. Bypassing of the BBB would not have been optimal for our study, since we were interested in the events preceding and after bacterial invasion. The same model was employed in paper II, to obtain tissue for co-localization assessment.

A rat model of intrathecal administration of bacteria directly into the cisterna magna with a micro-injection syringe pump, was used in paper III by our collaborators. Here, we required a model for a more advanced experimental meningitis, and an abundance of bacteria in the liquor. Anatomically, rats are larger than mice, which is advantageous for precise inoculation or sampling from small spaces in the brain [179]. Although mice can be used in behavioral tests to assess cognitive functions, rats are more tolerant to be handled by humans, and hence show less tendency of stress behaviors which can interfere with results [180]. In paper III, this was a favorable trait of the model species, since we were interested in the neurofunctional outcome after acute experimental meningitis episode. Rats which received antibiotic treatment after bacterial challenge, underwent habituation memory tests to assess meningitis-induced neurological damage.

In paper IV we established an intranasal immunization model with MPs in mice, which were subjected to intranasal pneumococcal challenge after 2 rounds of immunizations. Mucosal delivery of pneumococcal vaccines has been proposed to be advantageous in eliciting mucosal and humoral protective immune responses [181]. The intranasal route of infection was chosen to simulate the pneumococcal life cycle in colonization, and infection of the respiratory tract mucosa. A wide variety of genetically modified mice is available to study specific biomedical targets, representing a larger toolbox compared to rats [182]. In paper IV we used the muMt B-cell knockout mice to study the involvement of antibodies in prompting the immune response to the mucosal MP vaccine.

3.5 ETHICAL CONSIDERATIONS

The concept of animal welfare within research is not new, although the general awareness and concern has grown over the years. In 1959, Russel and Burch presented the principle of the 3 Rs; Replacement, Reduction and Refinement, of the use of animals in medical research. Essentially, the 3R principle encourages scientists to seek or develop alternatives to replace animals in their research, to design the studies carefully limiting the number of animals needed, and consider the health and wellbeing of the animals. Today, all research protocols which include working with laboratory animals must go through a strict review process by a governmentally mandated ethics committee. The committee evaluates whether the degree of severity of the proposal is proportionate to the expected value of the research. This is to ensure that the research conducted is within the framework of the law, in Sweden, the Animal Welfare Act (2018:1192). The application process requires researchers to obtain permission to include animals in studies, to apply the principle of the 3 Rs, to the study proposals. In the studies included in this thesis, we applied the first R, Replacement, by using *in vitro* models when possible. The second R, Reduction, has been addressed by limiting the number of mice per group included in experiments, with regards to statistical power, as well as storing tissue and materials for repetitions or future use. The 3rd R, Refinement has been considered in several ways. Firstly, all personnel working with the animals need to complete a training and obtain a certificate in laboratory animal handling. A satisfactory knowledge of the biology of the mouse or rat, and its normal behavior, is a prerequisite good husbandry practice. Suitable housing conditions for mice, in addition to access to food, water and having sufficient space, include materials that promote natural behaviors. Availability of nesting material, hiding places, and other cage enrichment stimulate building of nests, burrowing and foraging behaviors. In turn, this prevents negative stereotypical behaviors to develop, like barbing which is a sign of boredom. The experimental group set-ups of mice are designed with respect for their social nature and hierarchy, housing them together whenever possible. Handling by humans and experimental procedures can be distressing and painful for animals. It is necessary for the handler to recognize pain, suffering and distress in mice and use the appropriate anesthesia and analgesia to alleviate pain. Adhering to high standards in treating laboratory animals humanely, stands as an ethical argument on its own, as it is generally considered wrong to inflict pain on others, humans, and animals. Besides, distressed animals do not make good research subjects by increasing data variability, and thus should be diligently avoided.

