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PNEUMOCOCCAL INTERACTIONS WITH THE HOST: THREATS AND THERAPEUTIC APPROACHES

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Cover: Immunofluorescence microscopy image of A549 human alveolar epithelial cells incubated with extracellular vesicles from *Streptococcus pneumoniae* serotype 4 strain TIGR4. A549 cells are stained for actin with Alexa Fluor 594 phalloidin (red), nuclei are stained with DAPI (blue) and extracellular vesicles are stained for pneumolysin with Alexa Fluor 488-conjugated goat anti-mouse IgG antibody (green).

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PNEUMOCOCCAL INTERACTIONS WITH THE HOST: THREATS AND THERAPEUTIC APPROACHES

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A mio padre

For my father

ABSTRACT

Streptococcus pneumoniae (the pneumococcus) is a Gram-positive bacterium responsible for substantial morbidity and mortality worldwide. Apart from causing severe pneumonia, septicemia or meningitis, pneumococci are also major contributors to less severe diseases like otitis media and sinusitis. Pneumococcal autolysis was thought to be the main route that *S. pneumoniae* utilizes in order to deliver its virulence factors. Recently a new mechanism has been proposed, the release of extracellular vesicles (EVs).

Presence of adhesins and other virulence factors on EVs leads to cell responses after contact with vesicles. We observed that pneumococcal EVs are indeed a mechanism for the delivery of virulence factors to host cells, and that interactions of vesicles with dendritic cells lead to activation of cells and release of pro-inflammatory cytokines. Since EVs mimic the outside of a bacterium, they can play a role as decoys for the immune system. Tightly linked to this decoy function is the ability of EVs to promote immune evasion through binding of serum components. Indeed, we discovered that pneumococcal EVs are able to bind several components of the human complement system, leading to formation of the membrane attack complex on vesicles.

Outer membrane vesicles (OMVs) released from Gram-negative bacteria have been directly used as vaccines in numerous preclinical mouse models. We isolated pneumococcal vesicles and found that they are able to confer serotype-independent protection in mice. Moreover, these vesicles stimulate the production of antibodies directed against pneumococcal antigens. These antibodies are able to increase opsonophagocytosis of pneumococci by mouse macrophages, and are required for protection, as demonstrated by the absence of protection in mice that are not able to produce B lymphocytes. Moreover, in our model the vesicles are able to protect mice against an infection with a pneumococcal strain of serotype 3, to a higher degree than what we observed for the currently available pneumococcal vaccine PCV13. The protective effect in humans of PCV13 against IPD caused by serotype 3 is debated.

The structure of the pneumococcal capsule differs vastly between serotypes. We found that these differences have profound consequences in determining the disease progression in terms of pneumonia or septicemia, in mice. In particular, we observed that serotype 2 was quickly cleared from the lungs but migrated efficiently to the blood, while serotype 3 remained in the lungs, since the thick capsule made bacteria able to adhere less to cells and better avoid opsonization by the complement system.

Fate of pneumococcal disease is tightly linked to the immune response against pneumococci. We found that a compound used in traditional Chinese medicine is able to potentiate the response of dendritic cells against pathogens, as well as increase the antimicrobial activities of host cells.

Overall, the work in this thesis provides information on pneumococcal interactions with the host immune system and highlights the potential use of vesicles in future vaccination strategies.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following papers, which will be referred to by Roman numeral throughout the text:

I. **CODEMO M.**, MUSCHIOL S., IOVINO F., NANNAPANENI P., PLANT L., WAI S. N., HENRIQUES-NORMARK B.
Immunomodulatory Effects of Pneumococcal Extracellular Vesicles on Cellular and Humoral Host Defenses.
mBio, 2018 Apr 10, 9(2): e00559-18.

II. **CODEMO M.***, IOVINO F.*, MUSCHIOL S., NANNAPANENI P., HENRIQUES-NORMARK B.
Streptococcus pneumoniae microparticles evoke a heterologous serotype-independent protection towards invasive pneumococcal disease.
Manuscript

III. NORMAN M., PATHAK A., **CODEMO M.**, SENDER V., GALLOTTA M., NANNAPANENI P., BOOTSMA H. J., BROWALL S., JONCZYK M., HASTE L., HERMANS P., ANDREW P., HENRIQUES-NORMARK B.
Growth and defence strategies affect pneumococcal disease pattern: septicaemia versus pneumonia.
Manuscript

IV. XIE S., SPELMINK L., **CODEMO M.**, SUBRAMANIAN K., PÜTSEP K., HENRIQUES-NORMARK B.*, OLLIVER M.*
Cinobufagin Modulates Human Innate Immune Responses and Triggers Antibacterial Activity
PLoS ONE, 2016 Aug 16; 11(8): e0160734.

* The authors contributed equally to the work.

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LIST OF ABBREVIATIONS

AM	alveolar macrophage
AMP	antimicrobial peptide
AOM	acute otitis media
APC	antigen-presenting cell
ASC	apoptosis-associated speck-like protein containing a CARD
CAP	community acquired pneumonia
CARD	caspase recruitment domain
CBG	cinobufagin
CbpA	choline binding protein A
CCR	C-C chemokine receptor
CD	cluster of differentiation
CDC	cholesterol-dependent cytolysin
CLR	C-type lectin receptor
CR	complement receptor
CTL	cytotoxic T-cell
CXCL	chemokine (C-X-C motif) ligand
DAMP	danger-associated molecular pattern
DC	dendritic cell
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EV	extracellular vesicle
FH	Factor H
GAPDH	glyceraldehyde-3-phospate dehydrogenase
GM-GSF	granulocyte macrophage colony stimulating factor
hBD	human β-defensin
Hic	Factor H inhibitor of complement
HIV	human immunodeficiency virus
HNP	human neutrophil peptide
HRP	horseradish peroxidase

IFN	interferon
Ig	immunoglobulin
IL	interleukin
IPD	invasive pneumococcal disease
IRF	interferon regulatory factor
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
LRR	leucine-rich repeat
LT	lymphotoxin
LTA	lipoteichoic acid
MAC	membrane attack complex
MARCO	macrophage receptor with collagenous structure
MBL	mannose binding lectin
MCP	monocyte chemoattractant protein
MDP	muramyl dipeptide
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
MRC	macrophage mannose receptor
MV	membrane vesicle
MyD88	myeloid differentiation factor 88
NBD	nucleotide-binding domain
NET	neutrophil extracellular trap
NF-κB	nuclear factor-kappa B
NLR	NOD-like receptor
NOD	nucleotide-binding oligomerization domain
OMV	outer membrane vesicle
PAMP	pathogen-associated molecular pattern
PBP	penicillin-binding protein
PGN	peptidoglycan
pIgR	poly Ig receptor
PMA	phorbol myristate acetate

PRR	pattern recognition receptor
PspA	pneumococcal surface protein A
PspC	pneumococcal surface protein C
RANTES	regulated on activation, normal T-cell expressed and secreted
RIG-I	retinoic acid-inducible gene I
RIP	receptor interacting protein
RLR	RIG-I-like receptor
RNS	reactive nitrogen species
ROS	reactive oxygen species
SC	secretory component
SIGN-R1	SIGN related-1
SpsA	secretory IgA binding protein
SR-A	class A macrophage scavenger receptor
STING	stimulator of IFN genes
T_H-cell	helper T-cell
TIL	Toll IL-1
TLR	toll-like receptor
TNF	tumor necrosis factor
TRIF	interacts with TIR-domain-containing adapter inducing IFNβ
WTA	wall teichoic acid

1 INTRODUCTION

1.1 *Streptococcus pneumoniae*

The bacterium *Streptococcus pneumoniae* was first discovered in 1881 when Louis Pasteur and George Miller Sternberg separately reported the isolation of diplococci from blood of rabbits after injecting the animals with human saliva (1, 2). The two scientists proposed two different names for the bacterium: *Microbe septicemique du salive* and *Micrococcus pasteuri*, respectively. In 1886 the name *Pneumococcus* was first used by Albert Fraenkel, as a reference to the disease caused by this microbe. *Diplococcus pneumoniae* was the name proposed in 1920 after its microscopic morphology, discovered after the introduction of Gram staining. Only in 1974 the current name *Streptococcus pneumoniae* was used for this bacterium for the first time, as a result of the observation that this bacterium grows as long chains of cocci in liquid media (3). However, the pneumococcus is still a frequently used term to refer to this microbe.

Pneumococci are Gram-positive bacteria able to grow *in vitro* on blood agar plates at 37°C and with 5% CO_2 in the atmosphere. After overnight growth bacterial colonies appear greyish with a green zone around them, indicating α-hemolysis, the lysis of erythrocytes in the blood and oxidation of hemoglobin (Fig. 1). Laboratory techniques to distinguish between *S. pneumoniae* and other commensal streptococcal species such as *S. viridans* include sensitivity to the antibiotic optochin. However, pneumococcal isolates resistant to optochin have been reported (4).

Pneumococcal colonies on blood agar plates appear mucoid due to their characteristic polysaccharide capsule that surrounds the bacterial cell wall. Based on the composition of the pneumococcal capsule, different pneumococcal serotypes have been classified. So far at least 97 serotypes have been identified (5).

Pneumococci are able to naturally internalize deoxyribonucleic acid (DNA) and incorporate it into their genome, a process called transformation (6). It was first demonstrated by Frederick Griffith after inoculating mice with both a non-encapsulated avirulent strain and a heat killed encapsulated virulent strain. After bacterial challenge mice died due to the infection, and he could isolate and grow in culture an encapsulated virulent strain of *S. pneumoniae*. Griffith's hypothesis was that a protein was transferred from the heat killed bacteria to the live ones that would confer the ability to become virulent again (7). Only in 1944 Oswald Theodore Avery demonstrated that the factor responsible for this modification was not a protein, but DNA (8).

