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Studies on pneumococcal polysaccharides and their effect on immune cells

by

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Karolinska Institutet

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To my family

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Abstract

Streptococcus pneumoniae is a major cause of morbidity and mortality in children and adults worldwide. The spectrum of infections caused by *Streptococcus pneumoniae* is well-characterized. The bacteria is transmitted via droplets/aerosols to the nasal cavity, from which it is spread locally to ears, sinuses, bronchi, lungs, blood (septicemia) and CNS (meningitis). In the perspective of increasing global antibiotic resistance and the reported shift of pneumococcal types post vaccination, alternative preventive actions might be warranted in the future. In order to prevent or cure pneumococcal infection, it is therefore important to investigate the adhesion capacity of the bacterium to cells/receptors and also to investigate the response of the immune system to *Streptococcus pneumoniae*.

To investigate *Streptococcus pneumoniae* binding to human cell receptors, an ELISA was developed where the binding to two previously proposed receptors could be compared in the same test system. The results showed that Streptococcus pneumoniae adhered to the human cell receptor glycolipid asilao-GM1, in which the specific binding site was characterized as the disaccharide GalNAcβ1-4Gal. In response to the results showing asialo-GM1 as a human cell receptor for *Streptococcus pneumoniae*, further investigation aimed to determine the pneumococcal structure that would adhere to this receptor. One of the pneumococcal surface structures is the cell wall polysaccharide, CWPS, which contains phosphoryl choline.

To investigate whether CWPS was the pneumococcal ligand binding to asialo-GM1, carbohydrate material was extracted from an uncapsulated strain of pneumococci. The carbohydrate material in the extract was separated and fractions binding to the pneumococcal glycolipid receptor asialo-GM1 were detected. In nuclear magnetic resonance spectroscopy (NMR) analysis, these fractions exhibited a pattern consistent with a reference spectrum of pure CWPS. The purified CWPS adhered to asialo-GM1 without protein involvement as it was unaffected by CWPS exposure to proteinase K. Streptococcus pneumoniae grown under conditions where choline was replaced with ethanolamine did not bind to the host cell receptor asialo-GM1. This indicated that CWPS, with intact phosphoryl choline residues, is the ligand responsible for binding pneumococci to the glycolipid receptor asialo-GM1.

Having determined CWPS as the responsible ligand to host cell receptor asialo-GM1, the immune response toward this structure deserved more detailed analyses. This was investigated in regard to CWPS activation of a subset of immune cells. Apart from CWPS, *Streptococcus pneumoniae* also expose other important polysaccharides surrounding the bacterium, i.e. the capsule. The composition of the capsule differs between different *Streptococcus pneumoniae* bacteria and hence classifies these into different types. Different sets of capsules are used in commercial available pneumococcal vaccines. The present study examined CWPS as well as some capsular polysaccharides included in the pneumococcal vaccines, for their individual capacity to activate immune cells. CWPS, three different capsular polysaccharides (types 3, 9 and 23) and LPS (positive control) were used for in vitro stimulation of whole blood. CWPS and the three capsules activated the immune cells differently (measured as CD69 expression). Generally, NK cells and NK-like T cells exhibited the strongest activation followed by monocytes and T cells.

Among the three capsules, capsule type 23 induced the strongest activation and cytokine release, followed by type 9 and type 3. In these experiments, CWPS induction was well in the range of what was seen from type 23.

CWPS is also a Toll Like Receptor (TLR) 2 ligand, and was investigated together with other TLR2 and TLR4 ligands for their capacity to induce gene expression and cytokine release from isolated monocytes and NK cells. Incubation of isolated peripheral blood monocytes with CWPS induced transcriptional upregulation and subsequent secretion of several major proinflammatory cytokines and chemokines, similar to the other TLR ligands investigated. CWPS as well as the other TLR ligands exhibited significant upregulation of CXCL8 expression in isolated NK cells.
With the results showing that CWPS is the ligand responsible for binding pneumococci to the host cell glycolipid receptor asialo-GM1 and also having confirmed that CWPS is an effective activator of immune cells, its impact in clinical settings (smokers) was investigated.

Cigarette smoking is a well-known and high risk factor for infections of *Streptococcus pneumoniae.* The study investigated whether the TLR ligands (including CWPS) would induce a different immune response in smokers compared to non-smokers. In these experiments no difference in TLR gene expression could be detected between smokers and non-smokers in unstimulated cells.
Following CWPS incubation with isolated monocytes, cells from smokers showed an increased upregulation of pro-inflammatory mediators as compared to non-smokers. Monocytes from non-smokers downregulated the immune regulatory molecules IL-10 and SOCS-1 after CWPS stimulation, while this was not found in smokers. The results suggest that the transcriptional activation of pro-inflammatory genes after TLR activation is dysregulated in smokers.

SAMMANFATTNING

Infektioner orsakade av pneumokocker är en vanlig orsak till sjuklighet och dödlighet runt om i världen. Enligt WHO inträffade ca 14,5 miljoner episoder av allvarlig pneumokocksjukdom år 2000. Bakterien sprids som droppsmitta från friska bärare, som är koloniserade med bakterien i näshålan, samt från personer sjuka i pneumokocksjukdom. Från näshålan sprids pneumokockbakterien vidare till olika delar av kroppens lokalisationer. Vanligast är spridning till öron, bihålor och lungor men bakterien kan också spridas vidare till blod och hjärnhinnor.
På grund av resistensutveckling mot antibiotika hos bakterier samt det faktum att tillgängliga vacciner inte skyddar mot alla sjukdomsframkallande pneumokocker, är det viktigt att undersöka bakteriens bindning och kolonisation på humana celler. Genom att studera dessa mekanismer samt deras påverkan på immunförsvarets reaktioner för att eliminera pneumokocker ökas kunskapen om hur man i framtiden kan bekämpa denna bakterie.

För att närmare undersöka pneumokockers bindning till mänskliga celler var målet i arbete I att utveckla ett testsystem för att påvisa pneumokockers bindning till två föreslagna bindningsmolekyler, s.k. receptorer, och därefter jämföra påvisad bindningskapacitet hos pneumokocker till respektive receptor. Ett test utvecklades där de två föreslagna pneumokockreceptorerna (asialo-GM1 och lactotriaocylceramid), samtidigt kunde analyseras i ett och samma testsystem, vilket inte varit möjligt tidigare. Resultaten visade att pneumokocker band till båda receptorerna men att bindningen till en av dem (asialo-GM1 receptorn) var starkare. Testet möjliggjorde även fördjupad utredning av vilken struktur på pneumokocken som band till asialo-GM1 receptorn.

I arbete II var syftet att utreda med vilken struktur pneumokockerna band till receptorn, asialo-GM1. Pneumokocker odlades och separerades i två olika faser, en proteinfas och en sackaridfas. I sackaridfasen separerades de olika ingående sackariderna från varandra och testades därefter för bindningskapacitet till asialo-GM1 i det tidigare nämnda testet. Genom detta förfarande isolerades den specifika sackarid som band till asialo-GM1 receptorn. Materialet analyserades därefter med nuclear magnetic resonance spectroscopy (NMR), en metod som undersöker egenskaperna hos organiska molekyler, vilken visade att det framrenade ämnet överensstämde med en cellväggs polysackarid (CWPS), som pneumokocker exponerar på sin yta. Sackariden är gemensam för alla pneumokocktyper. För att närmare undersöka vilken struktur i CWPS som band till receptorn, odlades bakterierna i olika definierade odlingsmedium, innehållande antingen cholin eller etanolamin som infogas i CWPS under bakterietillväxt. Pneumokocker som odlats i cholin-innehållande medium band fortfarande till receptorn, till skillnad mot de etanolaminodlade pneumokockerna som inte band. Cholin behövs alltså för att kunna binda till receptorn, och förmodas vara den struktur med vilken pneumokocker binder till asialo-GM1.

I arbete III var syftet att närmare undersöka immunsvaret mot CWPS samt mot tre olika kapselsackarider. Pneumokocker förekommer i 97 stycken så kallade kapseltyper. Vilka av dessa kapseltyper som orsakar sjukdom varierar över tid, geografisk region, ålder och övriga sjukdomar hos individen. Kapslarna från sjukdomsframkallande

pneumokocker ingår i pneumokockvaccin. Det ena tillgängliga vaccinet består av 23 olika kapselpolysackarider och det andra vaccinet består av 7, 10 eller 13 olika kapselsackarider kopplade till ett protein (konjugerat vaccin). Vaccin som består av endast kapselsackarider ger ett rent B-cellssvar, dvs produktion av skyddande antikroppar. Det proteininnehållande, konjugerade vaccinet, ger förutom ett B-cellsvar även ett T-cellsvar, som förstärker B-cellssvaret samt genererar T-minnesceller.

I arbete III undersöktes aktiveringen av olika immunceller genom deras utsöndring av cytokiner (små proteiner som påverkar andra celler) efter exponering av CWPS och tre olika pneumokockkapslar, samtliga ingående i pneumokockvaccinet. Stimuleringen och mätningarna gjordes i blod från friska individer.
Både CWPS och kapslarna aktiverade immuncellerna, men i olika grad. Den högsta aktiveringsgraden uppvisade NK-celler och NK-liknande T-celler, följt av monocyter. CWPS aktiverade i regel cellerna i högre grad än vad kapslarna gjorde, undantaget monocyter. Kapslarna skiljde sig åt från varandra avseende förmåga att aktivera immunsvaret. Kapseltyp 23 aktiverade immuncellerna starkast, därefter följde kapseltyp 9 och lägst aktiveringsgrad uppvisade kapseltyp 3. Den uppmätta cytokinfrisättningen följde samma mönster som cellaktiveringen för kapslarna. Studien hjälper till att öka förståelsen för effektvariationer för pneumokockvaccin-komponenter och kan därför bidra till utveckling av förbättrade vacciner mot pneumokocker.

På flera celltyper i immunförvaret, såsom makrofager, dendritiska celler, neutrofiler, T- och B-celler finns Toll-lika receptorer (TLR) som bland annat känner igen CWPS hos pneumokocker. Bindningen till TLR initierar en kaskad av intracellulära signaler som resulterar i produktion av proinflammatoriska cytokiner. I arbete IV undersöktes genuttryck och även sekretion av proinflammatoriska mediatorer i isolerade monocyter och NK-celler, efter stimulering med substanser som aktiverar TLR (såsom CWPS, Pam3CSK4 och LPS). Studien visade att CWPS från *S. pneumoniae* ökar genuttryck för inflammatoriska mediatorer i isolerade humana monocyter och i isolerade NK-celler. Det uppmätta genuttrycket för de inflammatoriska mediatorerna var mer uttalat i monocyter från rökare. I monocyter från icke rökare uppmättes en nedreglering av immunreglerande molekyler, efter CWPS stimulering, vilket inte var fallet hos rökare. Denna studie visar också att cigarettrök påverkar immunförsvaret hos friska rökare med normal lungfunktion.

List of Abbreviations

CBA	Cytometric bead array
CbpA	Choline binding protein A
CD	Cluster if differentiation
CDC	Centers for Disease Control and prevention
COPD	Chronic Obstructive Pulmonary lung disease
CRP	C reactive protein
CWPS	Cell Wall Polysaccharide
DAMP	Danger associated molecular patterns
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immunosorbent assay
FACS	Fluorescence activated cell sorter
FEV1	Forced Expiratory Volume during one second
FITC	Fluorescein isothiocyanate
FSC	Forward scatter (cell size)
IPD	Invasive pulmonary disease
Ig	Immunoglobulin
IL	Interleukin
LPS	Lipopolysaccharide
LR	Laminin Receptor
LTA	Lipoteichoic acid
mAB	Mono clonal antibody
MFI	Median, or mean fluorescence intensity
MHC	Major histocompability complex
NET	Neutrophil extracellular traps
NK-cell	Natural killer cell
NKT	Natural killer T cell
NOD	Nucleotide- binding oligomerization domain
NLR	NOD like receptors
NMR	Nuclear magnetic resonance
OD	Optical Density
PBMC	Peripheral blood mononuclear cells
PCho	Phorohoryl choline
PCR	Polymerase Chain Reaction
PCV	Pneumococcal conjugate vaccine
PG	Peptidoglycan
PPV	Pneumococcal polysaccharide vaccine
PRR	Pattern recognition receptors
PAF	Platelet activating factor

PAMP	Pathogen associated molecular patterns
PIgR	Polymeric immunoglobulin receptor
PsrP	Pneumococcal serine-rich repeat protein
RIG	Retinoc acid-inducer
RLR	RIG-like-1 receptor
RNA	Ribonukleinsyra
SOCS	Suppressor of cytokine signaling
SSC	Side scatter (cell granularity)
STAT	Signal transducer and activator of transcription
TA	Teichoic acid
Th cell	T helper cell
TNF	Tumor necrosis factor
TLR	Toll like receptor
WHO	World Health Organisation

List of Publications

The present thesis is based on the following articles:

I. Marianne Sundberg-Kövamees, Tord Holme, AnnMargret Sjögren.
Specific binding of Streptococcus pneumoniae to two receptor saccharide structures.
Microbial Pathogenesis 1994; 17:63-68

II. Marianne Sundberg-Kövamees, Tord Holme, AnnMargret Sjögren.
Interaction of the C-polysaccharide of Streptococcus pneumoniae with the receptor asialo-GM1.
Microbial Pathogenesis 1996; 21: 223–234

III. Marianne Sundberg-Kövamees, Johan Grunewald, Jan Wahlström.
Immune cell activation and cytokine release after stimulation of whole blood with pneumococcal C-polysaccharide and capsular polysaccharides.
International Journal of Infectious Diseases 2016; 52: 1-8

IV. Johan Öckinger, Marianne Sundberg-Kövamees, Michael Hagermann-Jensen, Nik Kruisbergen, Muhammadd Hamza Bokhari, Johan Grunewald, Jan Wahlström.
Increased expression of inflammatory mediators in monocytes from smokers, after stimulation with cell wall polysaccharide from *Streptococcus pneumoniae* and other Toll Like Receptor ligands.
Manuscript

INTRODUCTION AND BACKGROUND

History

One-hundred and thirty-six years ago, in the year of 1881, *Streptococcus pneumoniae* was first isolated and described separately by two scientists, Pasteur in France and Sternberg in the United States. Pasteur isolated pneumococci and described what today is known as the capsule (1) while Sternberg described a micrococcus, 0,5µm in diameter, joined in pairs in the blood of infected rabbits (1). Soon afterwards, Friedländer, Fraenkel and Weichselbaum described the association of pneumococcus with pneumococcal disease (1). Seven years later Klemperers demonstrated a protective effect of antiserum against pneumococcal disease.

In the beginning of year 1900, Neufeld described the technique for typing of the pneumococcal capsule, which was named "the quelling reaction". This method is still used to distinguish between the different capsular types of pneumococci. However, the method was not used for identification of human pneumococcal capsules until 1931(1). In the 1920´s, Avery and Morgan concluded that the specific soluble substance of *S. pneumococcus* is a polysaccharide (1). Exploring that finding in 1945, a tetravalent vaccine composed of four different pneumococcal capsular polysaccharides was used in experiments for human immunization and subsequently prevented type-specific pneumococcal pneumonia (2).
In 1928, seventeen years before this proven protective effect of immunization against pneumococcal disease, penicillin was discovered by Alexander Fleming. Penicillin has been of the greatest importance for the treatment of pneumococcal disease. Together with Florey and Chain, Fleming was awarded the Nobel Prize in 1945, for "the discovery of penicillin and its curative effect in various infectious diseases" (3). From having been a true "wonder-drug", penicillin lately has been somewhat hampered by the increasing resistance in pneumococci and other bacteria.