4 RESULTS AND DISCUSSION

4.1 PAPER I

Receptor Blockade: A Novel Approach to Protect the Brain From Pneumococcal Invasion

The BBB separates the brain from the circulating blood and has critical functions in both protection and nutrient supply of the brain. Endothelial cells form the layer of the interior surface of the blood vessels. The pneumococcus has developed strategies to cross the BBB from the blood and invade the brain. How the bacteria achieve this, and invade the brain, through receptor-mediated endocytosis via pneumococcal pilus-protein RrgA interactions with the two endothelial receptors PECAM-1 and pIgR, was previously described [59].

In paper I we evaluate the efficiency of blocking the receptors PECAM-1 and pIgR on the blood-brain barrier endothelium as adjunctive meningitis therapy in mice with experimental meningitis. We used a murine bacteremia-derived meningitis model [55, 57] and a bioluminescent TIGR4 strain of *S. pneumoniae* to investigate whether blocking antibody treatment targeting the receptors could prevent the development of pneumococcal meningitis in mice.

Combined antibiotic and antibody treatment resulted in no or few viable bacteria in the brain and 100% survival in a 5- and 10-days infection. The control group treated with antibiotic only had a survival rate of 60%. The antibiotic blockage of the receptors was maintained throughout the duration of the study. Receptor blockade did not interfere with antibiotic permeability through the BBB. We also compared the degree of neuroinflammation between survivors and non-survivors using microglial morphology and Iba-1 signal intensity as readout. We observed a more prominent pro-inflammatory prone activity in non-survivors concluding that excessive microglial activation is associated with poorer overall survival.

Thus, blocking antibody treatment targeting the PECAM-1 and pIgR successfully prevented development of pneumococcal meningitis in mice, enhanced the chances of survival and prevented brain damage through dampened neuroinflammation. To answer whether these encouraging results can be reproduced in a treatment setting for humans, further research is needed. The optimal time window for treatment, duration of blockage beyond 10 days after administration and antibody binding to other sites in the body must be investigated.

In this study, an additional observation was made which inspired us to investigate pneumococcal interactions to other cell populations in the brain. Asymptomatic presence of low numbers of pneumococci was found in the brains of some survivors up until 10 days post infection. We speculate whether bacteria are efficiently

cleared by microglia and other phagocytes in the brain, or if they can survive within intracellular niches. A series *of vitro* assays to study microglial responses to the pneumococcus, their phagocytic activity, and gene expression profile have been set up. The differential responses of a murine microglial cell line to bacteria having or lacking specific virulence factors involved in bacterial meningitis pathogenesis such as NanA, the pilus-I protein RrgA, PspC, and Ply could be of importance. To study the relevance of microglial responses to *S. pneumoniae in vivo*, we plan to use the conditional microglia knockout model described here [183]. We have yet to explore this avenue fully.

4.2 PAPER II

Neuronal death in pneumococcal meningitis is triggered by pneumolysin and RrgA interactions with β-actin.

Vast neurological cell death collectively gives rise to brain sequelae, varying from cognitive and motor disabilities to hearing loss, which is developed in 50% of pneumococcal meningitis survivors [22]. Ply has been shown to indirectly induce neuronal cell death [78, 80], but thus far the literature lacks knowledge if pneumococci can directly interact with neurons, which virulence factors induce neuronal cell death, and what host factors they interact with. In paper II, we address this question.

The methods to study the interaction of the pneumococcus to neurons *in vitro* were in part based on the ongoing project on the pneumococci-microglia interplay. Here we used human primary neurons and SH-SY5Y human neuroblastoma cells differentiated into mature neurons using retinoic acid. Co-immunoprecipitation (IP) using recombinant His-tagged RrgA and Ply coupled with ni-NTA magnetic beads was applied to pull down interacting proteins from neuronal cell lysates. The major interacting protein for both RrgA and Ply was identified to be cytoskeletal β-actin. Ex vivo mouse brain tissue from mice challenged with *Streptococcus pneumoniae* containing these virulence factors in a bacteremia-derived meningitis model co-localized with β-actin.