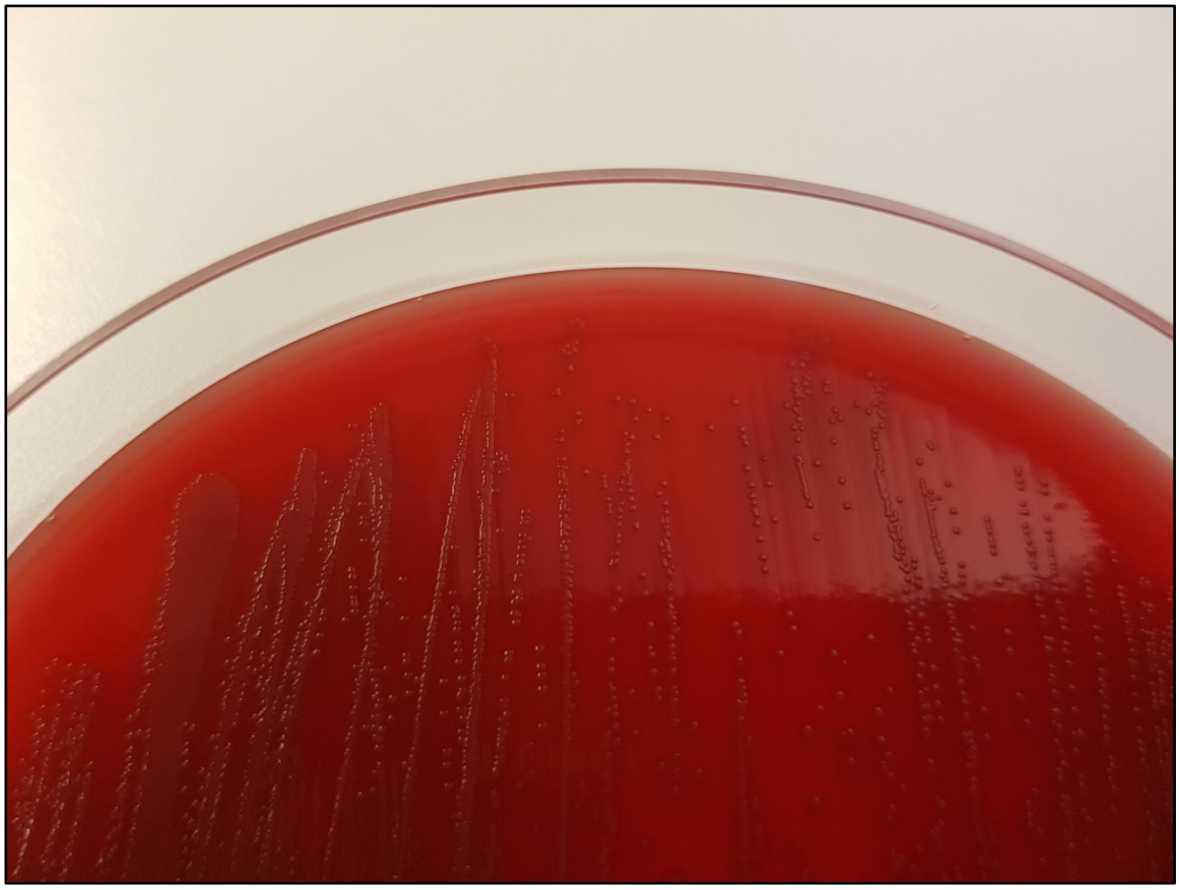

Figure 1. Colony morphology of the *S. pneumoniae* serotype 4 strain TIGR4. Bacteria were grown overnight at 37°C with 5% CO_2 in the atmosphere.

1.1.1 Colonization and disease

Pneumococci are both human-adapted colonizers of the nasopharynx and pathogenic bacteria. Diseases range from milder infections, such as otitis media and sinusitis, to severe diseases, such as pneumonia, bacteremia and meningitis. In this chapter, different manifestations of pneumococcal colonization and disease will be described.

Colonization

S. pneumoniae naturally colonizes the human nasopharynx asymptomatically. The bacterium is able to spread between individuals through aerosol droplets and thus can be found in up to 60% of children (9-11), while colonizing around 5% of adults (12, 13).

Most colonization episodes result in an asymptomatic presence of pneumococci in the nasopharynx, followed by clearance of the bacteria by the immune system. Variables accounting for duration of colonization include age of the host and pneumococcal serotype. It was observed that colonization can last between 2 and 368 days, with an average of 37 days (14). Children below the age of 5 had significantly longer colonization episodes, in comparison with older hosts. Moreover, the longest colonization time was observed for serotypes 6 and 23 (14).

In most cases colonization of the nasopharynx is required for pneumococci to translocate to other parts of the body and cause disease. The potential to colonize or cause disease greatly varies between pneumococcal serotypes and a significant inverse correlation between carriage and invasive disease has been observed (15). Serotypes 6B, 19F and 23F are among the ones frequently found in carriage episodes, while serotypes 1, 5 and 7 are frequently observed causing disease (15-17).

Otitis Media

Acute otitis media (AOM) is an inflammation of the middle ear that occurs very frequently in children, and is the most common disease caused by *S. pneumoniae*. Pneumococci account for 3.1 million cases of otitis media annually in children below 5 years of age, in the United States (18), and rank among the bacteria most commonly isolated from AOM cases (19). While AOM usually resolves spontaneously, persistent or recurrent otitis media can potentially cause complications and sequelae such as hearing loss, perforation of the tympanic membrane, facial paralysis or meningitis (20).

Sinusitis

Also known as a sinus infection or rhinosinusitis, sinusitis is the inflammation of the paranasal sinuses, four air-filled spaces present in the cranial bone around the nose. Although most sinusitis cases are caused by viruses, pneumococci represent one of the bacteria most frequently isolated from patients affected by sinusitis (21).

Pneumonia

Pneumococcal infection of the lungs leads to pneumonia, the inflammation of the lungs. In almost all countries *S. pneumoniae* is the most frequent cause of community acquired pneumonia (CAP), a disease common in children under 5 years and adults older than 65 years (22). Pneumonia mostly affects developing countries, where it causes 20% of deaths in children under 5 years, while it is responsible for only 2% in industrialized countries (23).

The risk of death from pneumonia greatly varies among pneumococcal strains, and it positively correlates with carriage frequency, while negatively correlating with the invasiveness of the serotype (24). Moreover, carriage frequency is negatively correlated with potential to cause pneumonia (24). For example, serotype 1 and 5 have low carriage frequency and risk of death from pneumonia, but high potential to invade. Instead, serotype 19F is characterized by a high carriage rate and risk of death from pneumonia, while having a low invasiveness (24).

Bacteremia and sepsis

Invasive pneumococcal disease (IPD) is defined as the infection of normally sterile sites of the body, such as the blood or the brain. These infections contribute to 11% of all deaths in children below 5 years of age (25) (Fig. 2).

Translocation of pneumococci to the bloodstream from different sites, such as the lungs or the inner ears, will cause bacteremia. This condition is defined as the presence of bacteria in the blood that are able to replicate. Infection of the blood can lead to sepsis, a life-threatening condition caused by an immune reaction to the infection, ultimately leading to damage to tissues and organs.

A study conducted in Denmark observed a 30-day mortality rate of 16% after pneumococcal bacteremia and sepsis, and a correlation between older age and increased 30-day mortality rate (26).

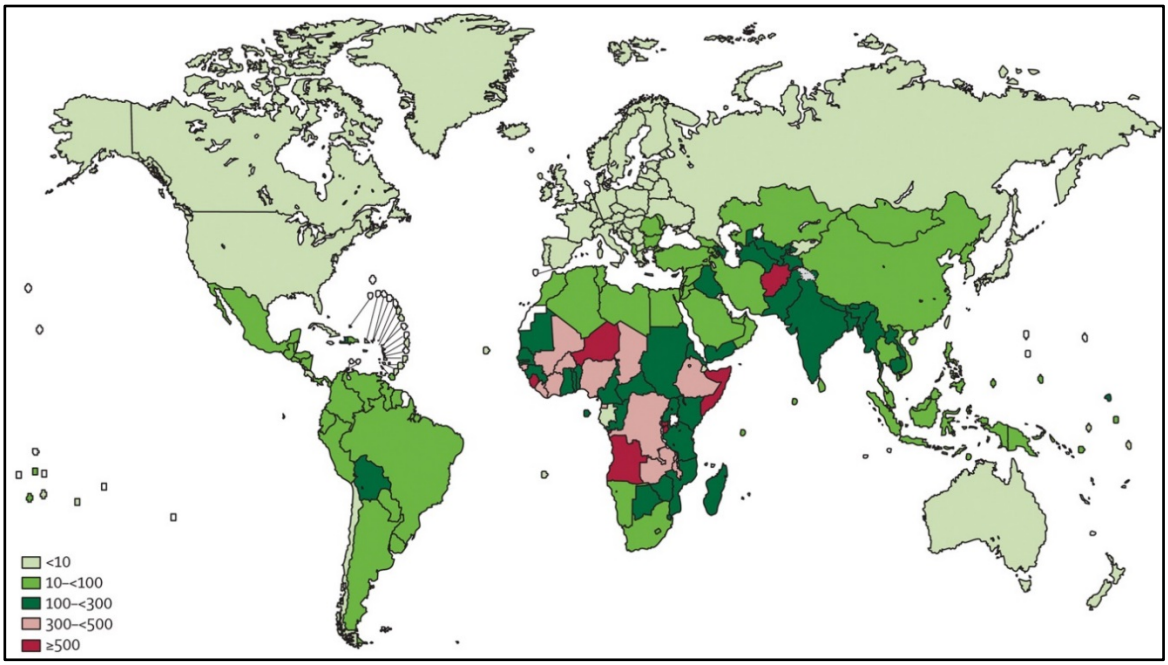

Figure 2. Global rates of mortality in children younger than 5 years caused by pneumococcal infections. Rates are shown per 100.000 children under 5 years of age. Adopted from (25).

Meningitis

The meninges are the three membranes, namely Dura Mater, Arachnoid Mater, Pia Mater, that surround the brain. Acute inflammation of these membranes, so called meningitis, can arise from infection by various microorganisms. Pneumococcal infections are the most common cause of bacterial meningitis in Europe and the United States, followed by meningococcal infections. These potentially lethal pneumococcal infections cause death in 16 to 37% of the patients and cause severe neurological sequelae in 30 to 52% of patients that survive the infection (27, 28). Sequelae include hearing loss, focal neurological deficits, and cognitive impairment (29, 30).

Risk factors

A fully developed and functional immune system is an important requirement for the prevention and clearance of infections caused by *S. pneumoniae*. Due to this reason, individuals with deficient immune systems, such as children under 2 years of age, and individuals with weakened immune systems, such as people above 65 years, have an

increased risk of contracting pneumococcal infections. Immunocompromised individuals, such as those affected by human immunodeficiency virus (HIV) infections, autoimmune diseases, primary immunodeficiencies or immunosuppressive therapies are also characterized by increased risk of pneumococcal infections (31, 32).

Moreover, immunocompetent adults can be at high risk of pneumococcal disease if they are affected by comorbidities such as cardiovascular and cerebrovascular disease, diabetes and alcoholism (31). Behavioral and environmental factors such as smoking, preceding viral respiratory infections (for example influenza A virus infection) and frequent contact with children can also increase risk for pneumococcal infections (32).

1.1.2 Pneumococcal virulence factors

Pneumococci have evolved many mechanisms to escape or modulate the host immune system. The polysaccharide capsule, and other virulence factors such as the proteins pneumolysin, the autolysin LytA and the Pneumococcal surface protein C (PspC) are contributing to those strategies. However, the host immune system has also developed its own mechanisms to fight pneumococcal infections, such as pattern recognition receptors (PRRs), the complement system and the adaptive immune system. In this chapter, pneumococcal immune evasion strategies will be discussed, while host responses will be the focus of the next chapter.