In the same year as the discovery of penicillin, the discovery of the natural transformation capacity in pneumococci was made by Griffith. In these experiments, mice were injected subcutaneously with a virulent capsulated pneumococcal strain type 3 that was heat killed, together with a living, non-virulent uncapsulated pneumococcal strain type 2. The combination of the two strains resulted in a virulent type 3 strain. This indicated that virulence could be transformed between the bacterial strains (4). Eventually, in 1944 Avery discovered that the transforming material responsible for the results in Griffths experiment was indeed nucleic acid (5).

Epidemiology

Infection due to *Streptococcus pneumoniae* is a well-known major cause of morbidity and mortality worldwide. In 2015, it was estimated that 1.5 million people worldwide died from pneumococcal pneumonia (6). Pneumococci are thought to be transmitted via droplets/aerosols, mostly from persons already colonized in the nasopharynx, healthy carriers, or by direct contact with patients suffering from pneumococcal disease (Figure 1). Among the healthy carriers, children are the most frequently infected with a peak incidence at the age of 2-3 years. Thereafter the colonization in the nasopharynx is reduced to < 10% in adults

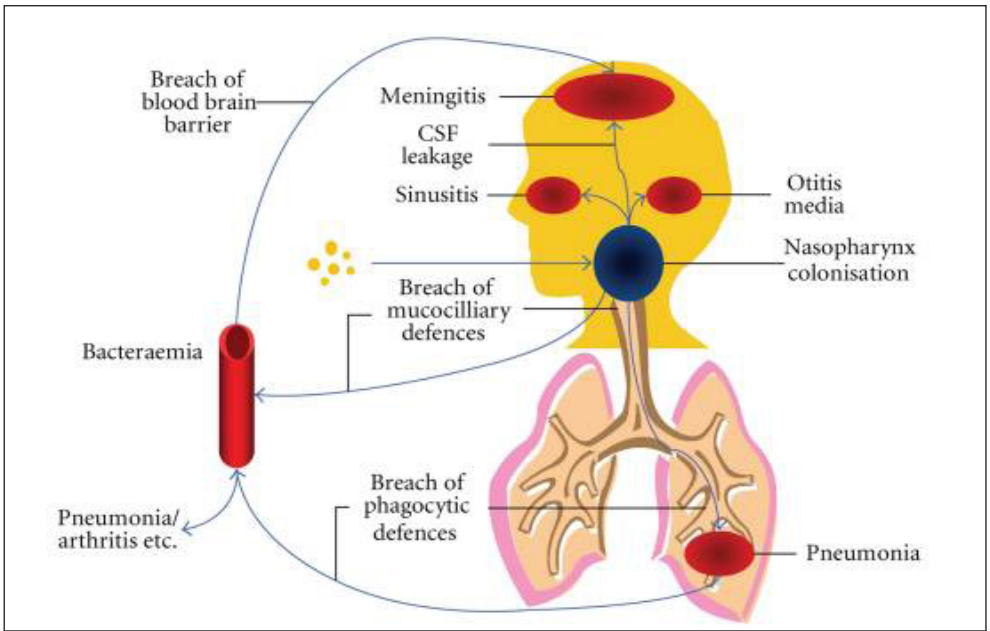

Figure 1. Progression of pneumococcal disease from the nasopharynx to sinuses, ears, lungs, blood stream and to the central nervous system (CNS). Modified from U. R. Goonetilleke et al., 2009, Interdisciplinary Perspectives on Infectious Diseases (108).

without children/close contact with children (7). Initially the bacteria enter the nasal cavity, where colonization in the nasopharyngeal epithelium takes place. From the primary infection site, the bacteria can then spread locally to bronchi, lungs, ears or sinuses. In addition, if bacteria cross the mucosal barrier, they enter the bloodstream resulting in bacteremia. Once established in the bloodstream, distal infections may occur and upon passage of the blood-brain barrier, the bacteria may cause meningitis (7). The specific pneumococcal serotypes, described below, differ between geographical region, age, disease syndrome/severity and also over time (8). In general, young children and elderly, as well as immunocompromised patients, have a high risk for being infected. However, also individuals with a recent history of influenza virus infections and patients with comorbidities, such as chronic lung disease, heart disease, malignancies and diabetes have a higher incidence of pneumococcal disease (9).

Microbiology

As indicated by its name and mentioned above, *Streptococcus pneumoniae* is a main agent for pneumonia, predominately of the lobar type. *Streptococcus pneumoniae* is a gram positive coccus. The bacteria most often grow in pairs, as the diplococci described by Sternberg in 1881, but may also occur in short chains or even as single cells. The individual size of the cells are between 0,5 µm and 1,25 µm in diameter. The bacteria are capable of growing in oxygen-rich as well as in oxygen-poor environments. Pneumococci are conveniently identified by alpha-hemolysis on blood-agar plates, or by optochin sensitivity. During alpha-hemolysis, the pneumococcal enzyme pneumolysin degrades hemoglobin in

the blood-agar, converting it into a green pigment, which can be easily spotted around the bacterial colonies. Optochin (ethylhydrocupreine hydrochloride) on the other hand, inhibits the growth of pneumococci in vitro and hence is used in the diagnostics of *Streptococcus pneumoniae* infection. Pneumococci also lack the enzyme catalase and are soluble in bile salts and by deoxycholate (10), exposing other possible ways to identify this specific agent. The bacteria are surrounded by a polysaccharide capsule. Each pneumococcal strain can be identified and typed by the swelling of the capsule upon its binding to homologous antibodies, the Quellung reaction (swelling reaction) (10). In cases of non-typeable pneumococcai, an additional possibility of identification is molecular diagnostics, which include detection of certain pneumococcal specific genes, such as autolysin (10).

Capsule

The pneumococcal cell surface is covered with capsular polysaccharides. Until today 97 different capsular serotypes have been described, exhibiting variations in their sugar composition and linkage (11, 12). The capsule is considered to be the major virulence factor of the *Streptococcus pneumoniae*. Clinical isolates causing invasive disease are all capsulated, loss of capsule will dramatically reduce virulence (8, 14). The capsule exerts important functions such as acting as a steric hindrance and preventing phagocytosis (7, 13). In a mouse model, Avery et al. demonstrated the importance of capsular virulence when this was reduced, after enzymatic digestion of the serotype 3 capsule (14). The virulence differs between pneumococcal strains depending on the capsular serotype (15, 16). The pneumococcal vaccines are based on different capsular pneumococcal polysaccharides.
Pneumococcal colonies can spontaneously undergo a phase variation from an opaque state, suitable for survival in blood, to become transparent, adapted for colonization in the nasopharynx (17). The increased virulence of opaque pneumococci is associated with increased expression of capsular polysaccharide and decreased expression of teichoic acids compared with the transparent phenotype (18). Serospecific antibodies are protective against respective capsule (19). In the pneumococcal chromosome, the genes coding for the biosynthesis and expression of capsules are closely linked (20).

Cell Wall

The cell wall of *Streptococcus pneumoniae* consists of an outer layer built up by peptidoglycan, teichoic acid and lipoteichoic acid (21) surrounding an inner layer consisting of a double phospholipid membrane. The peptidoglycan (PG) is associated with both teichoic acid (TA) and lipoteichoic acid (LTA). LTA and TA are chemically identical compounds, but differs in their attachment to the cell wall (21, 22) (Figure 2). LTA is anchored by its lipo part located into the cytoplasmic membrane, while TA is covalently bound to PG. Both TA and LTA contain phosphorylcholine (PCho), which is the binding site for choline binding proteins (23). PCho also adheres to platelet-activating factor receptor (PAFr) on host cells (24) and is also recognized by C-reactive protein (CRP), a protein in the immune system, capable of activating complement (25). CRP originally received its name due to the reactivity with pneumococcal teichoic acid, or C-polysaccharide, as pneumococcal teichoic acid was previously named (26). Cell wall polysaccharide (CWPS) is also known as a teichoic acid. Based on genomic analyzes, the surface proteins in the pneumococcal cell wall can be divided into one of three different groups; choline binding proteins, that are non-covalently

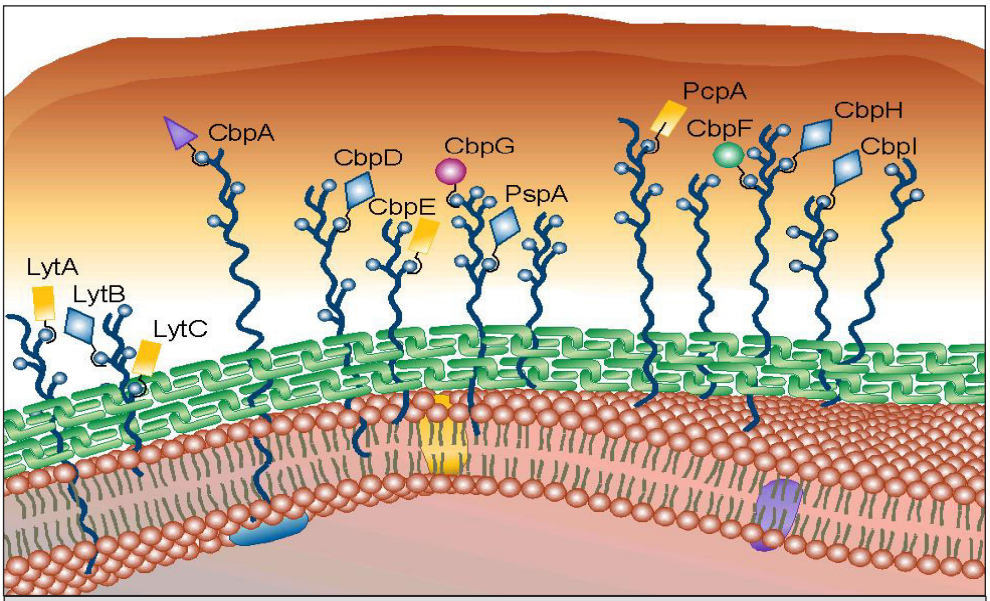

Figure 2. This stylized representation of the pneumococcal cell wall shows the 12 identified choline binding proteins (Cbps) bound via a conserved binding domain to the choline component of teichoic acid or lipoteichoic acid (blue circles on dark blue structures) which are in turn anchored to either peptidoglycan (green) or the plasma membrane. Modified from Jonathan A. McCullers et al., 2001, Frontiers in Bioscience (109).

linked to the cell wall, lipoproteins anchored in the cell wall peptidoglycan and proteins with a LP X TG motif covalently bound and anchored in the cell wall (21). Purified cell wall components and whole pneumococci result in the same inflammatory response (27). Cell walls induce inflammation by white blood cell activation and cytokine release (28).

Immune response

The human body has different ways of defending itself against microbes, these include physical barriers such as skin and mucous as well as protection of airways by the cough mechanism and sneeze reflexes which perform a mechanical clearance of the mucus, produced by epithelial cells in the respiratory tract. Microbes caught in this secretion are also transported away by cilia.

If the microbe succeeds to penetrate the physical barrier, it encounters the immune cells and molecules that are always present in tissues. The innate immune defense is comparatively unspecific, while the adaptive immune defense is highly specific and effective. Although more efficient, it is also slower, since T- and B-cells must undergo clonal expansion and differentiation after activation by the current microbe. The adaptive defense has a long-lasting memory, thereby often protecting from reinfections caused by the same microbe.

In the bone marrow, hematopoietic stem cell develops into either lymphoid stem cells which mature into lymphocytes or into myeloid stem cells, which in turn develop further

into monocytes, granulocytes, mast cells, red blood cells and platelets. Monocytes may further develop into macrophages and dendritic cells. The various white blood cells, monocytes, macrophages, lymphocytes and granulocytes as well as mast cells and dendritic cells together build up the cellular part of our immune system.

Cell types in the innate immune system

Monocytes, macrophages, dendritic cells
Monocytes develop in bone marrow and afterwards migrate into the blood and further into different tissues where they differentiate into macrophages or dendritic cells. These cells have antigen presenting molecules on their surfaces, the major histocompatibility complex (MHC) molecules. Most cell types in the human body express MHC class I, while MHC class II molecules are present on antigen presenting cells such as the previously described macrophages and dendritic cells. Macrophages and dendritic cells are found in all tissues of the human body. After phagocytosis of foreign substances by macrophages and dendritic cells, they are transported to lymphoid organs, where peptides derived from the foreign substances (antigens) are presented to T-helper cells. Monocytes, macrophages, and dendritic cells are all important producers of cytokines and initiates phagocytosis.

Granulocytes
Granulocytes are divided into three different subgroups. These are; neutrophilic, eosinophilic and basophilic granulocytes. In the blood, the neutrophilic granulocytes are the most common. These granulocytes have a phagocytic function and are short lived. They have the capacity to release cytokines and to start inflammation. Granulocytes are stored in large quantities in the bone marrow and may rapidly be released to the blood when needed in response to various stimuli.

NK cells
NK-cells also develop in the bone marrow. They have both activating and inhibitory receptors and capacity to secrete various cytokines. The NK-cell receptors can identify infected or abnormal cells and enable the NK-cells to quickly kill them by secretion of cytotoxic substances. They also have the ability to secrete cytokines that stimulate both the innate and the adaptive defense system via dendritic cells and Th1 cells.

Complement system
The complement system consists of more than 30 circulating or cell-bound proteins produced in the liver. When in contact with microbes these proteins trigger a cascade of reactions which help to defend the body (29). The main functions of the complement system are opsonization to promote phagocytosis and activation of neutrophil chemotaxis, eventually killing the microbe. Complement activation is a multistage process where the most important step is the cleavage of factor C3 into C3a and C3b. C3b can then opsonize and facilitate phagocytosis. The complement system may be activated by three different mechanisms. The classical complement activation pathway occurs by the recognition of an antigen-antibody complex or the complex between the antigen and non-antibody acute phase protein, for example CRP. The alternative activation pathway initiates the degradation of C3 to C3b, on the cell surface of microbes. Finally, the lectin activation pathway is initiated when lectin recognize carbohydrates bound to mannose on the microbe (29).

Cytokines and Chemokines
Cytokines are small proteins, mostly glycoproteins, with highly specific activity. They are produced by different cells in the immune system. They act as communicators between the immune cells by secretion and then binding to receptors on the immune cell membrane. This binding then initiates intracellular signals, which may have both activating and inhibitory effects. As a result, a certain cytokine may have several different effects and also different cytokines may exert the same effect. Cytokines are involved in the innate as well as the adaptive immune system. Pro-inflammatory cytokines are secreted by macrophages, dendritic cells, mast cells and granulocytes. Adaptive defense cytokines are secreted by lymphocytes, especially T-helper cells. Cytokines can affect the own cell (autocrine), the closest cells (paracrine) or cells far away from the producing cell (endocrine).
Chemokines are a distinct group of small signal proteins affecting migration of cells. They stimulate leukocyte movement and direction of migration.

Pattern Recognition Receptors, PRR
The cells in the innate immune system have receptors called pattern recognition receptor (PRR). They recognize specific molecules called pathogen associated molecule patterns, PAMPs, on microorganisms. Cells from humans or other mammals do not have these molecule patterns. There are three different groups of PRR; membrane-bound Toll-like receptors (TLRs), intracellular retinoic acid-inducer (RIG)-1-like receptors (RLRs), and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs).
TLRs are the most studied PRRs. LPS, found in gram-negative bacterial cell wall, is the most studied PAMP. C-reactive protein (CRP) is an example of PRR in soluble form.