Cells were infected with TIGR4 *S.pneumoniae* wild type and mutants lacking virulence genes coding for Ply and the pilus-I adhesin RrgA. Pneumococcal pilus-I RrgA was found to promote adhesion to neuronal cells by binding to cytoskeletal β-actin filaments, which can be exposed on the plasma membrane and protrude from the outer cellular surface. The presence of Ply and its non-pore forming cholesterol binding domain 4 (D4) was shown to further increase β-actin filaments exposure on the neuronal plasma membrane, which in turn enhanced RrgA binding to β-actin.

Both Ply and RrgA promoted invasion into neuronal cells and induced cell death through β-actin interactions. Ply caused disruptions of the filaments, which was

demonstrated in calcium imaging experiments. Ply, and RrgA to a lesser extent, caused an increase in cellular calcium influx which is an indicator for disruption of the actin cytoskeleton. STED super-resolution microscopy was employed to visualize the disrupted β-actin filaments in neurons infected with pneumococci expressing RrgA and Ply. Pre-treatment with β-actin antibody significantly reversed the effects, and protected the cells from being damaged and invaded by pneumococci.

This new interaction pathway of how the pneumocccus can directly bind to and invade neuronal cells, can contribute to a better understanding how neuronal sequele are formed during pneumococcal meningitis, and may provide novel targets to prevent them.

4.3 PAPER III

Dysfunctional glymphatic system with disrupted aquaporin-4 expression pattern on astrocytes causes bacterial product accumulation in the CSF during pneumococcal meningitis.

After addressing direct cellular interactions between *S. pneumoniae* and neurons, we continued to investigate indirect mechanisms of injury in pneumococcal meningitis. In addition to direct bacterial toxicity, hyperactivated inflammatory response and vascular complications leading to cerebral edema, contribute to cellular damage [79]. The precise mechanisms by which cerebral edema is induced by bacterial presence in the brain, are only party elucidated.

The CSF circulation functions as a sink for metabolic waste produced in the CNS, CSF is flushed throughout the cortex, where it mixes with interstitial fluid and is thereafter cleared out into the draining lymph nodes. This rapid fluid transport system is called the glymphatic system because it is dependent on glial cells, astrocytes, which facilitate the transport of fluid. Present on astrocytic end-feet are AQP4 water channels, responsible for the para-arterial influx of CSF into the brain parenchyma and efflux interstitial fluid and extracellular solutes from the interstitial compartments of the brain and spinal cord [98, 99].

The invasion of bacteria into the brain leads to increased debris and waste production which must be eliminated to regain CNS homeostasis. An impaired glymphatic system has been implicated in several neurological disorders [98]. Together with our collaborators Dr. Tatiana Barichello's group from the Health Science Center at Houston (Texas, USA) and The Universidade do Extremo Sul Catarinense (Santa Catarina, Brazil), we hypothesized that a malfunctioning glymphatic system could be the reason for buildup of waste and fluid in the brain in pneumococcal meningitis.

In paper III we studied the glymphatic systems functionality in pneumococcal meningitis using a meningitis rat model, with intracisternal administration of *S. pneumoniae* of serotype 3 into Wistar rats, previously established [184]. Evans blue-labelled albumin (EBA) tracer dye was administered together with bacterial

suspension into the cisterna magna using a micro-injection syringe pump. In this way, we could follow the trafficking of fluid from the CSF compartment, into the brain parenchyma as well as the returned fraction in the peripheral circulation, by measuring EBA levels in the serum and in the brain. We found that the glymphatic system was indeed functioning poorly in the rats subjected to pneumococcal meningitis, demonstrated by high levels of EBA retained in the brain and less EBA effluxed into the systemic circulation.

We then wondered what kind of pneumococcal waste products could be accumulated in the fluid, and in which compartment, the brain parenchyma or in the CSF. We found that Ply and the capsule were accumulating over time in the CSF during infection. Both products were detectable in the brain parenchyma but did not accumulate there. Thus, drainage between the two compartments reduces over time, as disease progresses.

Accordingly, increased astrogliosis, microgliosis, neuronal cell death and loss of synaptic connections were detected in the brains of the meningitis rats. This was also reflected in diminished normal memory functions of rats subjected to behavioral tests to test habituation memory.