The capsule

The pneumococcal cytoplasmic membrane is surrounded by a thick cell wall consisting of peptidoglycan, which is in turn covered by a polysaccharide capsule, characterized by a great variability in its saccharide composition. The classification of pneumococci in different serotypes and serogroups is based on the composition of the capsules and their serological properties. A serotype is characterized by a unique capsular chemical structure and immunologic properties, while a serogroup includes serotypes that share serologic properties, such as cross-reactive antibodies (5). Antibodies that are specific to a serotype or serogroup do not usually react to different serogroups.

The capsule, alongside with the genetic background of the strain (33), is responsible for reducing the amount of opsonization by the complement system, a process further described in chapter 1.2.1. This is achieved by shielding surface proteins and other potential ligands. Moreover, the usually negative charge of the surface of the capsule allows pneumococci to avoid being trapped by the mucus or neutrophil extracellular traps (NETs) (34, 35).

Proof of the fundamental role of the capsule in pneumococcal virulence is the observation that non-encapsulated strains of this bacterium rarely cause invasive disease, while being present in around 3% to 19% of asymptomatic carriage isolates (36). However, adherence to cells is a requirement for the progression of invasive disease, and the capsule reduces the ability of pneumococci to interact with host surfaces. To overcome this limitation *S.*

pneumoniae is able to modulate the thickness of the capsule, a phenomenon known as phase variation. The phenotype can vary between a transparent variant, able to colonize the nasopharynx (37), and an opaque variant, more virulent in a model of systemic infection (38).

Notably, capsule production is not the only difference between the two phenotypes. For example, teichoic acid quantity is increased in the transparent variant, and other pneumococcal virulence factors are differentially expressed in the two phenotypes (38).

Pneumolysin

When pneumococci are grown overnight on blood agar plates a green area of hemolysis is caused by the production and release of pneumolysin (Ply), a cholesterol-dependent cytolysin (CDC). This 53 kDa protein interacts with cholesterol on host cell membranes, forming rings of 30 to 50 monomers that create pores in the cell membrane, leading to disruption of its integrity and lysis of the cell (39, 40). Moreover, Ply can bind mannose and the sialylated blood group antigen LewisX on the surface of cells (41, 42).

Ply does not have a signal sequence that would allow it to be actively secreted in the extracellular environment (43). It is found in the cytoplasmic fraction of the bacterial cell and non-covalently attached to the cell wall fraction, as well as in culture supernatants (44). Pneumococcal growth in liquid medium *in vitro* is characterized by a lag phase, an exponential phase, a stationary phase and a subsequent autolysis, which is pneumococcal specific. Until recently Ply was considered to be released by pneumococci during the autolysis process. However, an autolysis-independent release has also been observed (45), suggesting that secretion of this protein might be regulated by different processes.

While high concentrations of Ply induce cell death mediated by formation of pores in cellular membranes and decrease in ciliary beating of human epithelial cells (46), sublytic concentrations have immunomodulatory effects, such as the induction and release of pro-inflammatory cytokines from host cells. There is conflicting evidence regarding the involvement of toll-like receptor (TLR) (see chapter 1.2.1) activated by pneumolysin. Some literature present evidence for the involvement of TLR4 (47-50), while other evidence supports a TLR4-independent release of cytokines (51).

Release of pro-inflammatory cytokines interleukin (IL)-1β and IL-17 by pneumolysin is mediated by activation of the inflammasomes NLRP3 and AIM2 (51-53). Pneumolysin in endolysosomes can form pores on the membranes of these organelles and lead to the release of peptidoglycan in the cytosol of host cells, which activates the NOD2 receptor (54).

While pro-inflammatory effects of pneumolysin are well known, anti-inflammatory functions have also been reported in the literature. Incubation of dendritic cells (DCs) (see chapter 1.2.1) with pneumolysin results in inhibition of DCs maturation and cytokine release (55).

The autolysin LytA

Pneumococcal autolysis is mediated by the protein LytA, a choline-binding amidase. This enzyme is a N-acetylmuramyl-L-alanine amidase. LytA cleaves peptidoglycan in the cell wall between the glycan strand and the stem peptide of the peptide chain (56). This leads to perturbation of the cell wall and lysis of the bacteria.

LytA lacks a signal peptide for extracellular localization and is mainly localized intracellularly during exponential phase, with only a small fraction associated to the cell wall, probably resulting from cell turnover during logarithmic growth (57). LytA activation is triggered when the cell wall synthesis machinery becomes inactive, due to stationary phase, or by activity of cell wall-targeting antibiotics. In this condition nascent peptidoglycan at the equatorial plane is exposed becoming available to LytA (57).

Contribution of LytA to pathogenesis is still not fully understood. One proposed hypothesis is that LytA indirectly mediates virulence by inducing lysis and release of other virulence factors, such as pneumolysin (58). Another theory suggests that LytA-mediated lysis would increase DNA exchange in competent pneumococcal populations, leading to easier exchange of genetic material between pneumococci (59). LytA also affects sensitivity of pneumococci to antibiotics, such as penicillin G and vancomycin, that act on the cell wall of bacteria, leading to penicillin and vancomycin resistance in clinical isolates with defective autolysis (60, 61). Moreover, this enzyme has been suggested to be involved in the bacterial response to the antimicrobial peptide LL-37 by affecting capsule shedding (62).

Pneumococcal surface protein C

Pneumococcal surface protein C (PspC), also called *Streptococcus pneumoniae* secretory IgA binding protein (SpsA), Choline binding protein A (CbpA) and Factor H inhibitor of complement (Hic), has an important role in the virulence of *S. pneumoniae*. This cell wall-associated protein is highly variable in its sequence among different pneumococcal serotypes. Sequence comparison led to the identification of 11 groups: 6 groups share a choline binding domain in the protein sequence, while 5 groups contain a LPxTG domain (63). PspC variants with choline binding domain are non-covalently bound to phosphorylcholine present in lipoteichoic acid (LTA) associated with the cell membrane or in cell wall teichoic acid (WTA). PspCs with a LPxTG domain are covalently linked to the amino group of cell wall peptidoglycan (64).

In some pneumococci two copies of PspC, one with choline binding domain and one with LPxTG anchor, can be found (63, 65).

Like other surface proteins PspC possesses many functions. The main role of PspC in pneumococcal virulence is to promote immune evasion by binding human Factor H (FH) (66) and therefore prevent deposition of the complement protein C3 (67), ultimately leading to a reduced complement attack and opsonophagocytosis by immune cells.

PspC is also one of the pneumococcal adhesins, binding to the secretory component (SC) of human secretory Immunoglobulin (Ig) A and poly Ig receptor (pIgR) (68, 69). This binding is required for pneumococcal invasion of the epithelium (70), as well as for translocation across epithelial cells (71). Adherence of pneumococci is also promoted by binding of PspC to extracellular matrix proteins such as vitronectin (72) and thrombospondin-1 (73).

PspC is responsible for the species-specific binding of human pIgR (74), SIgA (74) and FH (75), thus contributing to host specificity of pneumococci. Other proteins that contribute to host specificity include IgA1 protease, enolase and pneumococcal surface protein A (PspA) (76).

1.2 THE HUMAN IMMUNE SYSTEM

The human body has the ability to distinguish between external molecules (so called non-self molecules) and its own components (or self molecules) through the immune system. This process allows the body to fight pathogens and tumor cells. Different layers of protection that can be divided into the innate and the adaptive immune system constitute the human immune system.

The purpose of the innate immune system is to provide a fast and non-specific response to infections. It also stimulates the adaptive immune system, which provides a delayed but highly specific response to a specific pathogen, as well as an immunological memory to better fight the same infection in the future.

1.2.1 The innate immune system

Physical barriers are the first line of defense encountered by pathogens. Such barriers include the skin, mucus, the alveolar-blood barrier and the blood-brain barrier. If a pathogen is able to penetrate these barriers other components of the innate immunity begin to fight the infection. These components are cells such as monocytes, dendritic cells, macrophages and neutrophils, as well as soluble factors such as proteins of the complement system and antimicrobial peptides.

Pattern recognition receptors

Cells of the innate immunity are equipped with pattern recognition receptors (PRRs) on their membrane or in their cytosol. The function of these receptors is to recognize pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs). As the innate immune system has to provide a broad and non-specific response to infections, PAMPs are well conserved among bacterial species, and include bacterial DNA and peptidoglycan components (77). Examples of membrane-bound PRRs include Toll-like receptors (TLRs) and C-type lectin receptors (CLRs). Among the most important cytosolic receptors are nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) and retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs).

TLRs are transmembrane proteins whose function is to recognize extracellular or endosomal PAMPs. While 13 different TLRs are found in mice, only 10 were identified in humans (78). These receptors share a similar structure, consisting of an ectodomain constituted by leucin-rich repeats and responsible for the recognition of PAMPs, transmembrane domains, and a cytosolic Toll IL-1 (TIL) domain, responsible for signal transduction. Apart from TLR3, all TLRs use the adapter molecule myeloid differentiation factor 88 (MyD88) in order to amplify the signal, leading to activation of the transcription factor nuclear factor-kappa B (NF-κB) and production of inflammatory cytokines. The endosomal receptor TLR3, as well as TLR4, interacts with TIR-domain-containing adapter inducing IFNβ (TRIF), leading to activation of transcription factors NF-κB and interferon regulatory factor 3 (IRF-3), which in turn induce the production of type I interferon (IFN) (79).

The cytosolic receptors NLRs have a common structure consisting of a central nucleotide-binding domain (NBD). Most members of this family of receptors have a C-terminal domain consisting of leucine-rich repeats (LRRs) for the recognition of PAMPs and a variable N-terminal domain that transmit the signal. The NLRC subfamily is characterized by a N-terminal caspase recruitment domain (CARD) that interacts through homophilic CARD-CARD interactions with the adapter molecule kinase receptor interacting protein 2 (RIP2), leading to activation of NF-κB. Within this subfamily, NOD1 and NOD2 are involved in the recognition of different motifs of the peptidoglycan (PGN) molecule, such as muramyl dipeptide (MDP) (80). The NLRP subfamily contains a N-terminal pyrin domain. Members of this family such as NLRP3 are able to form multi-protein complexes, referred to as inflammasomes, that recruit the adapter protein apoptosis-associated speck-like protein containing a CARD (ASC), which in turn can bind the pro-form of caspase-1. Bound caspase-1 can undergo autocatalytic cleavage to produce the active subunits p20 and p10. This leads to cleavage of the pro-forms of IL-1β and IL-18 that are then secreted in their active forms (81). Inflammasomes are tightly regulated in both their transcription and activation by various stimuli, such as pore-forming agents, MDP, crystals, potassium efflux and extracellular ATP. Microbial DNA can induce the release of IL-1β and IL-18 through a different member of the inflammasomes, absent in melanoma 2 (AIM2) (81).