Toll-like receptors
TLRs recognize PAMPs from bacteria, parasites, fungi and viruses. The binding initiates a cascade of intracellular signaling, which results in production of proinflammatory cytokines and interferons. The intracellular signaling cascade is negatively regulated. One of the main regulators is suppressor of cytokine signaling-1, SOCS-1. In humans there are 10 known TLRs found on several cell types, such as macrophages, dendritic cells, neutrophils, T and B cells but also on epithelial cells and fibroblasts. TLR2 recognizes pneumococcal lipoteichoic acid (LTA) (29), bacterial lipopeptides, yeast ligands, parasitic and viral proteins (30). TLR4 is known as the LPS receptor but it also recognizes pneumolysin, a cytotoxin of pneumococci, as well as host heat shock proteins, fibrinogen and certain viral proteins. Pneumococcal DNA, like other bacterial DNA of the so-called CpG type, is recognized by TLR9.

NOD like receptors
NOD like receptors (NLRs) are a family consisting of more than 20 cytoplasmic receptors that are activated by both intracellular and extracellular pathogens. NLRs initiate signaling that promotes an immunological response (31). mRNA upregulation of NOD1 and NOD2 expression is seen after pneumococcal infection in mouse lung tissue and bronchial epithelial cell line (32). NOD2, in turn, recognizes peptidoglycan fragments from pneumococcai digested by lysosome in mice (33).

Cells in the adaptive immunity
The cells responsible for the adaptive immune system are B-lymphocytes and T-lymphocytes. Antigens are identified by B-cell membrane-bound antibodies and T-cell receptors. Each lymphocyte recognizes only one specific antigen. The B-cell membrane-bound antibodies (B-cell receptors) recognize parts of proteins, lipids, carbohydrates, nucleic acids and chemical groups. The T-cell receptors (TCRs) recognize peptides presented by MHC class I or class II molecules on antigen-presenting cells.

The adaptive immune response is activated and develops when it is exposed to antigen and the immunity created depends on the agents for which it is exposed. Although slower, the adaptive immunity is more specific and more powerful when exposed to the same antigen, compared to the innate immunity. The adaptive immunity also generates a valuable memory for antigen elimination. Antigens can be divided into eliciting T-cells dependent response, or T-cells independent response. Capsule polysaccharides generate an independent response (34). MHC II on antigen presenting cells are incapable to present polysaccharides, and hence no specific T-cell depending response is elicited. Antigens causing an independent response cannot conduct isotype switch for production of B cells with specific affinity and memory.

Lymphocytes
There are three different groups of lymphocytes; T cells, Natural Killer cells (NK cells) and B cells. The T cells develop in the bone marrow and then migrate to thymus where full maturity and function are reached. Here, most autoreactive T cells (i.e. reactive to substances in the body) are eliminated. Each T cell has a T cell receptor that recognizes only one specific antigen. In the body there are (before specific antigen encounter) only a few T cells bearing any particular, specific T cell receptor but the total population of T cells contains an enormous diversity of T cell receptors. In order for the T cell to differentiate and proliferate (undergo clonal expansion), the antigen needs to be presented together with an antigen presenting molecule, MHC. T cells are divided into subspecialized groups such as cytotoxic T cells / killer cells ($CD8^{pos}$), T helper cells ($CD4^{pos}$) and Regulatory T cells, T-reg cells.

T helper (Th) cells have the $CD4^{pos}$ receptor on their surface and bind to MHC class II on antigen presenting cells. Upon binding, they release cytokines and chemokines that activate and recruit other immune cells, such as macrophages, B lymphocytes, cytotoxic T lymphocytes ($CD8^{pos}$). The mature Th cells can be divided into different subclasses, Th1, Th2 and Th17. They have different cytokine profiles, thus affecting the immune system in different ways (35). The cytotoxic T cells (Tc) express the $CD8^{pos}$ receptor on their surface. The T Cell Receptor (TCR) of $CD8^{pos}$ cells bind to MHC class I on host cells infected with microbes. Upon binding, Tc secrete lytic proteins, after which the host cells are lysed. There are also regulatory T cells, T-reg cells, which are capable of inhibiting some immune responses.

B cells
The B cell is predetermined to produce one type of antibody for a specific antigen. Upon activation, the B cells increase in number, all with the same type of antibody production capacity. Some of the cells produce antibodies while others become memory cells for later

use. Antibodies are proteins that prevent the antigen from binding to its target cell by blocking or neutralization. B cells may also function as an antigen presenting cell to activate Th cells by presenting antigens on MHC class II. Some antigens composed of large molecules of repetitive carbohydrates, such as polysaccharide capsules from pneumococci, can activate T cell-independent B cells in the spleen, providing a rapid antibody response to such bloodborne pathogens.

Antibodies

There are several antibody isotypes; IgM, IgA, IgG, IgE and IgD. The different isotypes have different functions, eg neutralization, opsonization or complement binding. The B cells first express IgM and IgD but may after further activation switch to produce IgG, IgA or IgE with the exact same antigen specificity. Upon repeated exposure to the same agent, IgG (or IgA, IgE) response arise much faster and with early high affinity due to the memory B-cells. Most B-memory cells produce IgG antibodies. IgM consists of five antibodies linked together. IgM and IgG both have a binding site for complement factor C1. IgG exists in the largest amount of all antibodies and is able to pass over the placenta. In humans there are four IgG subclasses, IgG1-4. IgG1 and IgG3 activate complement and constitute antibodies to protein antigens, and IgG2 against polysaccharide antigens. Decreased amount of IgG2 subclass may provide poorer defense against encapsulated bacteria such as pneumococci. IgA is present in monomeric form in the serum and on various mucous membranes as secretory IgA and can be transferred to breast milk. IgA blocks the binding of bacteria, viruses and toxins in the mucous membranes. IgE is found in mast cells and connected to allergic and anaphylactic reactions. IgD predominantly exists in membrane-bound form but may be present in small amounts in serum. Its function is still unclear.

Pneumococcal immunity

The classical host defense mechanism against pneumococci is phagocytosis of bacteria opsonized by complement or antibodies. The classical pathway is the most important portion of the complement system in the response to pneumococci. The C-reactive protein, CRP, a soluble PRR, also activates complement by binding to the cell wall polysaccharide, CWPS (36). Several TLRs of host cells recognize pneumococcal PAMPs, and initiate an immunologic response. TLRs recognize different parts of pneumococci, e.g. TLR2 recognizes bacterial LTA, TLR 4 recognizes pneumolysin and TLR9 recognize pneumococcal DNA (29).

B-cells are important in the defense against pneumococci in producing anti-capsular antibodies. Antibodies against CWPS are thought to mediate protection against invasive disease (37, 38). Certain IL-17 producing T cells are protective against extracellular bacteria such as *Streptococcus pneumoniae* (39). The anti-pneumococcal defense involves a variety of cytokines, IL-6, IL-12, IL-17, IL-18, and TNF and IL-1 (29). NLRs are intracellular PRR of the host cell and one of the NRLs, NOD2, recognizes small fragments from pneumococcal peptidoglycan (31).

Pneumococci can escape the immune system in several ways. The capsule acts as steric hindrance and prevents phagocytosis. The capsule also prevents binding of both IgG and CRP to *S. pneumoniae* and thereby inhibits the classical complement activation pathway (13). Surface proteins that help to avoid the immune system are PspA, which reduce complement binding to the pneumococcus (40) and CpbA that counteracts complement (41). Pneumo-

cocci can also cause repulsion of neutrophil extracellular traps (NETs) by a positive charge on the surface of the bacterium due to the composition of LTA (42).

Prevention

Pneumococcal vaccines

Already back in 1914, initial experiments were performed regarding a pneumococcal vaccine containing whole killed bacteria (43). In 1945 MacLeod showed that type specific infections in humans could be prevented with a type-specific 4-valent pneumococcal polysaccharide vaccine (2). However, it was not until the 1970s that pneumococcal polysaccharide vaccine was licensed. In 1983 a vaccine containing 23 pneumococcal capsular polysaccharides (PPV 23) was introduced containing the most frequently occurring serotypes of invasive pneumococcal disease (IPD). The vaccine has also been shown to contain CWPS (44). The elicited immunological response to vaccination depends on the age of the patient and varies between different serotypes. PPV is a T cells independent antigen that generates B cells that activate and provide the antibody response. PPV elicits a weak immune response in children under the age of 2 years. Since 2000, there is also a conjugated pneumococcal vaccine (PCV) where the capsular saccharides are linked to a carrier protein, diphtheria carrier protein CRM. PCV contains T cell-dependent antigens that provides both B-cell activation and antibodies and a T-cell response, including T-cell memory. The first PCV was protective against 7 pneumococcal serotypes (4, 6B, 9V, 14, 18C, 19F, 23F). Since 2015, PCV7 has been replaced by PCV10 (PCV7 + 1, 3, 7F) or PCV13 (PCV10 + 19A, 6A, 3). Compared to PPV, PCV is more immunogenic and provides more effective protection against infection, even for children under 2 years of age. PCV effectively reduces the frequency of invasive pneumoccocal disease for the serotypes included in the vaccine (9, 45). The vaccine protects both against systemic and mucosal infection and prevents colonization in the nasopharynx. This also prevents dissemination in society. Between 2007 and 2009, the conjugated vaccine (PCV) was introduced in the Swedish childhood vaccination program. After vaccination with PCV reports have shown a change in carriage and IPD of pneumoccocal serotypes to types not included in the vaccine. This was observed in both the vaccinated and the non-vaccinated population (9).

Objective and Aims

- To develop an ELISA adhesion test, in order to investigate suggested host cell receptor molecules for their specific binding to *S pneumoniae*, and to compare the adhesive ability of the various receptors.

- To identify and characterize the pneumococcal ligand responsible for binding to host cell asialo-GM1, using a non-capsular pneumococcal strain.

- To investigate cell wall polysaccharide (CWPS) and individual capsular polysaccharides from *S pneumoniae* for their ability to activate immune cell subsets from healthy controls and to quantify cytokine levels after stimulation.

- To investigate gene expression and secretion of inflammatory mediators in isolated monocytes and NK cells from healthy individuals after stimulation with three different TLR ligands, including CWPS, and comparing smokers with non-smokers.

METHODS

Bacterial strains and culture conditions (Articles I and II)
The different pneumococcal strains used in Article 1 and 2 were CCSR-SCS-2 clone 1 (type 12) (46) (Article I), R36A (type 2 mutant without capsule) (Articles I and II), CCUG 6605 (type 19) (Article I) and CCUG 2987 (type 23) (Article I). The bacteria in Article I were grown on blood agar plates at 37º C overnight, and then diluted in buffer (TRIS buffered saline or anti hydrophobic PSM buffer) to OD 1.0 at 600 nm. In Article II, R36A grown on blood agar plates was inoculated into liquid brain heart infusion BHI, with 5% fetal calf serum or into a defined medium (47). The defined medium was further divided into two different variants where one contained phosphorylcholine, while the other version of the defined medium contained ethanolamine instead of phosphorylcholine. In order to secure that ethanolamine had been properly incorporated, the bacteria were cultured in this phosphorylcholine-free medium for four rounds. Prior to the experiments, all bacteria were cultured to OD 0.8 at 600 nm. After growth in liquid medium, the bacteria were washed in PBS and diluted in the test buffer.

Enzyme-linked immunosorbent assay, ELISA (Articles I and II).
Microtiter plates were coated with the receptors, asialo-GM1 (Articles I and II) and lacto-triaocylceramide (Article I) in concentrations ranging from 1 ng/ml to 10 µg/ml (Article I) and 10 µg/ml (Article II) in 100 µl methanol. The methanol was evaporated at 37º C for 18 h at room temperature (RT). The plates were then coated with 1% BSA in TBE for 1 h in RT. The bacteria, diluted in 1% BSA in TBE or in antihydrophobic PSM buffer, were then added to the microtiter plates in 1:2 dilutions spanning from OD 1.0 to 0.003 (Article I), and to OD 0.8 (Article II) at 600nm. In Article II, bacteria, purified saccharide and heat extracted soluble bacterial substances were also tested for binding to the receptor asialo-GM1. The saccharide material and bacteria were diluted in antihydrophobic solution. The heat extract was diluted in TBS. Bacteria, saccharide material and heat extract were added to coated wells (described above) and incubated for 1 h at 37º C. As negative controls, wells without receptor (Articles I and II) and wells coated with E. coli LPS (Article I) were used.

Detection of bound bacteria (Articles I and II), saccharide material (Article II) and heat extract (Article II) was performed either using a monoclonal mouse anti-phosphorylcholine antibody and subsequently a rabbit anti-mouse antibody or a polyclonal rabbit anti-pneumococcal serum (46). In both cases an alkaline phosphate conjugated swine anti-rabbit antibody was used. Analyses were done in spectrophotometer at 405 nm, after addition of phosphate substrate for 30 minutes.

Preparation of cell wall polysaccharide (Article II)
In the preparation of cell wall polysaccharide (CWPS), the bacteria were cultured to OD 1.0 at 600 nm in BHI supplemented with the addition of 5% FCS and 1% glucose. Bacteria were then washed in 0.9% NaCl before hot phenol-water extraction, which separates the saccharides and the proteins. The aqueous phase was dialyzed against running tap water to remove any phenol residues before freeze-drying. The saccharides were purified by gel permeation chromatography. The lyophilized material was dissolved in distilled water containing 1% butanol and run on a 100 ml Sephadex G-100 column with 0.5 ml saccharide

(10 mg / ml). From the column, 2.5 ml fractions were collected and every second fraction was tested in Elisa for binding to asialo-GM1. The procedure was repeated 16 times. The material from the fractions that bound to asialo-GM1 with an absorption > 0.2 at 405 nm, were pooled and freeze dried.

Extraction of soluble substances from whole bacteria (Article II)
To investigate the immunological properties of soluble substance from whole bacteria, bacteria were grown in medium to OD 1.0, at 600 nm. After washing in PBS, the bacteria were diluted to OD 1.0 at 600 nm and incubated in water bath at 65° C for 40 minutes. Following centrifugation, the supernatant was stored in 1/10 of concentrated polyethylene glycol 20 000.

Dot blot (Article II)
The purified CWPS preparation (described above) was examined using Dot blot. After saccharides were applied to the nitrocellulose filters, the filters were blocked with TRIS buffered saline containing 0.05% Tween-20, in TTBS for 1h. Then, after initial incubation with a monoclonal antibody to the repeating part of the C-polysaccharide (46), sequential incubations with a rabbit anti-mouse antibody and by an HPR-conjugated goat anti-rabbit antibody followed. The filters were then developed with the substrate (4-chloro-1-naphtol) and the reaction was stopped by applying tap water. Between each of the described steps the filters were washed in TTBS.

Treatment of purified saccharide with protease and dissociating agents (Article II)
To eliminate proteins from the purified saccharide preparation and also from the heat extract, 0.5 mg/ml saccharide and heat extract were treated with protease K (50 µg/ml) for 1 hour at 20° C. The reaction was stopped by boiling for 4 minutes. The material was dialyzed in PBS at 4° C overnight. The protease K treated and dialyzed material was examined in ELISA for binding to asialo-GM1 as well as on SDS-page and in Western blot. The lyophilized saccharide material (0.5 mg/ml) was also treated with either 1M urea for 30 minutes or exposed to 6M guanidine-HCl for 15 minutes at 20° C. The material was finally dialyzed in PBS at 4° C.