Finally, we assessed whether the levels of AQP4 were affected by meningitis and found that these were unchanged during the course of the disease, and between the meningitis group and uninfected controls. We then studied the expression pattern of AQP4 on astrocytes, and found that during meningitis, the astrocytes adjacent to the vasculature underwent extensive astrogliosis, disrupting the AQP4 organization away from the end-feet. This structural disorganization is an attribute for loss of AQP4 function, which leads to blockages in the glymphatic system and consequent buildup of waste and fluid.

We believe that a better understanding of how the brain rids itself of excess fluid and neurotoxic waste can hold potential for improved interventions towards resolution of disease, particularly to prevent neurological sequelae.

4.4 PAPER IV

Membrane particles evoke a serotype-independent cross-protection against pneumococcal infection that is dependent on the conserved lipoproteins MalX and PrsA.

Even though the PCVs have been quite effective in reducing the incidence of IPD in vaccinated children, the carriage rates have been less effected. The evident serotype replacement disease, with non-vaccine type serotypes causing more disease worldwide, highlights the need to develop new vaccine strategies that are universally cross-protective.

All living cells can produce and release EVs, enriched with various cargo acting as cues for cell-cell communication [185]. During colonization, pneumococci produce cytoplasmic membrane derived nano-sized EVs, which can carry different types of cargo involved in communications with other bacteria, and host immune response modulation [141]. Vesicles produced by bacteria grown in liquid are referred to as EVs, while we called vesicles spontaneously produced by plate-grown bacteria membrane particles (MPs).

In paper IV, we propose a possible application for pneumococcal MPs as a vaccine platform. In this context, vesicles possess advantageous features, since they can carry and deliver antigens in their native conformation, such as lipidated protein antigens. MPs are superior to EVs in prompting antibody responses and conferring protection after immunization. Also, they may pose adjuvant effects.

Mice were immunized intranasally with purified vesicles from TIGR4 S. pneumoniae of serotype 4 and then infected with either a serotype 1 or a serotype 3 strain intranasally. We found a cross-protection against this heterologous infection, 80% protection against the serotype 1 strains, and 50% against the serotype 3 strain. Immunization with MPs derived from serotype 3 pneumococci conferred a 100% homologous protection against infection with the same strain. The MPs evoked protection in an antibody-dependent manner since MP mediated protection was completely abolished in B-cell deficient mice.

Initially, we investigated whether the major antigens involved in evoking MP mediated protection were choline-binding proteins like PspA or PspC, since they have been suggested as vaccine candidates. Bacterial pellets of serotypes 1, 3 and 4, and a serotype 4 mutant lacking PspA, were choline washed with an excess concentration of choline to detach the choline-binding proteins. The eluded choline-binding protein containing fractions and the cell pellets, respectively, were tested for binding to sera from type 4 MP immunized mice in Western blot analysis. PspA antibodies specific for type 4, but not 1 and 3, were abundant in the immune sera, however the effects of removing PspA from the MPs were negligible in inducting heterologous protection.

Presence of Ply did not impact the protective efficacy of MP immunization against pneumococcal infection either, excluding Ply as an important antigen in mediating the cross-protection.

The two major antigens bound by the immune sera in the pellet fractions, which were important for the protection, were identified to be the lipoproteins MalX and PrsA, in a series of IP experiments. Both proteins were needed to evoke high percentage of cross-protection by MP immunization. Furthermore, the MP-raised antibodies displayed a higher binding capacity to PrsA and MalX than recombinant proteins, indicating that exposure of lipoproteins in their native conformation could be important. As demonstrated by live-cell imaging, of the binding of MP immune sera to live bacteria, the lipoproteins seem to be mostly exposed in the cell division sites. Both PrsA and MalX are highly conserved (99.5% and 98%) across pneumococcal strains and serotypes. Thus, the two proteins could serve as promising novel antigens to induce serotype independent cross-protection, if delivered in MPs.

5 CONCLUDING REMARKS

Here, the main findings of the four papers included in the thesis, will be outlined and discussed from a translational perspective. How our observations can impact continued research, as well as possibilities and limitations for clinical applications.