Different components of pneumococci are known to be recognized by various PRRs (Fig. 3). Components of the cell wall such as LTA, as well as lipoproteins, can bind the TLR2 receptor (82-84). Unmethylated CpG in pneumococcal DNA can be sensed by TLR9 present in endosomes (85). As described before, there is contrasting evidence regarding the involvement of TLR in the recognition of Ply. The receptor NOD2 senses the MDP fragments of pneumococcal PGN in a process that involves phagocytosis of bacteria, digestion in endolysosomes and delivery of fragments to the cell cytosol by pneumolysin activity (86). The inflammasomes NLRP3 and AIM2 are required for Ply-mediated release of IL-1β and IL-18 (51) (53). A still unknown receptor is responsible for the Ply-dependent sensing of pneumococcal DNA, leading to release of type I IFN in a process mediated by the adaptor molecule stimulator of IFN genes (STING) and the transcription factor IRF3 (87).

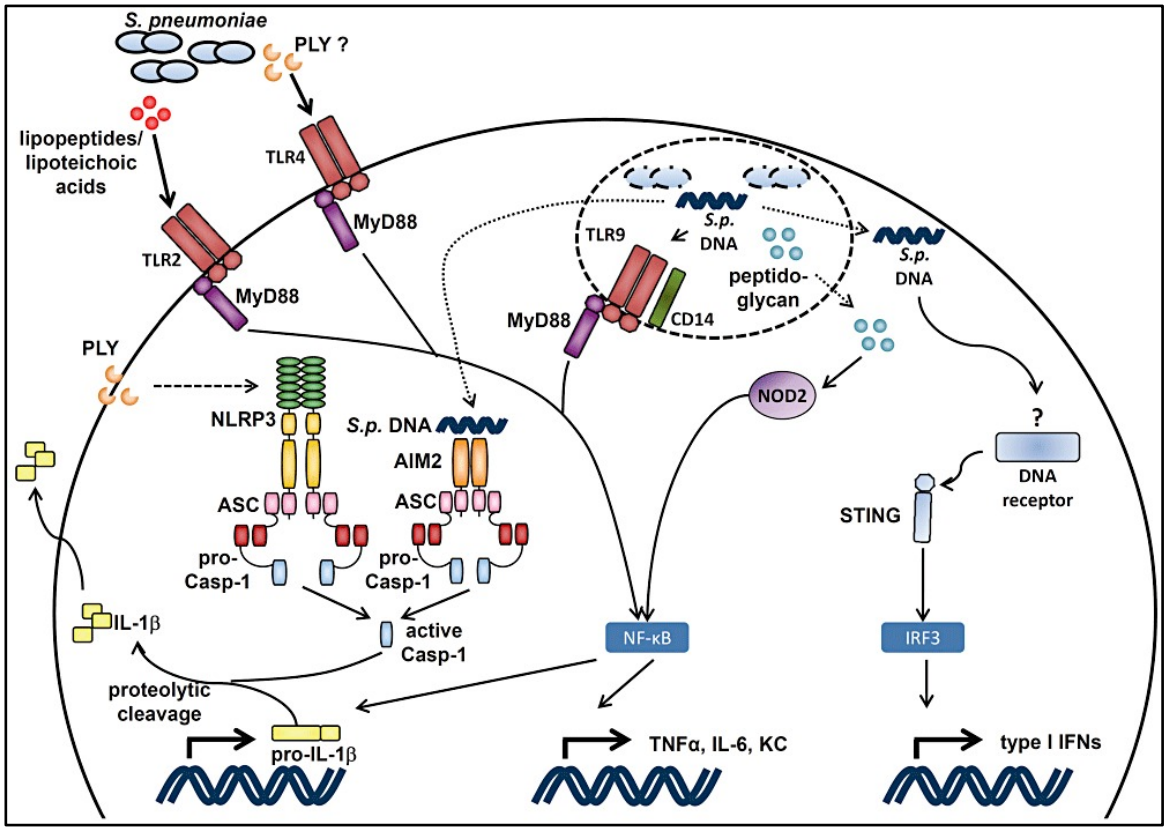

Figure 3. Signaling pathways involved in recognition of *S.* pneumoniae by PRRs. Pneumococcal LTA can activate TLR2, Ply is suggested to activate TLR4, and bacterial DNA is sensed by TLR9. Activation of these TLRs lead to production of inflammatory cytokines through the activity of the common adapter molecule MyD88 and the transcription factor NF-κB. The same pathway is activated when bacterial peptidoglycan bind the cytosolic receptor NOD2. Pneumococcal DNA is also proposed to bind an unknown DNA receptor, leading to the production of type I IFN through the adaptor STING and the IRF3 transcription factor, as well as the AIM2 inflammasome, leading to cleavage of pro-IL-1β that is released in its mature form. This pathway can also be activated by Ply through the NLRP3 inflammasome. Adopted from (87).

Macrophages

Macrophages are immune cells present in tissues that either differentiate from circulating monocytes or are resident in tissues since the early stages of fetal development (88). Alveolar macrophages (AMs) constitute 95% of leukocytes in the lungs, and their function is to recognize and internalize external particles, including pathogens, through a process called phagocytosis (89). Phagocytosis can take place after recognition of pathogens by different receptors present on the surface of macrophages, such as immunoglobulin receptors (Fcγ receptors - FcγRs), complement receptors (CRs), C-type lectins (like the mannose receptor) and scavenger receptors (like the macrophage receptor with collagenous structure – MARCO) (90).

Following internalization, pathogens are confined in an endosomal compartment, which then fuses with a lysosome to produce an endolysosome. In this compartment pathogens are digested by reactive oxygen or nitrogen species (ROS or RNS), such as hydrogen peroxide (H_2O_2), superoxide (O_2^-) or nitric oxide (NO) (91). Moreover, phagocytosis triggers the release of pro-inflammatory cytokines and chemokines, such as IL-8, monocyte

chemoattractant protein-1 (MCP-1) and RANTES (regulated on activation, normal T-cell expressed and secreted), leading to recruitment of neutrophils, activated monocytes and lymphocytes to the site of infection. While the contribution of macrophages to antigen presentation is limited, these cells can carry antigens to lymph nodes, where they are internalized by dendritic cells and presented to lymphocytes, leading to the initiation of the adaptive response (89).

The role of macrophages in fighting pneumococcal infections is well known. Important receptors in the phagocytosis of *S. pneumoniae* are complement receptor CR3 (92), macrophage mannose receptor 1 (MRC-1) (93), SIGN related-1 (SIGN-R1) (94), scavenger receptors MARCO (95) and class A macrophage scavenger receptor (SR-A) (96). As pneumococci are adapted to survive in presence of ROS, contribution of these molecules to killing of phagocytosed bacteria is limited, while RNS have a bigger impact in protecting from pneumococcal infections (97).

Dendritic cells

Dendritic cells (DCs) account for approximately 1% of immune cells in tissues or blood. The function of these antigen-presenting cells (APCs) is to scan the environment for non-self antigens, process them, expose them on the surface and present them to T-cells in order to start the adaptive immune response (98).

The internalization of antigens triggers activation of DCs, leading to presentation of the antigens on the cell surface, loaded on major histocompatibility complex class II (MHCII) molecules. Activation of DCs leads to upregulation and downregulation of different surface markers. Apart from MHCII, co-stimulatory molecules that are required for successful antigen presentation are also upregulated. Such molecules comprise cluster of differentiation (CD) CD86, CD40 and CD80. Receptors needed for endocytosis and phagocytosis are instead downregulated (99).

The key process in DC maturation is their migration from peripheral tissues to proximal lymph nodes, where they present antigens to T-cells. Migration is made possible by upregulation of a single chemokine receptor, C-C chemokine receptor type 7 (CCR7), making DCs sensible to gradients of macrophage inflammatory protein-3-β (MIP-3β) and/or 6Ckine produced by cells of the T cell zone in lymph nodes. Once in the T cell zone DCs can come in contact with the right antigen-specific T-cells and activate them (99).

Neutrophils

Neutrophils are short-lived cells produced in the bone marrow and released in the blood, where they represent the majority of leukocytes (100). Tissue inflammation leads to production of chemokine gradients with neutrophil chemoattractant activity, including release of IL-8, chemokine (C-X-C motif) ligand 1 (CXCL1 or KC), macrophage inflammatory protein 2 (MIP-2) or monocyte chemoattractant protein 1 (MCP-1) (101). Neutrophils subsequently leave the circulation and are attracted to the site of infection. There, their

function is to kill pathogens by releasing the content of their granules (to fight extracellular bacteria) or by fusing their intracellular granules with phagolysosomes containing internalized pathogens.

Apart from ROS, neutrophils granules contain antimicrobial peptides (AMPs) and proteases that help in the killing of bacteria (102). Another way for neutrophils to eradicate extracellular pathogens is the active formation of extracellular fibers named neutrophil extracellular traps (NETs), consisting of DNA and granule components (103).

Pneumococci developed several measures to evade killing by neutrophils. As described before, *S. pneumoniae* resists ROS (see above). The pneumococcal capsule protects the bacteria from being trapped in NETs (35), and bacteria produce endonuclease A that disrupts NETs by degrading DNA (104).

Complement system

The complement system consists of many plasma proteins that improve (complements) the activity of immune cells and antibodies in fighting non-self particles. Functions of this network of proteins are to opsonize pathogens, leading to improved phagocytosis by immune cells, induce inflammation at the site of infection and lyse bacteria by formation of the membrane-spanning pore named membrane attack complex (MAC). Following the first recognition of pathogens by this system, a cascade of proteolytic cleavage is activated, resulting in amplification of the first signal, that needs to be tightly regulated in order not to cause harm to host cells (105).

The classical pathway is a link between the innate and adaptive immunity and is started by binding of antibodies to pathogens. The C1q, C1r and C1s proteins (the C1 complex) then bind to two or more Fc regions of antibodies, ensuring that binding only happens when two or more antibodies are present in the same place on the surface of bacteria. Binding of the C1 complex triggers the C1r and C1s proteases to cleave C2 and C4 into larger (C2a, C4b) and smaller (C2b, C4a) fragments. The C4bC2a complex is the C3 convertase of the classical pathway and can be bound by the C3b fragment to form the C4bC2aC3b C5 convertase.

The alternative pathway can be activated by spontaneous hydrolysis of the protein C3. C3b bound on bacteria can recruit Factor B and D, forming the C3bBb C3 convertase. This leads to further proteolysis of more C3 to form the C3bBbC3b C5 convertase.