SDS gel electrophoresis and Western blot (Article II)
Bacteria, heat extract and purified pneumococcal saccharide material were also investigated in 12% SDS gel, for 45 minutes (100V, 54mA). The separated material in the gel was then transferred to nitrocellulose paper at 100V, 250mA for 30 minutes after which the filter was blocked for 2 hours in TTBS. To detect the material on the nitrocellulose paper, a rabbit anti-pneumococcal antibody or a mouse anti-phosphoryl choline monoclonal antibody was applied for 1 hour. For detection of the mouse monoclonal antibody, an additional antibody, a rabbit anti-mouse IgG, was used. Finally, the nitrocellulose filters were incubated with an HRP conjugated goat anti-rabbit IgG. For development, substrate 4-chloro-1-naphtol was added and the reaction was eventually stopped with tap water.

Subjects and blood samples (Article III and IV)
In Article III, healthy adults, non-pneumococcal vaccinated subjects aged 42 to 59 were enrolled. Venous blood was obtained in heparinized test tubes. 500 µl of whole blood was added to 12-well microtiter plates.

In Article IV (manuscript), 21 healthy non-smokers and smokers were recruited. Non-smokers had smoked less than 100 cigarettes in total and not smoked at all for the last 12 months. The smokers that were included had all a smoking consumption equal to or more than 5 "pack years", ie. the number of smoked cigarettes per day/20 x number of years. In Article IV, all subjects were examined by dynamic spirometry. At the time of sampling no subject had airway infection or allergy. Every subject donated 3x8 ml of venous blood. PBMC (peripheral blood mononuclear cells) were isolated by Ficoll-Paque separation.

Monocytes and NK cells were then isolated by negative selection using Pan monocyte and NK cell isolation kit prior to an auto MACS pro separator. There was no difference in the amount of cells obtained from smokers and nonsmokers. Respective cell type was put into a 96-well microtiter plates with the volume of 200 µl/well at a concentration of 200 000 cells/well.

Stimulation and recovery of cells (Article III and Article IV (manuscript))
In Article III, whole blood sample from test subjects was stimulated with pneumococcal capsular saccharides type 3, type 9 (9N) and type 23 (23F) and *Streptococcus pneumoniae* cell wall polysaccharide (CWPS) in concentration of 10 µg/ml and applying one capsule type per well. Incubation lasted for 4 h respective 12 h at 37 º C, in 5% CO2. As a positive control, LPS was used and as negative control, unstimulated whole blood. In Article IV, the isolated monocytes and the NK cells respectively initially rested for 2 hours, after which they were incubated with either medium or stimulated by different TLR ligands. The following were used in these experiments: TLR4 ligand; lipopolysaccharide from E coli K12 (LPS), 100 ng/ml, TLR 2 ligand: *Streptococcus pneumoniae* cell wall polysaccharide (CWPS), 10µg/ml or TLR2/4 ligand: Pam3CSK4, 200ng/ml. After 16 hours of incubation at 37º C, supernatants were aspirated and stored at -80º C, while the cells were lysed directly in the cell culture plate, and cell lysates stored at -80ºC.

Antibodies and flow cytometry (Article III)
To identify which cells that had been activated by the stimulation using capsular saccharides or cell wall polysaccharide as described above, the following labeled fluorochrome conjugated monoclonal antibodies were used; anti-CD3-Pacific Blue, anti-CD56-PE, anti-CD4-APC H7, anti-CD14-PerCP and anti-CD69-FITC. The antibodies were added to 50 µl of stimulated blood and incubated in darkness for 20 min, in RT. Thereafter, a Coulter multi-Q prep was used to illuminate red blood cells and to fix and stabilize white blood cells. The remaining sample from the stimulated blood, which were not used in the above tests, was centrifuged and the supernatant was stored at - 20ºC. The cells were analyzed by flow cytometry. To identify lymphocytes and different subgroups of lymphocytes, FSC and SSC were used together with the antibody pattern for the respective cell types; CD3 (T-cells), $CD3^{pos}$ $CD4^{pos}$, $CD3^{pos}$ $CD4^{neg}$, $CD3^{pos}$ $CD56^{pos}$ (NK-like T-cells) and $CD3^{neg}CD56^{pos}$ (NK cells). To identify monocytes, FSC and SSC were used together with CD14. Due to limitations in flow cytometry, we were not able to stain for CD8, instead the $CD8^{pos}$ cells were assumed to form the majority of $CD3^{pos}CD4^{neg}$ cells. In addition to the surface markers for cell lineages, CD69 expression was used as marker of cell activation. Data is presented as mean fluorescence intensity (MFI) for CD69 or relative MFI (MFI of CD69 for stimulated cells divided by MFI of CD69 for unstimulated cells).

Quantification of secreted cytokines/ inflammatory mediators (Article III)
In Article III cytokines in the supernatant from stimulated cells were quantified using Cytometric Bead Array (CBA). Cytokine specific antibodies coupled to micro-particles were incubated with the supernatant. Following the addition of an anti-cytokine antibody, the amount of the bound cytokine antibody could be measured using flow cytometry.

In Article III, the concentrations of TNF, IL-8, IL-10 and IFN-ɣ in supernatants were analyzed. Data was analyzed using cytometric bead array (CBA) flex set (BD, Franklin Lakes NJ, USA), a multiplex assay allowing simultaneous detection of several analysis in a small sample volume. The cytokines were detected using the Cytometric Bead Array (BD Biosciences) software, in a FACS Canto™ II flow cytometer (BD), and analyzed with FCAP Array Software version 1.01 (Soft Flow, Inc, St. Louis Park, MN, USA). The inflammatory mediators in Article IV; IL-1beta, IL-6, IL-8, IL-10, GM-SFS, TNF, CCL2, CCL5 and CXCL8 were analyzed in the CBA Magnetic Luminex Assay. The supernatants from the frozen cell stimulation experiments were thawed and diluted 1:2 before measured using the BioPlex 200 system, and data was analyzed using the Bio Plex Manager 6.1.

Analysis of gene expression (Article IV)
For the isolation of RNA, the Qiagen RNA easy Plus Micro kit was used, and for the subsequent cDNA synthesis, the High Capacity cDNA Reverse Transcriptase Kit, with 12 ng RNA/sample. To investigate the selected gene expressions, real-time PCR was performed on a CFX 384 Touch thermocycler. Expression of TNF, IL-1 beta, IL-6, IL-10, GM-CSF, TLR2, TLR4, CXCL8 and HPRT1 were quantified using Taqman assay. HPRT, which is expected to be constantly expressed (a so-called housekeeping gene), were used as a reference. Expressions of CCL-2 CCL-5, CD80, CD86, SOCS-1, SOCS-2 and SOCS-3 were quantified with specific primers designed “in house”, while CD14 and CD16 were analyzed using Prime PCR SYBR Green Assay (BioRad). All the described gene expression tests applied the iTag Universal SYBR green supermix for detection.

Statistics (Article III and Article IV).
In Article III, non-parametric variance analysis was used for multiple comparisons of continuous data. The non-parametric Wilcoxon-signed rank test was used to test statistical differences between two dependent observations. Descriptive statistics and graphical methods were used to characterize data. The study required tests of several hypotheses, where each hypothesis was analyzed separately and the occurrence of patterns and the consequences of the results were taken into account in the analysis. All analyzes were performed using the SAS system 9.3, (SAS Institute Inc., Cary, NC, USA) and 5, 1 and 0.1% levels were considered.

Graph Pad PRISM 5 was used for graphs (GraphPad Software, Inc., San Diego, CA, USA). P-values <0.05 were considered statistically significant.

In Article IV, gene expression and protein concentration was calculated by Graph Pad Prism 5. Non-parametric methods including Wilcoxon, Kruskal-Wallis and Spearman’s rank test were used as indicated, and p-values <0.05 were considered statistically significant.

RESULTS

Article I

Blocking of non-specific binding

A specific ELISA was developed in order to study the binding of *Streptococcus pneumoniae* to two different proposed pneumococcal receptors, asialo-GM1 and lactotriaocylceramide. Initially problem with highly unspecific binding of the pneumococci directly to the microtiter plate was encountered. Using E. coli LPS for coating in microtiter plates before pneumococci application, this unspecific binding could be blocked. With successively increasing concentrations of LPS, the described unspecific binding decreased. This indicates that the unspecific binding was of hydrophobic nature.

Comparison of two different media

In order to investigate the unspecific binding of pneumococci further, two different dilution media were compared. Pneumococcal strain R36A was diluted in either TBS-BSA or in ordinary blocking buffer, respectively. The unspecific binding described above was eliminated using pneumococci diluted in blocking buffer. As a control experiment, blocking buffer was also investigated without bacteria, which yielded completely negative results.

Comparison of two different receptors

Binding of R36A to asialo-GM1 could be observed at coating concentrations from 1 µg/ml. Binding to lactotriaocylceramide was detected at concentrations of 10 µg/ml. The results show that both these receptors are able to bind pneumococci, but compared to asialo-GM1, approximately 10 times higher concentration of lactotriaocylceramide is required. For lactotriaocylceramide, the binding capacity was half of the binding capacity of asialo-GM1 (Figure 3).

Binding of different pneumococcal strains to asialo-GM1

When testing different pneumococcal strains for binding to asialo-GM1, all tested strains bound; R36A, two strains with capsule (strain CCUG 6605 (type 19) and CCUG 2987 (type 23) and a C mutant strain. However, each strain exposed a different degree of binding. The C mutant strain, which is characterized by a small capsule of CWPS, bound to a lower degree (Table 1).

Table 1. Bindning of pneumococcal strains to asialo-GM1 in ELISA. The bacteria were suspensed in PSM-buffer to OD 1,0. Bound bacteria were detected using a polyclonal pneumococcal antibody. The coating dose of asialo-GM1 was 20 µg/ml.

Pneumococcal strains		Absorbance 405 nm
R36A	(ATCC 11733)	0.960
C-mutant	(CCSR-SCS-2)	0.487
Type 19	(CCUG 6605)	0.660
Type 23	(CCUG 2987)	0.536

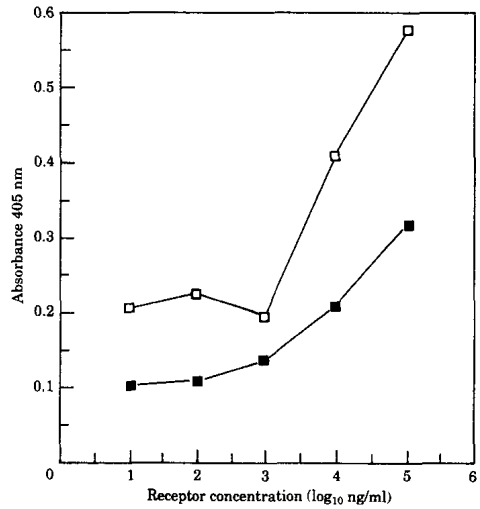

Figure 3. Comparison of the binding of Streptococcus pneumoniae, strain R36A, to two suggested receptor structures in an enzyme immunoassay. (□) Represents asialo-GM1 and (■) represents GlcNAcβ1-3Galβ1-4Glcβ1-ceramid.

Article II

Purification and characterization of CWPS

To characterize the structure with which the pneumococcal bacteria bind to asialo-GM1, the non-capsule pneumococcal strain R36A saccharides and proteins were separated by hot phenol-water extraction and purified by gel permeation chromatography. The resulting fractions from the Sephadex column were tested for binding to asialo-GM1 in ELISA, after which positive fractions were pooled and lyophilized. The freeze dried material was examined by nuclear magnetic resonance spectroscopy (NMR) and demonstrated good agreement with pure CWPS (22). In order to further confirm that the material purified indeed was CWPS, the material was also examined in Dot blot with an affinity purified rabbit monospecific antibody directed against an epitope containing sugar 2-acetamido-4-amino-2,4,6-trideoxygalactose (46). This is a repeating structure of sugar in CWPS. Analysis of the material in SDS-page and Western blot with the monoclonal anti-phosphorylcholine antibody yielded three to four bands in a step-like pattern. The bands had molecular weights between 20 and 30 kDa, the distance between the bands was about 2.2 kDa and the main band exhibited a molecular weight of about 22 kDa.
Depending on the amount of material added to the gel, Western blot showed four to six bands with a monoclonal anti-phosforylcholine antibody or a polyclonal rabbit anti-pneumococcal serum (49). To investigate whether CWPS contained any oligopeptides that would affected its binding to asialo-GM1, the purified material was treated with protease-K, urea and guanidine-HCL. No effect of binding capacity could be noted after this treatment (Figure 4).

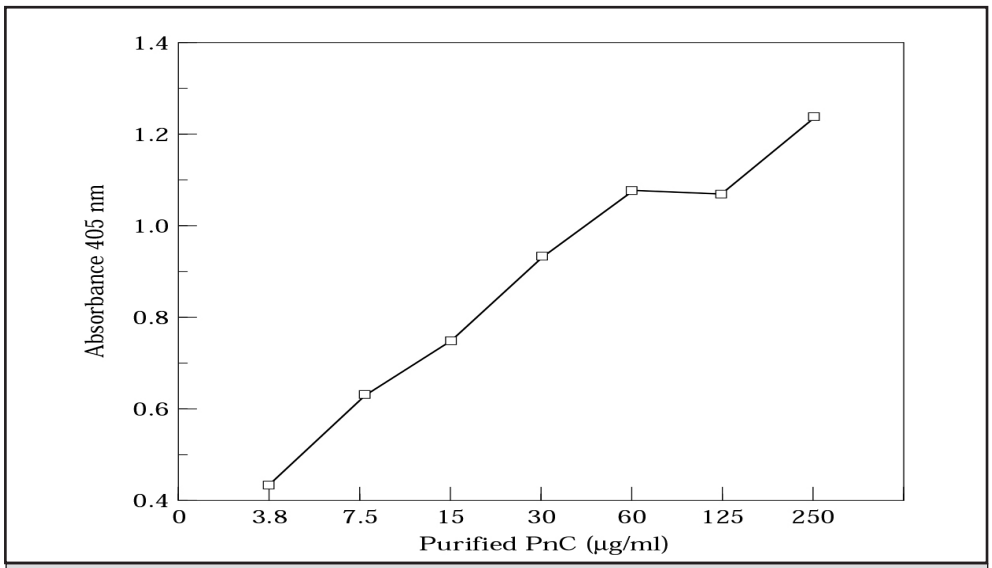

Figure 4. Binding of purified pneumococcal C-polysaccharide (PnC) to asialo-GM1 in ELISA. Detection of bound PnC was performed using an anti-pneumococcal polyclonal antibody.

Binding of solubilized surface components

By heat treatment of R36A at 65° C for 40 minutes, surface components, termed "heat extract", were extracted. The extract was examined in ELISA where the components that bound to pre-coated asialo-GM1 could be detected both with polyclonal antibodies as well as with monoclonal antibodies to phosphorylcholine. The latter indicates that the component in the heat extract that binds to asialo-GM1 contains CWPS. After heat extraction, the bacteria could still bind to asialo-GM1 in ELISA.

Bacteria harvested in different growth phases were investigated in regards to binding capacity. Bacteria harvested in log phase, growth phase, and stationary phase were compared. At equal cell concentrations, the resulting signal was higher in bacteria harvested in the log phase. This indicates that the bacterial surface exposes more binding material during the log phase. Heat extract from R36A was examined in SDS-PAGE and Western blot. Pure CWPS was used as control in the experiment. CWPS was identified with a monoclonal anti-phosphoryl antibody. The band pattern for the heat extract in Western blot with the monoclonal anti-phosphoryl antibody corresponded well to the band pattern from pure CWPS.