In paper I we show that blood-borne, piliated pneumococci enter the brain preferentially by receptor mediated transcytosis via the endothelial receptors pIgR and PECAM-1, and that antibody blockade of the two receptors efficiently inhibits brain invasion. PspC and the pilus adhesin RrgA were previously shown to be essential pneumococcal virulence factors that mediate the receptor interaction [59]. Like PspA, PspC, is highly variable in different strains of the pneumococcus, and only around 30% of isolates from colonization studies and invasive disease are piliated [169]. Hence, this interaction pathway of brain invasion could be strain specific to piliated strains. In the post PCV era, the presence of pilus-I has been found to be associated with hypervirulence, and is overrepresented in antibiotic resistant strains. Similar to the vaccine pressure induced clonal expansion of non-vaccine serotypes, the prevalence of piliated strains has shown an upward trend [169]. Therefore, continued surveillance and studying the disease-causing properties of piliated pneumococcal strains remains highly relevant.

The findings presented in paper I provide a proof-of-concept that understanding pneumococcal invasion mechanisms, and pharmacological modulation of host targets, can work as adjunctive therapy to antibiotics. Several fundamental limitations apply to receptor blockade of the two endothelial receptors to be translated directly into a clinical setting. Firstly, although the receptor homology of pIgR and PECAM-1 is preserved between mice and humans, and knockout mice lacking respective receptors are viable, the receptors are omnipresent on the endothelium in the body. A more specific target to the BBB would be preferred to avoid bodily wide side effects. Likewise, when a patient arrives to the clinic with pneumococcal meningitis, the brain is already overwhelmed with bacteria, and blockage of further entry from the blood is unlikely to contribute significantly to recovery.

In contrast, therapeutic intervention to prevent neurological sequelae in pneumococcal meningitis has a high beneficial potential. In paper II we show that the pneumococcus can both directly and indirectly interact with neuronal cells, inducing disruption of the cytoskeleton and cell death. Furthermore, we demonstrate that the bacteria can invade neuronal cells. We suggest neuronal β-actin as the main cellular target of both Ply and the pilus adhesin RrgA. In the study, pretreatment with antibodies against β-actin protect neuronal cells from bacteria induced toxicity. Again, β-actin is not a brain-specific target, it is abundantly expressed by all eukaryotic cells, which makes therapeutic application difficult. Whether a more targeted blockade of neuronal β-actin can be obtained by combination of antibodies, small molecules or nanoparticles, which can readily pass the BBB, is the objective of an ongoing project. We explore how neuronal cell damage is translated into loss of function *in vivo*, in paper III, by studying other damage inducing mechanisms of pneumococci in experimental meningitis. We show that the neurophysiological milieu is disrupted by the presence of bacteria in a cascade of events, involving the fluid drainage system of the brain. More specifically, the buildup of

fluid and bacterial components in the CSF during meningitis, is due to vast astrogliosis with disorganized AQP4 expression pattern, which in turn hinders normal fluid turnover to take place. The findings of the study, attest to the benefits of therapeutic intervention of relieving ICP, and dampen the self-amplifying neuroinflammatory response in bacterial meningitis patients. In this context, lumbar drainage, has been found to be a promising adjunctive therapy with corticosteroids and antibiotics, in reducing morbidity [186, 187].

In paper IV, we propose pneumococcal MPs as a novel vaccine platform to induce antibody mediated cross-protection against serotypes 1, 3 and 4 of *S. pneumoniae*. Furthermore, we identify the antigens involved in the cross-protection to be the conserved lipoproteins PrsA and MalX. Additional preclinical testing is needed to evaluate the safety and efficacy of MPs before moving onwards to clinical trials. For instance: evaluate the protective efficacy of MPs against a larger pool of serotypes, optimize MP isolation protocols, investigate potential cross-reactivity to commensal bacteria and explore other routes of immunization besides the mucosal route, and the need for adjuvants. Nevertheless, it is our assessment that bacterial vesicles hold a great potential as a tool to evoke a serotype independent immune response against pneumococcal infections and colonization.

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