The lectin pathway exploits recognition of carbohydrate PAMPs by the mannose binding lectin (MBL). Binding triggers the serine proteases associated with this lectin. Since these proteases are functionally similar to the fragments C1r and C1s, activation of these enzymes leads to cleavage of C2 and C4, as well as the same following steps of the classical pathway.

All the three different pathways that can be activated (Fig. 4) lead to formation of a C3 convertase. This protease cleaves C3 present in plasma into fragments C3a and C3b. While C3a is a chemokine that contributes to neutrophil attraction to the site of infection, C3b binds on the surface of bacteria. Moreover, association of the C3 convertase with C3b forms the C5

convertase, leading to recruitment of proteins C6 to C9 and formation of the MAC that lyse bacteria.

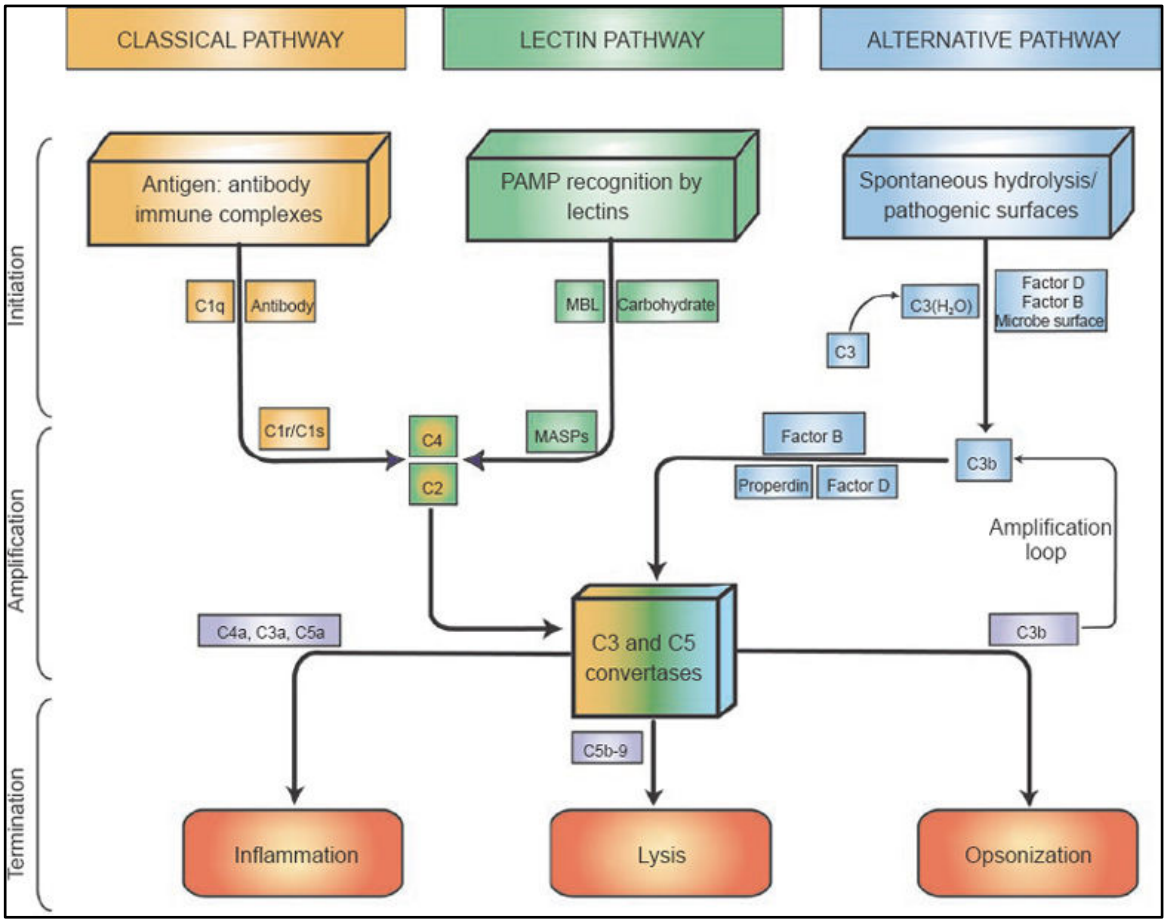

Figure 4. Schematic representation of pathways involved in activation of complement. Adopted from (105).

Pneumococci are able to evade the complement system through several mechanisms. The thick peptidoglycan layer physically protects all Gram-positive bacteria from the formation of the MAC complex. Due to this most of the contribution of the complement system in the killing of pneumococci is related to opsonization of bacteria to increase phagocytosis, rather than direct killing (106). However, the protein PspC of pneumococci, as described before, can bind the serum protein Factor H. This protein is important to avoid formation of complement complexes on host surfaces. It prevents binding of Factor B to C3b and acts as cofactor for the proteolytic cleavage of C3b by Factor I, to form the inactive iC3b, which can no longer form the C3 convertase (105, 107).

Antimicrobial peptides

Antimicrobial peptides (AMPs) are 6 to 59 amino acids long and contribute to the killing of various pathogens, such as Gram-positive and Gram-negative bacteria, enveloped viruses and fungi. While most cause lysis of bacteria by interfering with the cytoplasmic membrane, DNA, protein and cell wall synthesis are also common targets (108).

Major producers of AMPs in the respiratory tract are neutrophils and epithelial cells (109). Neutrophil α-defensins/human neutrophil peptides (HNPs), human β-defensins (hBDs), and

the cathelicidin hCAP18/LL-37 are the AMPs most commonly found in the lungs (110). Secretion of hBDs is either constitutive, as for hBD-1, or regulated by inflammatory signals, such as pro-inflammatory cytokines and TLR signaling (111).

AMPs involved in clearance of pneumococci include human α-defensins produced by neutrophils (112), hBD-2 and hBD-3 from lung epithelial cells (113) and hCAP18/LL-37 produced by mast cells (114).

1.2.2 The adaptive immune system

T-lymphocytes

T-lymphocytes are immune cells that derive from thymocytes in the thymus and migrate to the blood and lymphatic tissues. T-cell receptors on these cells are antigen-specific and are activated by different MHC molecules. MHC I antigens, present on all cells, activate the $CD8^+$ subset of T-cells, forming cytotoxic T-cells (CTLs). MHC II antigens, present on APCs, activate the $CD4^+$ subset, leading to effector helper T-cells (T_H-cells).

The function of CTLs is to directly bind and kill infected cells by producing perforin and granzyme B. Perforin creates pores in the membrane of target cells that allow granzyme B to enter and activate caspases, inducing apoptosis. Additionally, CTLs express the molecule Fas on their surface, which binds to its ligand FasL on the surface of target cells inducing caspase activation.

T_H-cells develop into different subtypes depending on the cytokine stimuli that they receive. Subtypes include T_H-1, T_H-2 and T_H-17 cells. IFN-γ and IL-12 drive the differentiation of T_H-1 cells, which in turn produce more IFN-γ, IL-2, tumor necrosis factor (TNF) and lymphotoxin-α (LT-α) leading to killing of intracellular pathogens.

IL-4 and IL-10 are involved in the development of T_H-2 cells, which secrete IL-4, IL-5, IL-10 and IL-13, contributing to B-cell maturation and production of immunoglobulin (Ig) G, IgA, and IgE, essential in the killing of extracellular pathogens.

IL-23 drives the differentiation of T_H-17 cells, which produce the pro-inflammatory cytokine IL-17. Interaction of IL-17 with different cell types leads to the release of different cytokines and chemokines, such as IL-6, CXCL8, CXCL2 and granulocyte macrophage colony stimulating factor (GM-GSF) promoting attraction of neutrophils and macrophage to the site of infection (115).

T_H-1 and T_H-17 cells are found to be particularly important in fighting pneumococci. The disappearance of T_H-1 cells from the circulation during pneumococcal infection is thought to be a sign of their migration to the site of infection (116). T_H-17 cells are proposed as mediators of an antibody-independent protection from pneumococci (117), resulting from recruitment of phagocytes to the site of infection (118).

B-lymphocytes

B-lymphocytes, or B-cells, mature in the bone marrow and are then found in the spleen or lymph nodes. These cells can bind antigens circulating in the lymph and present them on their MHCII molecules. After interaction with a T-cell specific for the same antigen the B-cell can differentiate into a plasma cell, which produces high affinity IgGs, IgAs or IgEs, or into a memory B-cell (119).

Some antigens like polysaccharides cannot be loaded on MHC molecules. Therefore, they activate B-cells in a T-cell independent manner, by cross-linking B-cell receptors. However, B-cells activated through this process cannot undergo memory B-cells differentiation and only differentiate into short-lived plasma cells producing low affinity IgM. Moreover, children under the age of 2 years do not possess fully developed B-cells and therefore cannot activate them in a T-cell independent manner. Due to this the early 23-valent pneumococcal polysaccharide vaccine (PPSV23) is no longer recommended for children, in favor of vaccines containing polysaccharides linked to a carrier protein that can be presented by MHCII molecules, leading to T-cell dependent maturation of B-cells (120).

1.2.3 Prevention and treatment of infections

Prevention

In order to prevent infection by the most common serotypes causing IPD 4 different vaccines are currently available, a pneumococcal polysaccharide vaccine (PPSV23) and pneumococcal conjugate vaccines (PCV7, PCV10 and PCV13) (Table 1).

Table 1. Currently available pneumococcal vaccines (121)

Name	Manufacturer	Year licensed	Serotypes included
PPSV23 Pneumovax®	Merck	1983	1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, 33F
PCV7 Prevenar®	Pfizer	2000	4, 6B, 9V, 14, 18C, 19F, 23F
PCV10 Synflorix®	GSK	2009	1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, 23F
PCV13 Prevenar 13®	Pfizer	2010	1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 23F

The polysaccharide-based PPSV23 was introduced to the market in 1983. This vaccine elicits an antibody response targeting the 23 capsular polysaccharides included in the formulation, promoting opsonophagocytosis of bacteria. As described before, this vaccine is poorly immunogenic, especially in young children (122).

Vaccines containing polysaccharides conjugated to a protein were then introduced in order to develop a T-cell dependent B-cell response in vaccinated subjects (123). While PCV7 and PCV13 contain a non-toxic variant of the diphtheria toxin, PCV10 contains protein D from *H.*

influenzae. Serotypes included in PCV7 were chosen because they were the dominant serotypes causing IPD in the United States. Since the serotype distribution varies between countries, serotypes covered by PCV7 represent fewer than 65% of IPD cases in Latin America and Asia (124). Additional serotypes in PCV10 and PCV13 should counterbalance the different global serotype distribution.