To further characterize the binding material, the extract was subjected to separation by SDS-PAGE after which the gel was cut into five different horizontal discs. Each disc was eluted in PBS and subdivided into two portions. The eluate was then tested in ELISA for binding to asialo-GM1 and also examined in SDS-PAGE and Western Blot to determine the molecular weight of the substances that bound to asialo-GM1.

Samples exposing binding activity to asialo-GM1, as detected with the polyclonal anti-pneumococcal antibody, were those containing a phosphorylcholine determinant. No reduced binding to asialo-GM1 in ELISA was observed when protease K-treated extract was analyzed.

Phosphorylcholine residues and binding of CWPS

To further investigate the phosphorylcholine in CWPS and its importance for binding to asialo-GM1, R36A was cultured in a defined medium in two different variants; one batch containing choline while choline was substituted with ethanolamine in the second batch. Bacteria grown in the choline containing medium bound to asilao-GM1 in Elisa, while ethanolamine-derived bacteria did not bind. Bacteria and heat extracts from both cultures were also investigated in SDS and Western Blot. Choline containing bacteria and extracts showed band patterns as for CWPS with a monoclonal anti-phosphoryl choline antibody. Ethanolamine containing bacteria exhibited no such bands when exposed to the same

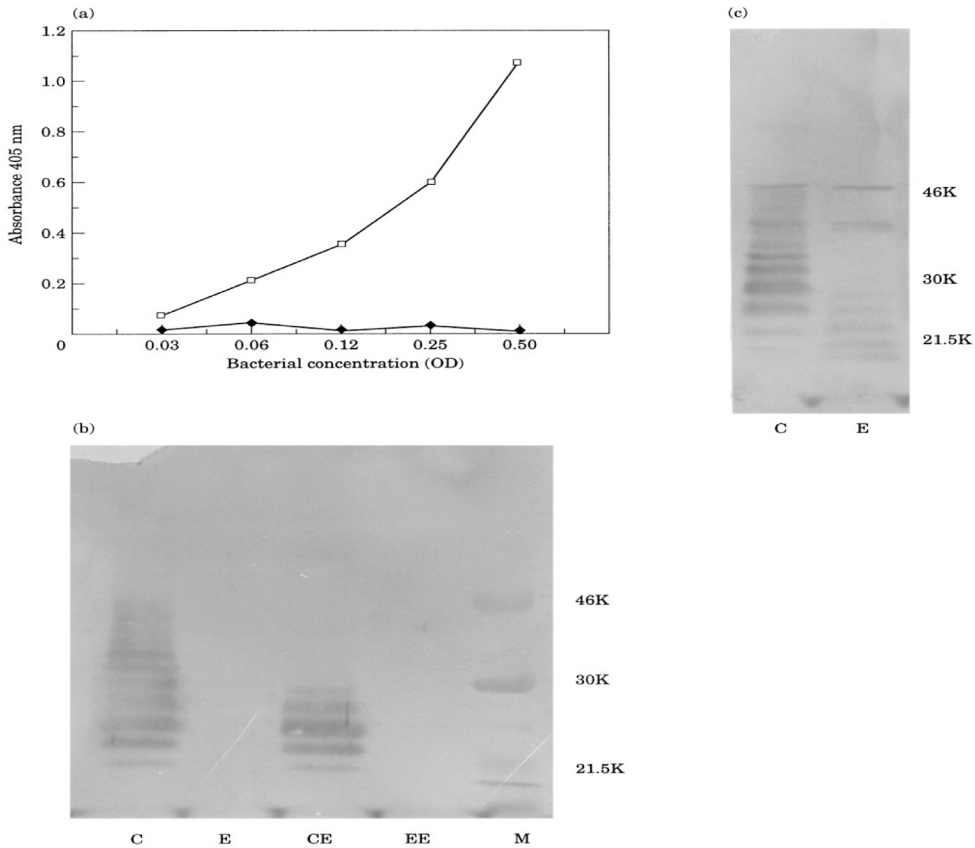

Figure 5. Pneumococcal cells (strain R36A) were grown in defined media containing either choline or ethanolamine. (a) Bacterial cells were tested for binding to asialo-GM1 in ELISA. —□—: bacteria grown in choline-containing medium; —♦—: bacteria grown in ethanolamine-containing medium. For detection anti-pneumococcal polyclonal antibody was used. (b and c) Bacteria and undiluted extracts were run in SDS-PAGE and analysed by Western blot. Extracts were obtained by heat-treatment of a suspension of bacterial cells (OD 1.0 at 600 nm). (b) Western blot using an antiphosphoryl choline monoclonal antibody. (c) Western blot using an antipneumococcal polyclonal antibody. M: molecular weight marker; C: bacteria grown in choline-containing medium; E: bacteria grown in ethanolamine-containing medium; CE: extract from bacteria grown in choline-containing medium; EE: extract from bacteria grown in ethanolamine-containing medium.

antibody. However, both choline and ethanolamine cultured bacteria exhibited bands in Western Blot with polyclonal anti-pneumococcal antibodies (Figure 5).

Article III

CD69 expression after stimulation of whole blood

CD69 was used as a marker for activated leukocytes in stimulation experiments where whole blood was stimulated with CWPS and with three different capsule types from pneumococci; type 3, type 9 and type 23. Leukocyte cell types; $CD4^{pos}$ T-cells, $CD4^{neg}$ T-cells, NK-like ($CD56^{pos}$) T-cells, NK cells and monocytes were analyzed for CD69 expression after 4 or 12 h stimulation respectively. The capsules activated all cell types analyzed, but to a different extent, as shown for NK-cells in Figure 6. Following CWPS stimulation, NK cells had the highest CD69 expression (measured as relative MFI value). The second highest values were observed in $CD56^{pos}$ T-cells, followed by monocytes and $CD4^{neg}$ T-cells and finally $CD4^{pos}$ T-cells (Figure 7). Overall, the CWPS stimulated immune cell subsets to a higher degree than observed for the capsules. However, CWPS is not included in the statistical comparisons since it was used as positive control. Instead, the ability of the three capsules to stimulate the different leukocyte cell types after 4 respective 12 h was compared. Regarding the pneumococcal capsules, type 23 stimulated the leukocytes strongly, followed by type 9 and type 3. There was a statistically significant difference observed between type 3 and type 23 in eight out of nine (8/9) tests and this was also observed between type 3 and type 9 in seven out of nine (7/9) tests. However, between type 9 and type 23, there was only a significant difference observed in three of nine (3/9) tests. The results were consistent for 4 h and 12 h stimulation, with only minor

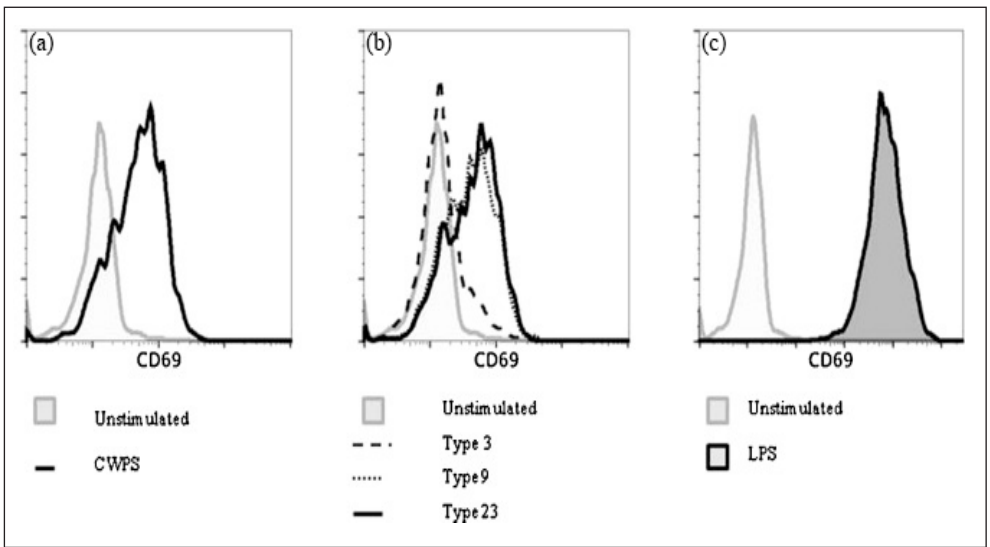

Figure 6. Cell activation was assessed as expression of CD69 by flow cytometric analysis. The histograms show results after whole blood of healthy controls was stimulated by CWPS and three different pneumococcal capsular polysaccharides. The figure depicts CD69 expression on NK cells where (a) shows CWPS and negative control, (b) shows type 3, type 9 and type 23 capsules and negative control and (c) shows LPS and negative control. CWPS; pneumococcal cell wall polysaccharide, LPS; lipopolysaccharide. (Number of individuals included, N = 9).

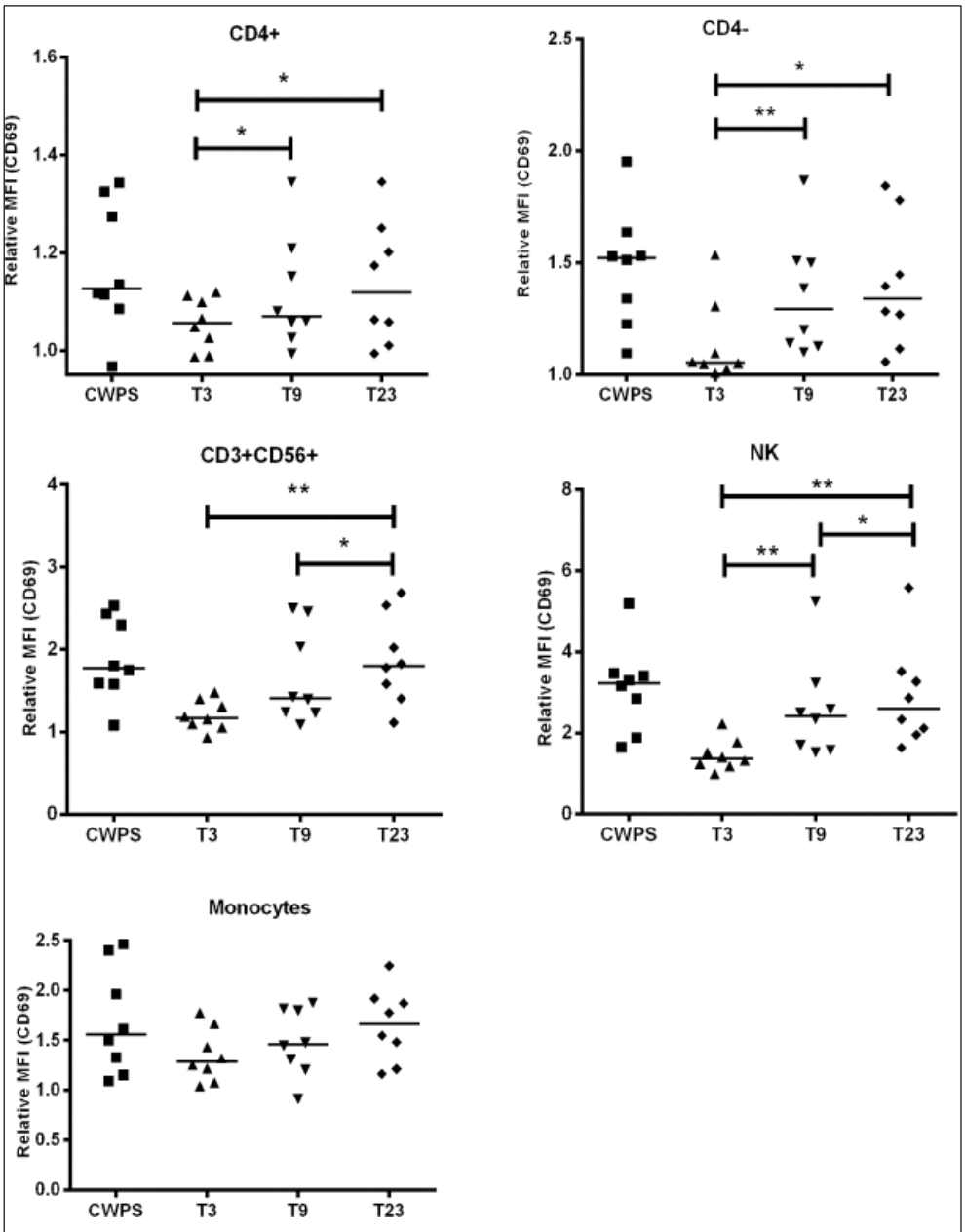

Figure 7. CD69 expression in cell subsets. CD69 expression was determined as a measure of cell activation after in vitro stimulation. Whole blood from healthy non-pneumococcal vaccinated subjects was stimulated for 4 h (Figure part A, N = 8) with pneumococcal C-polysaccharide (CWPS) and pneumococcal capsular polysaccharides type 3, type 9 and type 23. Unstimulated whole blood was used as negative control and stimulation with LPS as positive control (data not shown). Graphs show CD69 expression in stimulated versus unstimulated cells, expressed as relative MFI, for the respective cell subsets indicated above each graph. Statistical comparisons were only carried out between the three capsular polysaccharides (type 3, type 9 and type 23).

variations observed.

Quantification of secreted cytokines
Supernatants of unstimulated cells and cells stimulated with; CWPS, type 3, type 9 or type 23 pneumococcal capsules were compared for cytokine release. Stimulated cells released IL-8, TNF, IL-10 and IFN-ɣ. Exceptions from this were seen in type 3 for IFN-ɣ and IL-10, and in type 9 for IFN-ɣ. Stimulation of cells with CWPS showed that the IL-8 cytokine concentration was the highest, followed by IL-10, TNF and INF-ɣ. Stimulation with the capsules type 9 and type 23 yielded the highest concentration of IL-8, which was followed by TNF, IL-10 and IFN- ɣ. The cytokine pattern for the type 3 capsule gave rise to a higher value of IL-10 than TNF, which is similar to the described CWPS pattern.
The type 9 and type 23 capsules stimulated cytokine release to a much greater extent than what was observed for the type 3 capsule. There was a significant difference in the release of cytokines IL-8, TNF and IL-10 observed between capsule type 3 versus type 23 and type 9 respectively. Between the type 9 and type 23 capsules no significant difference was observed. There were no significant differences observed from stimulated cells exposed to the different capsules in regard to IFN-ɣ.

Article IV (manuscript)

This study includes two different groups of healthy subjects, smokers and nonsmokers, with normal lung function measured as $FEV_{1.0}$ and without any signs or symptoms of airway disease, lung disease or inflammatory disease, and with identical distribution of sex and age. Although spirometry did not show any signs of obstruction, the median $FEV_{1.0}$ in smokers was reduced compared with non-smokers.

CWPS from *S. pneumoniae* induces gene expression of pro-inflammatory mediators from monocytes
The ability of pro-inflammatory mediators to induce gene expression and secretion of inflammatory mediators in isolated blood monocytes after stimulation with cell wall polysaccharide, CWPS, from *Streptococcus pneumoniae* was investigated.
Monocytes were isolated from peripheral blood collected from twenty-one healthy subjects (11 smokers and 10 non-smokers), and the gene expression and secretion of inflammatory mediators were analyzed after stimulation with pneumococcal cell wall polysaccharide (CWPS), and other TLR ligands.
The results show that the gene expression of the cytokines *IL-1β* and *IL-6*, and the chemokines *CCL2* and *CXCL8* was induced by CWPS stimulation. Furthermore, the analysis showed that the expression was dependent on the concentration of CWPS used (1 or 10 µg/ml) and that the higher concentration (10 µg/ml) induced a significant increase compared to unstimulated monocytes. Therefore, the subsequent analyses in this study were performed using a CWPS concentration of 10 µg/ml.

Pro-inflammatory gene expression in monocytes and in NK-cells after stimulation with the TLR ligands CWPS, Pam3CSK4 and LPS
TLR2 ligand CWPS, TLR1/2 ligand Pam3CSK4 and TRL4 ligand LPS were all examined for their capacity to elicit gene expression of pro-inflammatory mediators and co-stimulatory molecules in isolated monocytes from twenty-one healthy subjects. Results

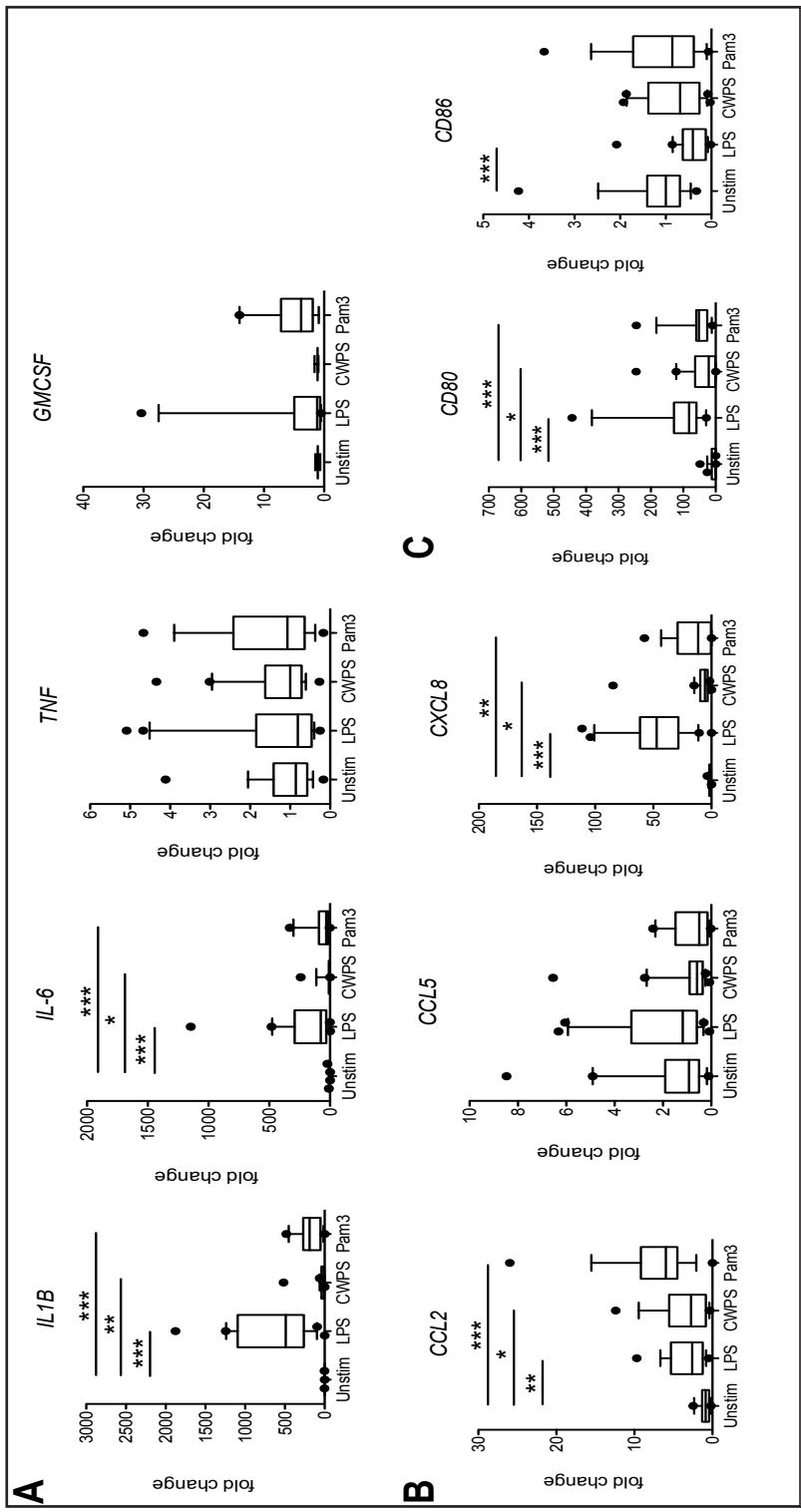

Figure 8. CWPS from S Pneumoniae indudes distinct expression of inflammatory mediators. Gene expression of selected cytokines (A), chemokines (B) and costimulatory molecules (C) measured by qPCR in isolated peripheral blood monocytes, after stimulation with LPS (10 ng/ml), CWPS (10 µg/ml), Pam3CSK4 (200 ng/ml) or media alone (unstim) for 16 h. Gen expression expressed as fold change relative to the median ΔCT in the unstimulated cells. Horizontal lines: median, boxes: interquartile range, whiskers: 10-90% range. Statistics calculated using Kruskal-Wallins non-parametric test wih Dunn's post test, compared to unstimulated cells, for each gene , *: p<0,05, **: p<0,01 ***: p<0,001

were compared to the findings in unstimulated monocytes (Figure 8). The concentrations of Pam3CSK4 and LPS used in the study were previously known to induce a pro-inflamatory response in monocytes. As expected all three ligands significantly elevated the pro-inflammatory cytokines *IL-1β, IL-6*, as well as the chemokines *CCL2 and CXCL8*, in monocytes. Gene expression of the co-stimulating molecules *CD80* and *CD86* was also regulated by TLR stimulation of monocytes. In the present experiments, all TLR ligands upregulated CD80, with the strongest upregulation shown for LPS, followed by Pam3CSK4 while CWPS elicited the weakest response. In contrast, LPS downregulated *CD86*, while the TLR2 ligands did not affect this gene expression after stimulation.

A previous study (Article III) indicated that CWPS activates NK-cells. Thus, isolated NK-cells from peripheral blood were also stimulated with the TLR ligands CWPS, Pam3CSK4 and LPS. All three ligands upregulated *CXCL8* expression significantly, but no other significant increase in gene expression was detected for the other inflammatory mediators, *IL-1β, IL-6, TNF, CCL2, CCL5*, included in the study.

Cytokine and chemokine secretion in monocytes after stimulation with TLR ligands
To analyze the secretion of pro-inflammatory mediators, the concentration of selected cytokines and chemokines was analyzed in the cell supernatant of stimulated monocytes from twenty-one healthy individuals. The TLR ligand CWPS induced secretion of IL-1β, IL-6, TNF, CCL2 and CXCL8, but not of GM-CSF or CCL5. The TLR ligands Pam3CSK4 and LPS both induced cytokine secretions of all the cytokines and chemokines investigated; IL-1β, IL-6, TNF, GM-CSF, CCL2 and CXCL8. The secretion of CCL2 and CXCL8 was strongly induced by all TLR ligands, whereas CCL5 secretion was not affected by TLR stimulation.

Cigarette smoke is a well-known risk factor for infection with *Streptococcus pneumonia* both systemically and in the airways. In order to investigate differences in the TLR-response, including the response to CWPS, based on cigarette smoking habits, the cohort was divided two groups; eleven non-smokers and ten smokers. They were then investigated for differences in the expression of proinflammatory mediators and secretion of cytokines and chemokines. The groups showed no significant difference in cytokine or chemokine production when the monocytes were incubated with medium alone (unstimulated). On the other hand, there was a difference detected between non-smokers and smokers in stimulated monocytes. Stimulation with Pam3CSK4 reduced CCL5 secretion significantly in smokers, while LPS stimulation instead resulted in a significant increase in CXCL8 secretion in smokers. CWPS stimulation however did not induce any difference in the secretion of cytokines or chemokines in the supernatant between non-smokers and smokers.

Gene expression of mediators in isolated monocytes from smokers and non-smokers
The groups were also analyzed for differences in gene expression after stimulation with the TLR ligands CWPS, Pam3CSK4 and LPS. In unstimulated isolated monocytes, no difference was found in the expression of selected genes between non-smokers and smokers. However, monocytes from smokers stimulated with CWPS induced a stronger inflammatory response, compared to cells from non-smokers. This difference was significant for the expression of *IL-6, TNF, CCL5* and *CD80*. Monocytes stimulated with Pam3CSK4 induced a significantly stronger *TNF* expression in smokers compared to non-smokers, whereas LPS-stimulated monocytes from smokers expressed a similar, but not significant, trend

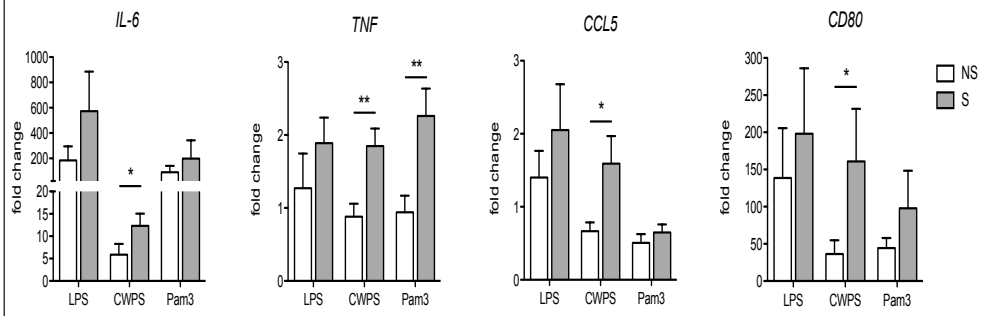

Figure 9. Stimulation with TLR-ligands induces a different response in monocytes between non-smokers and smokers. Gene expression of selected cytokines, chemokines, and co-stimulatory molecules was measured by qPCR in isolated peripheral blood monocytes, after stimulation with LPS (100 ng/ml), CWPS (10 µg/ml), Pam3CSK4 (200 ng/ml) for 16 h. Gene expression presented as fold change relative to the unstimulated cells, for each individual. Bars indicate mean, and error bars SEM. Statistic calculated in Mann Whitney test, for each stimulation gene. *: p<0,05, **: p<0,01.

(Figure 9). The gene expression of *CXCL8* in NK-cells exhibited no significant difference between smokers and non-smokers. Following LPS stimulation of TLR, the cytokine expression between smokers and non-smokers differed which may indicate a specific regulation of gene expression. While the expression of inflammatory mediators was higher in cells from smokers, no significant correlation was observed between the higher gene expression and smoking habits.

Gene expression for *TLR2* and *TLR4* in non-smokers and smokers
The study also analyzed whether the gene expression of *TLR2* and *TLR4* were associated with the difference in the gene expression for the inflammatory mediators observed between smokers and non-smokers. Monocytes incubated in medium, i.e. unstimulated, were analyzed to approximate the gene expression seen *in vivo*. In unstimulated cells there were no differences observed in gene expression for *TLR2* and *TLR4* between non-smokers and smokers.

Monocyte marker CD14 are expressed differently in smokers and non-smokers
The surface markers CD14 and CD16 are used to identify and characterize peripheral blood monocytes. Analysis of the gene expression for the surface markers showed a significantly reduced gene expression of *CD14* in smokers, but no difference in the gene expression for CD16 between nonsmokers and smokers. In unstimulated monocytes, there was a positive correlation of gene expression for both *CD16* and *TLR2* with the gene expression of *CD14*, i.e. individuals with high *CD14* expression also showed a high expression of *TLR2* and *CD16*.

Expression of immune-regulatory molecules induced by TLR stimulation, in monocytes of smokers and non-smokers
Immune-regulatory signaling can be affected by TLR stimulation. The monocytes were therefore investigated both in terms of gene expression and secretion of anti-inflammatory cytokine IL-10, in non-smokers and smokers, respectively. Secretion of anti-inflammatory

IL-10 was observed after stimulation with LPS and Pam3CSK4, while IL-10 concentration was unchanged after CWPS stimulation. CWPS stimulation of isolated monocytes induced downregulation of the gene expression of *IL-10* which was significantly more pronounced in non-smokers versus smokers.

Suppressors of cytokine signaling (SOCS) are a family of immunoregulatory compounds. To investigate differences between non-smokers and smokers, gene expression of *SOCS-1, SOCS-2* and *SOCS-3* was investigated in isolated monocytes (unstimulated cells and after TLR stimulation). In unstimulated cells from smokers, the analysis indicated a lower expression of *SOCS-1* and *SOCS-3*, compared to non-smokers. Expression of these two genes, correlated positively with CD-14 expression. Expression of SOCS-1 is downregulated after TLR2 stimulation compared to what is observed in unstimulated cells.

When comparing the groups of smokers and non-smokers, results show a *SOCS-1* downregulation only in cells obtained from the non-smokers. *SOCS-2* expression is upregulated by LPS, while *SOCS-3* expression is upregulated by either LPS or Pam3CSK4, however, no difference between smokers and non-smokers was detected. SOCS-1 and *SOCS-3* correlated positively with the gene expression for *CD14.*

DISCUSSION

Article I

Glycoconjugates on lung and epithelial host cells constitute a common binding site for pathogenic bacteria (24). The cell receptors, GalNAcbeta1-4Gal, GlcNAcbeta-1-4Gal are common binding sites for various bacterial pulmonary pathogens, including *Streptococcus pneumoniae* (48, 49).

When the present project was started, two different disaccharides from glycolipids had been proposed as epithelial cell receptors for the binding of pneumococci (48, 50). The binding sites on the glycolipids were proposed to be the disaccharides GlcNAcβ1-3Gal and GalNAcβ1-4Gal. Previous studies of these receptors were performed by different methods. One of the earlier described methods included an inhibition test to determine the pneumococcal binding specificity to nasopharynx cells while yet another method detected the binding of bacteria to ganglioside receptors that had been separated in thin layer chromatography. Using the described tests, the binding affinities of pneumococci to the various receptors were difficult to compare. In the thin layer chromatography system, no pneumococci binding could be detected to GlcNAcβ1-3Gal due to restrictions in receptor concentrations. This was not the case for GalNAcβ1-4Gal affinity, which exhibited good pneumococcal binding in the test (48). Neither were these two different methods comparable with regard to the amount of receptors needed for pneumococcal adhesion. When using the thin layer chromatography method, concentrations of less than 10 µg/ml GalNAcβ1-4Gal could easily bind bacteria (48) while for the epithelial cells inhibition test, 100 µg/ml of lactoneotetraocylceramide containing GlcNAcβ1-3Gal was needed to gain a 50% inhibition of pneumococci adhesion to epithelial cells (50).

In the current study, the aim was to directly compare pneumococcal binding to the different receptors using the identical method for both receptors. For this reason an ELISA was developed where the two receptors GlcNAcβ1-3Gal and GalNAcβ1-4Gal were coated. Initially, there was a major problem with non-specific binding of the pneumococci to the plastic in the ELISA plates. During the development of this ELISA, it was found that the non-specific binding could be markedly reduced by coating the plastic surface with the glycolipid receptors.