After PCV introduction, the incidence of IPD in vaccinated children reduced dramatically (125). In the United States IPD cases decreased by 77% in children under 5 years after introduction of PCV7 and the hospitalization rate for pneumococcal pneumonia in children under 2 years decreased by 65% (126, 127). However, expansion of IPD cases caused by non-vaccine types (NVT) have been observed (128-130), also in non-vaccinated age group such as the elderly. In 2016 in Sweden more than 70% of all IPD cases in the elderly were causes by NVTs. Moreover, an increase in carriage of NVTs 11A and 22F was observed in the Stockholm area (129) and more than 95% of all carriage isolates were of non-vaccine types post PCV introduction (131).

To overcome this serotype replacement, future pneumococcal vaccines should provide broad coverage against a larger pool of serotypes. In order to achieve this, protein vaccines are currently being developed. Targets include PspC and pneumolysin (132, 133). Whole cell vaccines of killed pneumococci are also currently being explored (134, 135).

In Gram-negative bacteria, outer membrane vesicles (OMVs) have been proposed as vaccine candidates. OMVs are spherical membranous bodies released by outward pinching of the outer membrane of Gram-negative bacteria. They harbor a cargo of periplasmic proteins surrounded by a lipid bilayer. Currently, an OMV-based vaccine for *Neisseria meningitidis*, Bexsero® (GSK), is licensed and others are currently being tested in clinical trials (136). Similarly to Gram-negative bacteria, Gram-positive bacteria release membrane vesicles (MVs, or extracellular vesicles, EVs) constituted by a cytosolic cargo surrounded by the cytoplasmic membrane. Recently, EVs from pneumococci have been isolated and their immunogenicity tested in a homologous challenge model (137). In this thesis, immunomodulatory properties of pneumococcal EVs are analyzed and their potential as vaccine candidate conferring broad protection is explored.

Treatment

Antibiotic therapy is the treatment of choice for pneumococcal infections. β-lactams, such as penicillin, are specially effective by interfering with penicillin-binding proteins (PBPs), leading to inhibition of cell wall synthesis, which in turn results in death of pneumococci.

However, mutations in PBPs or the cell wall muropeptide branching enzyme MurM lead to development of resistance to β-lactams, found already in 1967 (138). The prevalence of resistant pneumococci varies between countries. In Sweden, where the antibiotic use is low, 7.9% of invasive pneumococcal isolates had reduced susceptibility to penicillin in 2014 (139).

Pneumococci that are resistant to β-lactams are currently being treated with fluoroquinolones or macrolides. Fluoroquinolones bind to type II topoisomerase enzymes, such as DNA gyrase, impairing DNA replication. Macrolides bind to the 50S ribosomal subunit, inhibiting protein synthesis (140). Pneumococcal isolates resistant to macrolides are commonly found in Europe (139).

New therapeutic strategies currently being evaluated *in vivo* include blocking the mechanisms exploited by pneumococci to cross cell barriers in the body (69, 141).

2 AIMS

The aim of this thesis was to study the interactions of the human immune system with *S. pneumoniae*, focusing on the characterization of the immunomodulatory properties of EVs released by the bacteria and their potential use as a serotype-independent vaccine candidate. Moreover, we aimed at studying the relationship between different pneumococcal serotypes and disease progression, and to provide new putative future therapeutic approaches.

2.1 SPECIFIC AIMS

Paper I

To characterize extracellular vesicles (EVs) released by pneumococci and analyze their interactions with host cells and the complement system. The roles of EVs in pneumococcal pathology have been evaluated, focusing on induction of inflammation and immune evasion.

Paper II

To assess the potential use of pneumococcal EVs and microparticles (MPs) as serotype-independent vaccine candidates using *in vivo* models. Antibody responses to EVs and MPs were analyzed, as well as the ability of antibodies to increase opsonophagocytosis of bacteria *in vitro*.

Paper III

To investigate disease progression and immune responses of two pneumococcal strains belonging to different serotypes. *In vivo* mice models were used as well as *in vitro* bacterial adherence assays, and studies of phagocytosis and evasion of complement deposition.

Paper IV

To assess the effects of the natural compound cinobufagin (CBG) from Chinese medicine on innate immune responses and antimicrobial activity.

3 METHODOLOGICAL CONSIDERATIONS

3.1 BACTERIA

Strains of pneumococcal serotypes 1, 2, 3, 4 and 6B were used in this thesis.

The invasive strain of serotype 4, TIGR4 (T4) (ATCC BAA-334) (142), was used in paper I, II and IV. Its mutants lacking the *ply* (T4Δ*ply*) (55) or *lytA* (T4Δ*lytA*) (57) genes were used in paper I and II as controls for immunoblot detection of pneumococcal proteins in EVs and MPs. The isogenic mutant T4Δ*pspC* was used in paper I as a control for immunofluorescence detection of Factor H binding to PspC. The isogenic mutant in the capsule T4R (143) was used in paper I, II and IV. Previously it has been shown that T4R leads to similar phagocytosis rates and cytokine release as opsonized wild-type T4 *in vitro* (55).

The invasive serotype 1 strain BHN733 was used in paper II for the *in vivo* challenge of mice, as well as to assess the presence of pneumo-specific antibodies in mouse sera and to analyze the opsonophagocytic activities of antibodies. Strain BHN428 of serotype 3 was used in paper II to isolate MPs, challenge mice and analyze antibodies in the sera. Serotype 6B strain BHN191 was used in paper II for immunofluorescence assays to determine presence of antibodies in sera.

In paper III the serotype 2 strain D39-L and the serotype 3 strain Sp3-BS71 were used, as well as non-encapsulated mutants of serotypes 2 and 3, strains R6 and BHN1237.

3.2 IN VITRO MODELS

3.2.1 Cells

The focus of this thesis is interactions between *S. pneumoniae* and its host. Since pneumococci are human-adapted bacteria (76), most cell types used in this study have human origins. More specifically, human monocyte-derived dendritic cells were used in paper I since cytokines released by human and mouse dendritic cells greatly varies (55).

In paper III most data collected are from *in vivo* experiments with mice. For this reason, murine macrophages were used to evaluate adherence and phagocytosis of bacteria. Data were confirmed using human cell lines.

The following cell types were used in this thesis:

A549 cells

A549 are immortalized human alveolar epithelial cells isolated from a lung adenocarcinoma (American Type Culture Collection [ATCC], Manassas, VA) (144). These cells were used in paper I to evaluate internalization of pneumococcal EVs and in paper III to evaluate adherence of pneumococci to cells.

Human monocyte-derived dendritic cells

Human monocyte-derived dendritic cells were used in paper I and IV. These cells were differentiated from human monocytes, isolated from buffy coats of healthy donors, provided by Karolinska University Hospital. The RosetteSep human monocyte enrichment cocktail (StemCell Technologies) was used to isolate $CD14^+$ monocytes after a gradient centrifugation with Ficoll-Plaque Plus (GE HealthCare). Isolated monocytes were differentiated into dendritic cells for 6 days with IL-4 and GM-CSF (Peprotech). DC differentiation was assessed by staining cells for CD1a and CD11c markers, followed by flow cytometry analysis.

Murine RAW 264.7 macrophages

RAW 264.7 cells (American Type Culture Collection [ATCC], Manassas, VA) are a macrophage-like, Abelson leukemia virus transformed immortalized cell line derived from BALB/c mice (145). The cells were used in Paper II to assess opsonophagocytosis of bacteria and in paper III to evaluate adherence and phagocytosis of bacteria.

Human THP-1 derived macrophages

Human THP-1 monocytes (American Type Culture Collection [ATCC], Manassas, VA) are immortalized cells isolated from a patient affected by acute monocytic leukemia (146). For the differentiation into macrophages, cells were incubated with phorbol myristate acetate (PMA, Sigma) for 48 hours. Cells were used in paper I and III to evaluate opsonophagocytosis of bacteria.

Human HL-60 cells

HL-60 promyelocytes (ECACC98070106) are immortalized cells isolated from a patient affected by acute promyelocytic leukemia (147). Cells were differentiated into granulocytes by incubation with 1.5% dimethyl sulfoxide (DMSO, Sigma) for 5 days and were used for opsonophagocytosis killing assays (OPKA) in paper I.

Human Detroit 562 cells

Detroit 562 nasopharyngeal epithelial cells (American Type Culture Collection [ATCC], Manassas, VA) are immortalized cells isolated from the pleural fluid of a patient diagnosed with a primary carcinoma of the pharynx (148). Cells were used in paper III to evaluate adherence of bacteria to cells.

Human neutrophils

Human neutrophils were used in paper IV. Cells were isolated from buffy coats of healthy donors. Blood was layered on Biocoll (Biochrom AG) and centrifuged. Neutrophils were enriched with EasyStep human neutrophil enrichment kit (StemCell Technologies).

Neutrophil phenotype was assessed by staining cells for CD16 and CD66b markers, followed by flow cytometry analysis.

3.2.2 Cell toxicity measurements

Different methods for the assessment of cell toxicity were used in this thesis.

In paper I dead A549 cells and dendritic cells were stained irreversibly with fixable viability dye (FVD, EBioscience), in order to allow fixation of cells in the staining protocol.

In paper IV dendritic cells were instead stained with the intercalating agent propidium iodide (PI) (BD Pharmingen). Dendritic cells in paper I and IV were also stained with Annexin V to assess the percentage of apoptotic cells, using the Annexin V-FITC Apoptosis Detection Kit (BD Pharmingen).

In paper IV dendritic cell supernatants were analyzed with the Cytotoxicity kit (Roche) to detect the presence of the intracellular enzyme lactate dehydrogenase (LDH).

3.2.3 Adhesion and phagocytosis assays

In paper I and III opsonophagocytosis of bacteria by THP-1 derived macrophages was assessed. Differentiated cells were incubated with bacteria previously treated with human serum. Adherence of bacteria was assessed by washing cells, lysing them and plating bacteria. Phagocytosis was assessed by adding the antibiotic gentamicin to separate wells after incubation with bacteria, followed by plating colonies. Killing of intracellular bacteria by macrophages was assessed by further incubating macrophages after antibiotic treatment, followed by lysis of cells and plating bacteria.

Phagocytosis of bacteria by RAW 264.7 murine macrophages was assessed in a similar way in paper III. However, bacteria were not treated with serum prior to incubation with cells.

In paper III adhesion of non-opsonized bacteria to epithelial cells was evaluated, using A549 alveolar and Detroit 562 nasopharyngeal epithelial cells.

Opsonophagocytic killing of bacteria by differentiated HL-60 granulocytes was analyzed in paper I, similarly to what was described previously (149). Bacteria were incubated with human serum for 30 minutes, differentiated cells were then added and incubation was followed for 1 hour. Bacteria were plated before and after the assay and survival was calculated.