In addition, a dose-dependent inhibition of the non-specific pneumococcal binding could then also be achieved in the ELISA plates when pre-coated with different amounts of LPS, indicating a hydrophobic attachment as the cause for pneumococci non-specific binding. By addition of an anti-hydrophobic solution to the test system, this unspecific binding was effectively reduced, without affecting the ELISA otherwise. The described ELISA measures the binding of pneumococci to the purified receptor glycolipids, asialo-GM1 and lactotriaocylceramide, without any interference of other receptors.

Eventually, the results gained showed that pneumococci are capable of binding to the two proposed receptors GalNAcβ1-4Gal and GlcNAcβ1-3Gal, but with distinct different affinity. The bacteria bind with higher affinity to GalNAcβ1-4Gal than to GlcNAcβ1-3Gal, which is consistent with previous results.

Strains of the bacteria Escherichia coli have also been shown to bind to the two receptors GalNAcβ1-4Gal and GlcNAcβ1-3Gal (51)**.** As in the case of pneumococci, the E. coli strains bind stronger to GalNAcβ1-4Gal and with lower adherence to GlcNAcβ1-3Gal. Mutants of these E. coli strains which are negative in binding tests to an enteropathogenic LA cell line simultaneously showed significantly reduced binding to asialo-GM1. The α-streptococci bacteria, which are closely related to *Streptococcus pneumoniae*, also bind to more than one receptor with different affinities (52). In the present publication, the capsule did not prevent adhesion. This was later confirmed by Geelen et al., using the same pneumococcal strain (53).

GalNAcβ1-4Gal is identified in lung tissue. It is usually sialylated. Many viruses and also pneumococci contain the enzyme neuraminidase that possess the ability to cut away the sialic acid located on glycoconjugates of the host cell, thereby exposing previously hidden receptors (54). Pneumococcal pneumonia frequently occurs as a complication after viral infections in the upper respiratory tracts, which in part may be explained by the described ability of viral neuraminidase to expose the receptor (55). Neuraminidase enzymatic capacity also has the ability to reduce mucus viscosity which has been shown to further facilitate adhesion, colonization and biofilm formation on the host cell surface (7, 56).

GlcNAcβ1-3Gal has been isolated in breast milk. The receptor inhibits the binding of pneumococci to human pharyngeal and buccal epithelial cells, which may indicate that this receptor molecule may coat the outside of the bacteria resulting in a reduction of the binding to the epithelial cells (50). However, it should be noted that the presentation of the receptor molecule used in solution versus the presentation in solid phase may differ (57).

Following the publication of this work, several additional pneumococcal receptors on host cells have been described and examined. These include the platelet activating factor receptor (PAFr) found on epithelium in lung, bronchial and alveolar tissue, as well as in other cell types. Pneumococci can bind to PAFr, and be transported through the cell into the blood stream (24, 58). *Streptococcus pneumoniae* has also been shown to bind to the polymeric immunoglobulin receptor (pIgR) involved in the adhesion of pneumococci to human nasopharyngeal cells (59). The receptor plgR is also expressed on brain endothelial cells, and Iovino et al. found that pneumococci adhere to this receptor in the blood brain barrier (BBB) endothelium (60). Another receptor that is important for pneumococcal adhesion and invasion is the Platelet Endothelial Cell Adhesion Molecule 1 (PECAM-1) receptor also expressed on BBB endothelial cells, which mediates pneumococcal adhesion (61). The Laminin receptor (LR), found on endothelial cells also has the ability to bind *Streptococcus pneumoniae*. Finally, pneumococcal surface proteins can bind to Vitronectin (62).

Article II

Pneumococci adhere to human host cells through the interaction with several receptors (53, 63), including glycolipid receptors. It has been shown that pneumococci bind to the surface glycolipids of nasopharynx cells containing GlcNAcβ (1-3) Gal, as well as to lung and vascular endothelial cells containing GalNAcβ (1-4) Gal and GalNAcβ (1-3) Gal (48, 50, 64). An ELISA for the detection of pneumococci binding to asialo-GM1, which contains GalNAcβ (1-4) Gal has been developed and described in Article I (65). In a previous study

(Article I) it was shown using ELISA that both capsulated and uncapsulated pneumococci bind to asialo-GM1, which contains GalNAcβ (1-4) Gal (65). In addition, pneumococcal binding to type II lung cells (LC) was not prevented by different capsule types (64).

The aim of the current study (Article II) was to investigate the pneumococcal ligand responsible for the binding to asialo-GM1. Earlier reports indicated that pneumococcal binding to endothelial cells could be inhibited by soluble cell wall components from pneumococci. Since the maximum inhibition from soluble cell wall components described was only 60%, it could be assumed that there are additional adhesion mechanisms involved (53). The results in study II indicate that purified CWPS binds to asialo-GM1.
CWPS constitutes the major cell wall teichoic acid of the pneumococci. It is covalently bound to the cell wall and exposed on the pneumococcal surface (66). CWPS consists of ribitol-containing, repeating pentasaccharide units with two phosphorylcholine substituents linked to the acetylated galactosamine residues (22).

In the present study we show that purified CWPS bind in a dose-dependent manner to asialo-GM1 and that protease K treatment does not affect adhesion, i.e., protein components are not involved in the detected binding. Together this indicates that CWPS most likely is involved in the binding to the receptors asialo-GM1 and asialo-GM2, both containing GalNAcβ (1-4) Gal. Following heat treatment of pneumococcal bacteria at 65° C, material (”heat extract”) is released. This “heat extract” subsequently adhered to asialo-GM1 in ELISA. The substance which adhered to asialo-GM1 reacted with both a rabbit anti-pneumococcal polyclonal antiserum and an anti-phosphorylcholine monoclonal antibody (46). This indicates CWPS involvement in the binding to asialo-GM1. The heat extract was also separated on SDS PAGE from which fractions were eluted. It was shown that the SDS-PAGE fraction adhering to asialo-GM1 could be identified in Western blot as CWPS using a monoclonal anti-phosphorylcholine antibody. In the described experiments, treatment with protease K did not affect adhesion.
“Heat extract” and purified CWPS separated on SDS PAGE and analyzed in Western blot showed identical band patterns (unpublished data). The band pattern indicated different chain lengths of CWPS where each band contained different numbers of repeating units. Molecular weights for CWPS were estimated to range between 20-30 kDa, which corresponds well to the value of 26.4 kDa previously reported (67). The distance between the individual CWPS bands corresponded to approximately 2.2 kDa, which is believed to represent the difference in molecular weight for more than one repeating unit.

It has previously been shown that purified CWPS may be linked to fragments of peptidoglycan (68), which could explain the obtained results. However, in the present study the CWPS identity was confirmed by H-NMR as well as in Dot blot using a monoclonal antibody with specificity against the repeating units specific to the 2-acetamido-4-amino-2,4,6-trioxygalactose epitope. This epitope is independent of the phosphorylcholine moiety (46). When culturing pneumococci under laboratory conditions, choline may be replaced with ethanolamine or other amino alcohols in the culture medium. Ethanolamine is then incorporated where choline usually is situated (47). When ethanolamine was incorporated, the pneumococci lost their ability to transform and autolys. After cell division, the cells are associated with each other (47). In the case where choline was replaced by ethanolamine, these bacteria did not bind to asialo-GM1. This indicates that the presence of phosphoryl-

choline residues is necessary for the binding of pneumococci to asialo-GM1.

Both bacteria and “heat extract” from bacteria adhered to asialo-GM1 when the pneumococci were cultured in choline. The results show that CWPS, containing phosphorylcholine, is the ligand responsible for pneumococcal binding to the receptor asialo-GM1.

Interactions between carbohydrates and bacteria have been described previously. In Pseudomonas aeruginosa, LPS and pili specifically bind to glycolipid asialo-GM1 (69). The outer protein membrane of Chlamydia trachomatis is glycosylated and binds to the glycan of HeLa Cells. This binding is inhibited by D-galactose, D-mannose and N-acetylglucosamine, which indicates that the glycan portion of the outer membrane protein is involved (70).

Apart from asialo-GM1 it is known that phosphorylcholine is involved in binding to yet another receptor, the platelet activating factor receptor, PAFr. As revealed by the name, this receptor binds the platelet activating factor, PAF, a common cytokine. PAFr is found in lung, vascular and brain cells as well as in leukocytes (24). PAF, like pneumococci, exposes phosphorylcholine and both PAF and pneumococci bind to PAFr through the interaction of phosphorylcholine. The magnitude of interaction with PAFr may be affected by the amount of phosphorylcholine present in the pneumococcal cell wall (71). Via PAFr, the pneumococcus is taken up into an intracellular vacuole and by this mean transported to the blood stream (58). It has been shown that the expression of PAFr may be induced on lung cells and vascular endothelial cells following cytokine stimulation (24, 53). The choline replacement by ethanolamine in pneumococci decreased the binding to the PAF receptor (24).

Streptococcus pneumoniae binds to human cells through the action of several ligands. CbpA is one of the family members of the choline binding proteins (Cbp), anchored to phosphorylcholine on the cell wall teichoic acid (CWPS). Binding of pneumococcal CbpA to the plgR receptor on human nasopharyngeal cells, generates uptake of the bacterium into the cell (59). Furthermore, CbpA is able to bind to Laminin receptor, LR, on human vascular endothelial cells (72). CbpA also bind to human Vitronectin (73). Other pneumococcal proteins also function as adhesins, for example, other choline binding proteins (74) and pilus proteins (7, 75). Biofilm formation is reported to be mediated by Pneumococcal serine-rich repeat protein (PsrP) (76).

Article III

The aim of this study was to analyze the activation of immune cells as well as cytokine secretion after stimulation with *Streptococcus pneumoniae* saccharides, in blood from healthy individuals. The saccharides used for the study were C-polysaccharide (CWPS) and three different capsular saccharides; serotype 3, serotype 9 and serotype 23. All these capsules are included in the 23-valent pneumococcal vaccine and in the conjugated vaccines, with the exception of the 7-valent conjugated vaccine where serotype 3 is not included. The immune cells analyzed for activation (measured as CD69 expression) were monocytes, NK-cells, $CD4^{pos}$ T-cells, $CD4^{neg}$ T-cells and $CD56^{pos}$ T-cells.
The cytokine secretion in the supernatant was assayed for TNF, IL-8, IL-10 and IFN-ɣ.
All immune cell subsets analyzed were activated by stimulation of whole blood with

both CWPS and all of the capsules, with few exceptions. The responding immunocellular activation was, however, of varying amplitudes. The activation response from the stimulation was strongest for NK-cells, NK-like T-cells ($CD56^{pos}$ T-cells) and monocytes. In contrast, $CD4^{neg}$ and $CD4^{pos}$ T-cells exhibited the lowest degree of activation.
The three types of capsules differed from each other in their ability to induce immune cell activation: Type 23 achieved the highest activation, followed by type 9 while the lowest degree of activation after stimulation was observed with capsule type 3.
The same pattern was observed for cytokine secretion post-stimulation, in that the highest concentrations were induced by type 23. Again this was followed by type 9 capsules while the lowest values were observed from type 3 capsules.

In this study, CWPS activated monocytes, T-cells and NK-cells to a higher degree, compared to the capsules. CWPS is a teichoic acid (22) which is a known TLR2 ligand (77). TLRs recognize conserved patterns of different pathogens and are a part of the innate immune system. CWPS has a pattern of positive and negative charges in repeating units and has a so-called zwitterionic polysaccharide (ZPS), which enables direct stimulation of T cells (78). ZPS also act as ligands for TLR2 (79). CWPS induces B-cell proliferation by the identical subunits capable of crosslinking the B cell receptor. The degree of activation (CD69 expression) post stimulation was highest in the NK-cells. NK-cells are capable of stimulating B-cells to antibody release and isotype switch, which can be observed after vaccination against pneumococci (80-82). It has also been shown that no isotype switch of antibodies occurs in the absence of NK-cells (82).
Regarding analysis of $CD8^{pos}$ T cells, FACS limitations prohibited us from including anti-CD8 in the staining protocol and we therefore measured the activation of the $CD4^{neg}$ T-cell subset. However, in parallel experiments we found that almost 90% of $CD4^{neg}$ T-cells were $CD8^{pos}$, indicating that both CWPS and the three capsules activated $CD8^{pos}$ cells.

It has been shown that $CD8^{pos}$ T-cells are important for the protective pneumococcal antibody response (83). Antibodies to pneumococcal polysaccharides cannot be produced in mice lacking $CD8^{pos}$ (84).

Exposure of pneumococcal capsule type 23 activated all the investigated immune cells. This response was followed by type 9 and finally by type 3 that elicited the smallest degree of immune response among the studied capsules.

Capsule polysaccharides are known to be B-cell antigens. Therefore they are used in vaccines to induce protective antibodies. However, since the capsular saccharides are not TLR ligands, the mechanism behind this immune cell activation is unclear. Direct stimulation occurs in cells expressing TLR, which are monocytes, NK-cells (85), and also NK-like T-cells (86), but not $CD56^{neg}$ $CD4^{neg}$ T-cells or $CD4^{pos}$ T-cells. However, the serotype 1 pneumococcal capsule (not used in this study) is the only capsule capable of activating TLR. Studies indicate that there might be a small amount of CWPS even in highly purified capsular polysaccharides from pneumococci (87). In the present trials, therefore it cannot be completely excluded that CWPS might be involved in activation and cytokine secretion. The amount of CWPS bound in the different pneumococcal capsule types may vary. Furthermore, the repeating units in CWPS may either contain one or two phosphorylcholine residues (88). In summary, the mechanism behind capsule-induced activation of various immune cell subsets

remains to be clarified. The capsules generate antibody production by crosslinking to B-cell receptors. The antibodies elicited by the capsules have been shown to protect against fatal pneumococcal infections.

Today there are two different types of *Streptococcus pneumoniae* vaccines on the commercial market. One of these products is a saccharide vaccine containing 23 different *Streptococcus pneumoniae* capsules. The other commercially available product is a conjugated vaccine, where the capsular saccharides are attached to a protein. This vaccine contains either seven, ten or thirteen different capsular saccharides. In the body, after injection, the protein is degraded into peptides which are presented by MHC class II molecules on B-cells. These peptides are presented to T-cells, which are activated and start to proliferate as well as differentiate into effector cells that can provide B-cell help, thereby they generate differentiation and production of both B and T memory cells. In this conjugated vaccine, T-cell cytokines contribute to inducing various immunoglobulin (IgG) subclass patterns (89). The T-cell cytokine profile and IgG subclass response are dependent on the pneumococcal serotype (89). The capsular (non-conjugated) saccharide vaccine is also able to induce an immunoglobulin class switch from IgM to IgG, which would not be expected from a T-cell independent antigen. This could indicate that the different capsular saccharides may have different ability to induce immunoglobulin class switch via T-cells, NK-cells and monocytes. The capsule composition in the conjugated vaccine may affect the levels of the protective antibodies after vaccination. Pneumococcal pulmonary inflammation can be associated with difficulty to increase the antibody concentration against specific serotypes after vaccination (90).

The results from the cytokine release experiments exhibited a pattern consistent with the immune cell activation measured as CD69 expression, i.e., CWPS induced cytokine release to the highest extent (with the exception of IL-8 where type 9 and type 23 were more potent), followed by capsule type 23 and in a descending scale type 9 and type 3. The results show that the capsules differed in their ability to trigger cytokine release after in vitro stimulation of whole blood. The cytokines included in the study were chosen in order to analyze a broad spectrum of leukocyte functions, albeit with a limited number of cytokines
In the study of whole blood stimulation, TNF most likely activated a variety of cells, such as monocytes, neutrophils, $CD4^{pos}$ T-cells and NK-cells. TNF is known to be involved in acute phase reactions and has many proinflammatory effects.