3.2.4 Measurement of cytokines

In paper I, III and IV cytokines, chemokines and antimicrobial peptides in cell-free supernatants were quantified using enzyme-linked immunosorbent assays (ELISAs) kits according to the manufacturers' instructions.

In paper II ELISAs were used to determine EVs-, MPs- and pneumo-specific IgG titers in mice sera. Briefly, EVs, MPs or heat-killed bacteria were immobilized in wells of optical plates overnight. Non-specific binding was then blocked and wells were incubated with mice sera. IgGs were then detected using an anti-mouse IgG horseradish peroxidase (HRP) antibody (GE Healthcare).

3.2.5 Complement deposition and visualization

In paper III complement deposition on bacteria was analyzed after incubation with human or mouse serum. C3 bound to bacteria was detected by incubation with appropriate antibodies and flow cytometry analysis.

In paper I binding of complement proteins to pneumococcal EVs was assessed by ELISAs. Briefly, wells of optical plates were coated with EVs overnight. Non-specific binding sites were blocked and human serum was added to wells. Binding of complement proteins C3, C5b-9 and Factor H was then detected with specific secondary antibodies.

Complement binding to EVs was also visualized by immunofluorescence microscopy in paper I. EVs were dried on microscope slide and incubated with human serum. Slides were then incubated with the lipid dye Nile red, as well as antibodies for Ply, C3, C5b-9 and Factor H. Binding of antibodies was detected with appropriate secondary antibodies. Binding of C3 to opsonized bacteria in paper III was visualized by immunofluorescence using the same antibodies.

3.3 IN VIVO MODELS

Despite the well-known human host specificity of pneumococci (76), various animal models can be used to study pneumococcal infections (150). Among mouse models, commonly used inbred strains are BALB/c, C57BL/6 and CBA/Ca mice. While BALB/c and CBA/Ca mice are characterized by low and high susceptibility to pneumococcal infection, respectively (151, 152), C57BL/6 mice display intermediate susceptibility (153). This feature makes C57BL/6 mice ideal for the study of both highly pathogenic and non-pathogenic strains, along with the availability of a large number of genetically modified mice in this background.

In paper II and III mice were infected intranasally to mimic the natural route of pneumococcal infection. However, only part of the inoculum would reach the lower respiratory tract. In order to deliver the whole inoculum to the lower airways, mice were infected intratracheally in paper III in separate experiments.

To study the contribution of antibodies in the protection in paper II muMt knock-out mice in the C57BL/6 background were used. These mice are homozygous for a mutation of the μ chain gene, leading to a block in the formation of the pre-B cell receptor (pre-BCR) and in the maturation of B-lymphocytes.

In paper III the contribution of macrophages to disease progression was assessed by administering liposome encapsulated clodronate intratracheally. This routinely used method leads to a depletion of around 80-94% of alveolar macrophages (154).

3.4 ETHICAL CONSIDERATIONS

In vivo experiments were performed according to ethical permits by the local ethical committee Stockholms Norra djurförsöksetiska nämnd. Mice were subjected to 12 hours light and dark cycles with water and food provided *ad libitum*. The health status of mice was checked according to ethical permits.

4 RESULTS AND DISCUSSION

4.1 PAPER I

Immunomodulatory Effects of Pneumococcal Extracellular Vesicles on Cellular and Humoral Host Defenses

The release of outer membrane vesicles (OMVs) is a phenomenon known since the 1960s in Gram-negative bacteria (155). In more recent years extracellular vesicles (EVs), also called membrane vesicles (MVs), released by Gram-positive bacteria were also observed (156). The functions of OMVs and EVs include the delivery of virulence factors to the host, induction of pro-inflammatory responses and immune evasion (157). EVs released by *S. pneumoniae* serotypes 1,2, 6B, 8 and 23F were recently studied (137). In the first study of this thesis we isolated EVs from the pneumococcal serotype 4 strain TIGR4 (T4) and characterized them using different molecular methods, such as electron microscopy, atomic force microscopy, mass spectrometry, SDS-PAGE and western blotting, as well as studied their effects on innate immune responses.

The analysis of electron micrographs of negatively stained purified EVs revealed membranous bodies with a diameter ranging from less than 25 nm up to 250 nm. The majority of the vesicles were characterized by a diameter ranging between 20 to 80 nm, in line with EVs previously characterized from different serotypes (137). Transmission electron micrographs of non-encapsulated pneumococci revealed vesicles budding from bacteria, with no preferential point of origin on the bacterial surface.

We analyzed the protein content of EVs by mass spectrometry and classified the proteins found by their predicted subcellular localization. Similarly to EVs analyzed previously, mostly cytosolic and transmembrane proteins characterized the protein cargo of T4 EVs. Immunoblotting was further used to probe for specific pneumococcal proteins and virulence factors in EVs. While pneumolysin (Ply) was particularly enriched, the cytosolic enzyme glyceraldehyde-3-phospate dehydrogenase (GAPDH) was not, suggesting a selective enrichment of proteins in EVs. Among cell wall-associated proteins choline-binding proteins, such as LytA and PspC, were enriched, while the LPxTG major pilin subunit RrgB was not, possibly indicating low abundance of peptidoglycan in the EV preparations. Supporting this hypothesis, among known surface-associated virulence proteins most choline binding proteins were present in EVs, while only few peptidoglycan-linked LPxTG proteins were detected.

It has been described that EVs from Gram-positive bacteria such as *Staphylococcus aureus* are able to deliver virulence factors to host cells and induce toxin-dependent cell death (158, 159). To test this hypothesis, we then incubated human epithelial and dendritic cells with pneumococcal EVs to assess if EVs had similar effects on the cells. While EVs were internalized by both A549 lung epithelial and dendritic cells in a dose-dependent manner, no cell death was detected in A549 cells. A slight pneumolysin-dependent increase of toxicity

was observed in DCs incubated with EVs. However, cell death was considerably lower than what was observed upon incubation with live bacteria.

To assess the ability of EVs to induce DC activation we analyzed the expression of MHCII and the costimulatory molecule CD86 on the surface of the cells and we observed a pneumolysin-independent increase of both markers. Activation of DCs led to release of pro-inflammatory cytokines such as IL-6, IL-8 and TNF, and anti-inflammatory cytokine IL-10. IL-1β and IL-12 secretion was not induced by EVs. Since EVs isolated from the mutant T4Δ*ply* induced cytokine release to the same extent as wild-type EVs, we suggest that DC activation and cytokine release are dependent on different pneumococcal components, such as teichoic acids and lipoproteins, known activators of TLR2.

In many bacterial species OMVs and EVs function as decoy for antimicrobial peptides or bacteriophages (160) and thus promote immune evasion by bacteria. Additionally, immune evasion is achieved by protease-mediated degradation of complement proteins (161) or binding and sequestration of complement proteins (162). We incubated pneumococcal EVs with human serum and investigated binding of complement proteins C3, C5b-9 (the so-called membrane attack complex – MAC) and Factor H (FH) to EVs by immunofluorescence microscopy. We observed co-localization of all three proteins with the lipid staining Nile red, indicating binding of complement to the EVs. Binding was confirmed by immunoblot analysis, ELISAs and mass spectrometry. Moreover, FH binding was dependent on the presence of the FH-binding protein PspC. The results collectively suggest that the plasma membrane of EVs and nucleophilic targets for the complement are freely accessible from the outside, leading to activation of the complement cascade and formation of the membrane attack complex (MAC) on the vesicles.

To evaluate if complement binding by EVs could impair opsonophagocytosis of pneumococci by immune cells we incubated differentiated THP-1 macrophages with opsonized bacteria, after pre-treating human serum with pneumococcal EVs. Incubation of serum with EVs led to a decrease in bacterial uptake by the cells. Moreover, we performed an OPKA using differentiated HL-60 cells. Bacteria incubated with human serum pre-treated with EVs were able to escape killing by granulocytes, suggesting that sequestration of complement components by EVs leads to reduced complement deposition on bacteria and reduced binding to complement receptors on phagocytic cells.

In summary, we provide evidence for the release of EVs by pneumococcal serotype 4 strain T4. The vesicles are enriched for specific proteins, including the major pneumococcal toxin pneumolysin. EVs can be internalized by host cells and evoke activation of DCs and pro-inflammatory cytokines release. Complement proteins can bind to vesicles and can be activated, leading to formation of MAC on the surface of EVs. Sequestration of complement by vesicles leads to reduced opsonophagocytosis of bacteria by host immune cells. Based on these findings we suggest that EVs can play an important role in pneumococcal pathogenesis. During early stages of infection in the lungs vesicles could promote an inflammatory environment leading to tissue damage and bacterial spread. During bacteremia complement sequestration by EVs could enhance bacterial survival.

4.2 PAPER II

***Streptococcus pneumoniae* microparticles evoke a heterologous serotype-independent protection towards invasive pneumococcal disease**

Concurrently with the use of pneumococcal vaccines a rise in IPD cases caused by non-vaccine types (NVTs) strains has been observed (128) and the efficacy of the PCV13 vaccine against certain serotypes, such as serotype 3, is being debated (129, 163). Hence, new preventive strategies are needed. In Paper I we observed that pneumococcal EVs are able to induce DC activation, a fundamental step in the process of antigen presentation and induction of an adaptive immune response. Moreover, intramuscular administration of EVs in mice has proven to be successful in protecting the animals against a challenge with the same pneumococcal serotype (137). In paper II we assessed whether pneumococcal EVs could provide a serotype-independent protection *in vivo*. We also developed a method to isolate vesicles from plate-grown pneumococci to mimic *in vivo* biofilm growth (164), leading to a different type of blebs that we referred to as microparticles (MPs).

MPs were characterized as described in paper I. Interestingly, MPs isolated and purified from plate-grown bacteria showed differences in terms of size, protein composition and protein enrichment as compared to the previously characterized EVs.

In order to study induction of serotype-independent protection in mice we isolated EVs and MPs from the pneumococcal serotype 4 strain T4 (TIGR4), immunized C57BL/6 mice twice intranasally and infected them intranasally with the invasive serotype 1 strain, as a model for IPD. While immunization with EVs resulted in 65% survival, MPs provided 80% survival. In accordance with these data, we analyzed the bacterial load in the lungs of mice post sacrifice and observed a significantly lower number of bacteria in mice immunized with MPs. In particular 9 mice out of 20 immunized with MPs had a number of pneumococcal colonies below the detection limit, while all mice immunized with EVs displayed presence of bacteria in the lungs.

We then studied the antibody response in immunized mice and observed IgG specific for EVs or MPs in the sera, using ELISA assays. Antibodies were detected already after the first immunization. Antibodies specific for whole heat-inactivated T4 and its non-encapsulated mutant T4R were also detected, in higher levels in sera of mice immunized with MPs, however, this difference was not statistically significant. We further used sera from immunized mice as primary antibodies in immunofluorescence experiments to visualize antibody binding to pneumococcal serotypes 1, 3 and 6B. For all serotypes we detected signals on the surface of bacteria, and the signal was in all cases higher using sera from mice immunized with MPs.

Taken together our data suggest the production of specific IgG after immunization with pneumococcal MPs. The triggered antibody production appears to be independent of the bacterial capsule since we observed serotype-independent protection and binding of antibodies to serotypes 1, 3 and 6B. Based on the immunofluorescent staining results, targets

of the antibodies appear to be proteins exposed on the surface of bacteria since we detected binding of antibodies to the non-encapsulated mutant T4R in ELISAs assays.

We then evaluated whether the antibodies produced by immunized mice would affect opsonophagocytosis of bacteria. We incubated serotype 1 pneumococci with sera from immunized mice, following incubation with RAW 264.7 murine macrophages. Bacteria incubated with sera from immunized mice adhered significantly better to cells and survived significantly less once internalized by phagocytes.

As described before, current data suggest that PCV13 does not give a good protection against IPD caused by serotype 3. We therefore assessed if MPs would confer protection against serotype 3. While MPs isolated from serotype 4 conferred 50% survival, all mice immunized with MPs from serotype 3 survived the challenge. In comparison immunization with PCV13 conferred 75% of protection using our infection model. Our data also correlates well with the observed bacterial load in the lungs of mice at sacrifice. In fact, mice immunized with MPs from serotype 3 had significantly lower bacterial loads compared to control mice. While mice immunized with MPs from serotype 4 had lower number of colonies in the lungs than control mice, the difference was not statistically significant. Overall, these data suggest that pneumococcal MPs could provide a better protection *in vivo* compared to the currently available PCV13 vaccine.

We then made use of the muMT mice, which are not able to produce B-lymphocytes, in order to assess whether protection conferred by serotype 3 MPs against the same serotype was antibody-dependent. Indeed, while all wild-type mice survived the infection, all knock-out mice had to be sacrificed before the end of the experiment. Moreover, the bacterial load in knock-out mice was similar to that of non-immunized wild-type mice, suggesting that the protection is antibody-dependent.

In conclusion, in this study we display evidence supporting the potential future use of pneumococcal MPs as vaccine candidates. MPs elicited a serotype-independent protection in mice. Moreover, immunization with MPs led to production of antibodies recognizing surface antigens common to different pneumococcal serotypes. Antibodies were able to enhance opsonophagocytosis by murine immune cells and proved to be necessary for protection.

4.3 PAPER III

Growth and defence strategies affect pneumococcal disease pattern: septicaemia versus pneumonia

Differences in virulence between pneumococcal serotypes are mostly attributed to the capsule (165, 166). In this study we characterize differences in disease progression and pathology between two clinically relevant serotypes that use their capsular defense system in different ways.

Both strains analyzed in this study, D39 of serotype 2 and Sp3-BS71 of serotype 3, displayed similar mortality rates after intranasal infection of C57BL/6 mice. However, following intratracheal administration of bacteria, numbers of serotype 3 bacteria in the lungs remained similar, while most serotype 2 bacteria were cleared 24 hours post infection (p.i.). Despite the clearance from the lungs more mice infected with serotype 2 were bacteraemic and displayed higher bacterial loads in the blood, at the same time points.

To investigate this difference in the clearance of bacteria we depleted alveolar macrophages using liposome-encapsulated clodronate. Significantly more bacteria were found in the lungs of mice infected with either serotype 2 or 3, suggesting an involvement of macrophages in the clearing difference. Moreover, less mice were bacteraemic after clodronate treatment and infection with serotype 2. Taken together, these data suggest an involvement of uptake by macrophages in the spreading of serotype 2 pneumococci from the lungs to the blood.

Analyzing the cytokine responses in the lungs 24 hours p.i. we observed increased levels of pro-inflammatory cytokines, such as IL-1β, IL-6, IL-17a and TNF, and decreased levels of the anti-inflammatory cytokine IL-10 in mice infected with serotype 3. Histologic analysis of lungs confirmed these data and demarked areas of inflammation and pulmonary consolidation in the lungs of mice infected with serotype 3 were observed.

Functional measurements of the lung capacity of the mice further confirmed the data from the histologic analysis. While no differences were found in residual volume (RV), functional residual capacity (FRC) and total lung capacity, mice infected with serotype 3 displayed a reduced forced expiratory volume (FEV) compared to serotype 2 infected mice.

In vivo data on clearance by macrophages and bacterial loads in the lungs were confirmed by *in vitro* adherence and phagocytosis assay. Serotype 3 adhered significantly less to nasopharyngeal and alveolar epithelial cells. Moreover, significantly less serotype 3 bacteria adhered to and were internalized by mouse or human macrophages, in presence or absence of opsonization.

Another possible explanation for the differences in disease progression was observed after analyzing C3 deposition in the strains of the two serotypes. Significantly more C3 was deposited on serotype 2 bacteria, after incubation of bacteria with either mouse or human serum. These data were confirmed by immunofluorescence analysis of bacterial capsule and C3 staining for the two serotypes. While serotype 3 released large quantities of capsule in the supernatant, all bacteria stained positive for the capsule, and only few bacteria had C3 signal

on the surface. A possible explanation for this phenomenon could be that non-covalent anchorage of the capsule in serotype 3 allows the bacteria to release the excess capsular polysaccharide, as suggested by another study (167). However, extensive production of capsule by serotype 3 could explain the reduced bacterial growth during initial stages of infection, leading to lower bacterial load in the lungs.

In conclusion, we observed that differences in disease progression between these two virulent pneumococcal strains of different serotypes are mostly caused by how they use their capsular virulence factor.

4.4 PAPER IV

Cinobufagin Modulates Human Innate Immune Responses and Triggers Antibacterial Activity

The traditional Chinese medicine Chan-Su is derived from secretions of the toad *Bufo gargarizans*. Among the active components of this medicine are bufadienolides which are responsible for its positive effects on heart function (168). Chan-Su is also used in combination with traditional therapies against cancer (169). It is believed to function as an inducer of apoptosis of cancer cells *in vitro* by its component cinobufagin (CBG), through caspase mediators (170). Since Chan-Su is also commonly used to treat infections that can cause tonsillitis (171), in this study we assessed whether Chan-Su and its components CBG could have a direct or indirect antimicrobial effect.

Direct microbial killing was observed for Chan-Su and CBG only at concentrations many times higher than physiological concentrations, so direct antimicrobial effects were ruled out. We then investigated whether the component CBG could function indirectly by modulating the innate immune responses and specifically focused on DCs.

Incubation of DCs with lipopolysaccharide (LPS) from *Escherichia coli* in combination with CBG led to decreased DC maturation with increasing concentrations of CBG. In accordance with downregulation of DC maturation, we observed downregulation of the release of cytokines, such as TNF, IL-6, IL-8, IL-10 and IL-12p40.

We then investigated the effects of CBG on cell death and induction of apoptosis. While no necrosis was observed by either LDH assays or flow cytometry, a significant increase in the number of apoptotic Annexin V^+ cells was detected after incubation with CBG, in absence or presence of LPS. After measuring the activation of caspases in DCs incubated with CBG, we observed increase of poly-caspase positive cells, which was LPS independent.

Despite the downregulation of cytokines that we observed, CBG increased the production of pro-IL-1β by LPS-stimulated cells. In accordance with these data, active caspase-1 was upregulated by CBG, in the absence or presence of LPS.

Our data on cytokine release by CBG stimulation suggest that this compound has both anti- and pro-inflammatory effects, similarly to cytokines such as TGF-β (172) or antidepressants (173).

Next, we assessed whether the immunomodulatory properties of CBG would have effects on the production of AMPs by immune cells. While incubation of DCs with CBG only led to upregulation of hBD-2 and hBD-3 at the RNA level, stimulation of neutrophils with CBG resulted in upregulation of secretion of HNP1-3 and hCAP18/LL-37. We then proceeded to evaluate if this release of AMPs would result in increased killing of pneumococci incubated with CBG-treated neutrophils. Indeed, we could observe a statistically significant reduction in the survival of bacteria after this assay.

The release of AMPs by neutrophils possibly involves intracellular calcium levels, small GTPases as well as MAPK kinases (174). More studies are needed to investigate if CBG activates any of these pathways.

In summary, in this study we provide evidence that CBG can regulate the innate immunity response in different ways during a bacterial infection. While this compound downregulates DC maturation and cytokine release, it upregulates caspase-1 activity and IL-1β release. Moreover, CBG increases antimicrobial responses by neutrophils, suggesting a potential therapeutic use for the treatment of pneumococcal infections, possibly in combination with antibiotics.

5 CONCLUDING REMARKS

S. pneumoniae is the etiological cause of IPD which is one of the leading causes of death among bacterial infections worldwide. The current use of vaccines and antibiotics to prevent and treat IPD has its shortcomings. The limited range of serotypes covered in current vaccines gave rise to serotype replacement, hence increasing vaccine inefficacy. Furthermore, affordable vaccines are needed for more countries. The emerging occurrence of antibiotic-resistant pneumococci poses a serious threat and new antimicrobial compounds and therapeutic strategies are needed.

In paper I we characterized EVs isolated from pneumococci and studied their immunomodulatory roles, providing novel insights on their contribution to pneumococcal pathology. While they could increase inflammation during early stages of infection, when bacteria would reside in the lower respiratory tract, EVs can provide a mechanism for immune evasion during bacteremia, when pneumococci are constantly under attack by the complement system.

In paper II we provided insights on the possible use of pneumococcal MPs as future vaccine candidates. MPs were able to induce a serotype-independent protection in mice and elicited an adaptive immune response characterized by production of protective antibodies. Presence of many antigens targeted by these antibodies in MPs could be a successful characteristic for future vesicles-based vaccines to prevent IPD.

Paper III investigated how different pneumococcal serotypes use their capsule to gain advantages against the human immune system, and how those strategies also have drawbacks that could impact disease progression. Massive production of excess capsule guaranteed serotype 3 bacteria with a clever way to avoid complement deposition and phagocytosis by macrophages, but drastically impaired bacterial growth.

In paper IV we evaluated the immunomodulatory properties of the compound CBG derived from the Chinese medicine Chan-Su. While this molecule had general anti-inflammatory effects on DCs, it also induced the release of the pro-inflammatory cytokine IL-1β. Moreover, CBG directly induced the release of AMPs from neutrophils. Overall this compound has promising properties for future therapeutic approaches.

In conclusion, this thesis studied less known aspects of the interactions between pneumococci and the host and provided new insights on potential future preventive and therapeutic approaches to fight pneumococcal infections.

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