INF-ɣ has been shown to be produced by activated T-cells and NK-cells and in turn stimulates macrophages, leading to increased cytokine synthesis, increased phagocytosis and increased antigen presentation. IL-8 may be produced by monocytes and attracts neutrophils. IL-10 can be produced by T-cells and monocytes and also additional cells. It is an anti-inflammatory cytokine that inhibits e.g. T-cell responses. In vivo, pneumococcal components have been shown to be capable of activating immune cells to release cytokines, for example macrophages. The ability to induce IL-10 differs between the different pneumococcal capsules. Increased IL-10 may have a negative effect on the host immune response to vaccination.

Generally, when evaluating the effect of vaccination, the overall IgG concentration is measured in serum. In the presented study a more detailed analysis is offered of how the different immune cells and cytokine secretion patterns are affected by the various pneumococcal capsules and CWPS. As shown, CWPS activates the immune cells to a greater extent than the capsules. In turn, the capsules activate the immune cells differently. Cytokine release followed the same pattern as capsule-induced cell activation. As the three capsules studied were randomly selected, we believe that the proven differences in ability to stimulate immune cells can be observed also for other capsules.

In the current study we have included analysis of blood monocytes, which may be expected to mirror aspects of the activation of other TLR-expressing cells, such as dendritic cells and macrophages in tissue, in conjunction with pneumococcal infection or vaccination.This study increases the understanding of how pneumococcal vaccination and pneumococcal exposure affect human immune cells. There were observed variations in immune stimulation capacity among the various vaccine saccharide components. The results shown may partly explain variations in the effectiveness of the different capsule components of the vaccine.

Article IV (manuscript)

In this study, isolated monocytes and NK-cells were studied for their regulation of inflammatory mediators and other related immune mechanisms induced by LPS, Pam3CSK4, and CWPS from *S. Pneumoniae*.

The results showed that stimulation with CWPS upregulates the gene expression of several pro-inflammatory key mediators, including *IL-1β, IL-6, CCL2* and *CXCL8*, in a dose-dependent manner. This finding is consistent with previous studies that have shown that NFkβ regulate the pro-inflammatory response of pneumococci via TLR2 (91). CWPS, a TLR2 ligand, was compared to other TLR ligands, e.g. LPS (TLR4) and Pam3CSK4 (TLR2) respectively, for gene expression of pro-inflammatory genes in isolated monocytes. The results show that all the TLR ligands examined induce upregulation of *IL-1β*, *IL-6*, *CCL2, CXCL8* and *CD80* in monocytes, i.e. the ligands exhibit similar gene expression profile. In a previous study, we have shown that NK-cells are strongly activated after whole blood incubation with CWPS (92). Here we can confirm that CWPS induces gene expression for *CXCL8* in isolated NK-cells, demonstrating a direct CWPS activation of the NK-cells, presumably via TLR2. However, the overall effect of CWPS activation of NK-cells needs to be further investigated.

We have also shown previously (92) that CWPS activate monocytes, after whole blood stimulation. We thus investigated if CWPS and the other TLR ligands induced secretion of inflammatory mediators, in isolated monocytes. All TLR ligands induced released of IL-1β, IL-6, TNF, and the chemokines CCL2 and CXCL8 in the supernatant. This confirmed our previous finding of TNF secretion following CWPS stimulation in whole blood (92). One additional observation in the current study is that none of the TLR ligands induced detectable gene expression for *TNF* in monocytes, however TNF was nevertheless found in the supernatant. Since *TNF* is a known early response gene, it is thus likely that the upregulation of the *TNF* gene had already occurred, and again normalized, at the time of analysis.

We further studied the CWPS and the two other TLR ligands to analyze if they induced different responses between smokers and non-smokers. The results showed that the gene expression for *TNF* differed significantly between smokers and non-smokers. TLR ligands induced upregulation of the *TNF* gene in cells from smokers. After TLR2 stimulation the smokers remained unchanged. CWPS stimulation of isolated monocytes from smokers showed higher expression of *IL-6, CCL5* and *CD80*. Other TLR ligands, e.g. LPS and Pam3CSK4, exhibited similar but non-significant results in monocytes from smokers, with increased gene expression for inflammatory mediators.

However, no other inflammatory mediators showed increased concentration in supernatants from smokers, compared to non-smokers, thus showing a distinct different pattern compared to the gene expression. It is therefore likely that the separate mechanisms that regulate expression and secretion, are differently affected by cigarette smoking. Stimulation of monocytes with LPS induced a significantly higher concentration of CXCL8 in the supernatant of cells from smokers, compared to non-smokers, which could increase the recruitment of inflammatory cells.

Between smokers and nonsmokers, there was no observed difference in gene expression of *TLR2* and *TLR4*, although differences might not be directly associated with receptor expression. In contrast, a recently published study showed that smokers expressed higher levels of TLR2 after stimulation of PBMC, compared to non-smokers (93).

CD14 expression differ significantly between smokers and non-smokers, but *CD16* expression was unchanged. The surface markers *CD14* and *CD16* are used for the identification and characterization of peripheral blood monocytes. It has previously been shown that there are different monocyte populations, the classical (CD14 high) and the non-classical (CD14 low). The non-classical population (about 10% of the total monocyte population) yields higher TNF levels after TLR stimulation (94, 95)**.** The reduction of CD14 expression from smokers in unstimulated monocytes indicated an imbalance between classical monocytes. In this study, binding to CD14 or CD16 was not used in the selection of the monocytes, as such procedures may lead to cell activation (96, 97), and instead a method based on a negative selection was used. The monocytes can however also be activated by handling, isolation and incubation with medium only (98, 99).

TLR signaling in innate immune cells can be indirectly regulated by the SOCS (suppressors of cytokine signaling) family (100). SOCS 1, 2, and 3 are inhibitors of the TLR signaling, primarily by binding to molecules downstream of myD88-dependent NF-kB activation (101-103). Cytokine expression induced by TLR is negatively correlated to SOCS-1 expression. It is believed that the lower the cytokine expression detected, the lower is the SOCS1 expression, which is shown in alveolar macrophages in patients with COPD (104).
In the current study, where monocytes from smokers were stimulated by TLR ligands, the relative gene expressions for both *SOCS-1* and pro-inflammatory mediators (including *IL-6, TNF* and *CCL5)* were higher in smokers compared to that observed in non-smokers.
This suggest that smoking induce a dysregulation downstream of the TLR activation in order to affect common mechanisms inducing the expression of both SOCS-1 and proinflammatory mediators. This may suggest that smoke exposure could also affect the SOCS-1 mediated inhibition of NF-kB activation, making the inhibition less efficient.

Monocyte upregulation of pro-inflammatory mediators in smokers may provide clues for the increased pneumococci infection risk in this population. Human epithelial cells in the respiratory and nasopharynx tracts, as well as in other tissues, express surface proteins including platelet activating factor receptor (PAFr), polymertic immunoglobulin receptor (plgR), and plaelet endothelial cell adhesion molecule-1 (PECAM) (59, 61, 105), which act as host cell receptors for pneumococci, and thereby may enable ahesion and invasion. Pro-inflammatory cytokines, including TNF and IL-1, have been shown to upregulate the transcription of PAFR and plgR expression (24, 106, 107). In the present study, we have described an upregulation of pro-inflammatory mediators, including TNF, in smokers. This may lead to an increased receptor expression for adhesion and subsequent invasion of pneumococci.

We have shown here that CWPS from *S. Pneumoniae* is an effective activator of monocytes and NK cells, and that it induces transcription and secretion of pro-inflammatory mediators, similar to other TLR ligands investigated. Monocytes from smokers and non-smokers showed a different expression of inflammatory mediators and the cells from smokers exhibited a pronounced expression. Systemic innate immune response is affected by cigarette smoke, even in moderate, young smokers with normal lung function.

FUTURE PERSPECTIVES

As shown in this thesis, capsular saccharides generate different immune responses. It would be of interest to investigate the immune response to all capsules included in the pneumococcal vaccine. This may be of importance for future vaccine development. In addition, examination of CWPS impact for the respective capsule could be of interest.

The upregulated immune response in smokers would be of interest to study further. Even though the results in the present work detected an imbalance in the system, the true impact of smoking and pneumococcal disease was not fully described. Investigating proposed pneumococci receptors on cell lines for the upregulation of adhesions molecules after cytokine or smoke exposure could better clarify the mechanisms.

Special population cohorts are risk groups for IPD. Among other groups immunocompromised patients are frequently represented. This population (e.g. lung transplanted patients) should be investigated according to immunologic responses. Also, what vaccine yields best protective results and also what are the immunological responses to vaccination in this cohort could be studied.

CONCLUSIONS

- An ELISA was developed in order to evaluate the binding of *S Pneumoniae* to two previously suggested receptors.
- An uncapsulated *S Pneumoniae* strain adhered to a greater extent to asialo-GM1 than to lactotriaocylceramide receptors.
- The uncapsulated strain of *S Pneumoniae* adhered with high efficiency to asialo-GM1, while two capsulated pneumococcal strains adhered with lower efficiency. This indicated that both capsulated and uncapsulated *S Pneumoniae* are capable of binding to asialo-GM1.
- The adhering ligand from *S Pneumoniae* responsible for the binding to asialo-GM1 was purified and identified as cell wall polysaccharide (CWPS).
- Phosphoryl choline residues in the pneumococcal CWPS are essential for the interaction between CWPS and the receptor asialo-GM1.
- Among the three capsules investigated, capsule type 23 induced the strongest activation and cytokine release, followed by type 9 and type 3.
- After stimulation with the different capsules, NK cells and NK-like T cells exhibited the strongest activation, followed by monocytes.
- CWPS is an effective activator of immune cells that induces transcription and secretion of pro-inflammatory mediators, similar to other TLR ligands investigated.
- After incubation with CWPS, monocytes from smokers exhibited an increased upregulation of pro-inflammatory mediators compared to non-smokers.
- Non-smoker monocytes showed downregulation of immunoregulatory molecules IL-10 and SOCS-1, following CWPS stimulation, while this was not detected in cells from smokers.
- Immune response may directly upregulate the transcription of pneumococcal receptors expressed on the host cell surface, which could contribute to increased binding of pneumococci.

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References

1 Austrian R. Pneumococcus: the first one hundred years. Rev Infect Dis. 1981;3(2):183-9.

2 Macleod CM, Hodges RG, Heidelberger M, Bernhard WG. Prevention of Pneumococcal Pneumonia by Immunization with Specific Capsular Polysaccharides. J Exp Med. 1945;82(6):445-65.

3 Lobanovska M, Pilla G. Penicillin's Discovery and Antibiotic Resistance: Lessons for the Future? Yale J Biol Med. 2017;90(1):135-45.

4 Griffith F. The Significance of Pneumococcal Types. J Hyg (Lond). 1928;27(2):113-59.

5 Avery OT, Macleod CM, McCarty M. Studies on the Chemical Nature of the Substance Inducing Transformation of Pneumococcal Types : Induction of Transformation by a Desoxyribonucleic Acid Fraction Isolated from Pneumococcus Type Iii. J Exp Med. 1944;79(2):137-58.

6 Mortality GBD, Causes of Death C. Global, regional, and national life expectancy, all-cause mortality, and cause-specific mortality for 249 causes of death, 1980-2015: a systematic analysis for the Global Burden of Disease Study 2015. Lancet. 2016;388(10053):1459-544.

7 Henriques-Normark B, Tuomanen EI. The pneumococcus: epidemiology, microbiology, and pathogenesis. Cold Spring Harb Perspect Med. 2013;3(7).

8 McCullers JA, McAuley JL, Browall S, Iverson AR, Boyd KL, Henriques Normark B. Influenza enhances susceptibility to natural acquisition of and disease due to Streptococcus pneumoniae in ferrets. J Infect Dis. 2010;202(8):1287-95.

9 Galanis I, Lindstrand A, Darenberg J, Browall S, Nannapaneni P, Sjostrom K, et al. Effects of PCV7 and PCV13 on invasive pneumococcal disease and carriage in Stockholm, Sweden. Eur Respir J. 2016;47(4):1208-18.

10 Obregon V, Garcia P, Garcia E, Fenoll A, Lopez R, Garcia JL. Molecular peculiarities of the lytA gene isolated from clinical pneumococcal strains that are bile insoluble. J Clin Microbiol. 2002;40(7):2545-54.

11 Henrichsen J. Six newly recognized types of Streptococcus pneumoniae. J Clin Microbiol. 1995;33(10):2759-62.

12 Geno KA, Gilbert GL, Song JY, Skovsted IC, Klugman KP, Jones C, et al. Pneumococcal Capsules and Their Types: Past, Present, and Future. Clin Microbiol Rev. 2015;28(3):871-99.

13 Hyams C, Camberlein E, Cohen JM, Bax K, Brown JS. The Streptococcus pneu-

moniae capsule inhibits complement activity and neutrophil phagocytosis by multiple mechanisms. Infect Immun. 2010;78(2):704-15.

14 Avery OT, Dubos R. The Protective Action of a Specific Enzyme against Type Iii Pneumococcus Infection in Mice. J Exp Med. 1931;54(1):73-89.

15 Sandgren A, Albiger B, Orihuela CJ, Tuomanen E, Normark S, Henriques-Normark B. Virulence in mice of pneumococcal clonal types with known invasive disease potential in humans. J Infect Dis. 2005;192(5):791-800.

16 Brueggemann AB, Peto TE, Crook DW, Butler JC, Kristinsson KG, Spratt BG. Temporal and geographic stability of the serogroup-specific invasive disease potential of Streptococcus pneumoniae in children. J Infect Dis. 2004;190(7):1203-11.

17 Serrano I, Melo-Cristino J, Ramirez M. Heterogeneity of pneumococcal phase variants in invasive human infections. BMC Microbiol. 2006;6:67.

18 Kim JO, Weiser JN. Association of intrastrain phase variation in quantity of capsular polysaccharide and teichoic acid with the virulence of Streptococcus pneumoniae. J Infect Dis. 1998;177(2):368-77.

19 Chudwin DS, Artrip SG, Korenblit A, Schiffman G, Rao S. Correlation of serum opsonins with in vitro phagocytosis of Streptococcus pneumoniae. Infect Immun. 1985;50(1):213-7.

20 Paton JC, Morona JK, Morona R. Characterization of the capsular polysaccharide biosynthesis locus of Streptococcus pneumoniae type 19F. Microb Drug Resist. 1997;3(1):89-99.

21 Bergmann S, Hammerschmidt S. Versatility of pneumococcal surface proteins. Microbiology. 2006;152(Pt 2):295-303.

22 Fischer W, Behr T, Hartmann R, Peter-Katalinic J, Egge H. Teichoic acid and lipoteichoic acid of Streptococcus pneumoniae possess identical chain structures. A reinvestigation of teichoid acid (C polysaccharide). Eur J Biochem. 1993;215(3):851-7.

23 Rosenow C, Ryan P, Weiser JN, Johnson S, Fontan P, Ortqvist A, et al. Contribution of novel choline-binding proteins to adherence, colonization and immunogenicity of Streptococcus pneumoniae. Mol Microbiol. 1997;25(5):819-29.

24 Cundell DR, Gerard NP, Gerard C, Idanpaan-Heikkila I, Tuomanen EI. Streptococcus pneumoniae anchor to activated human cells by the receptor for platelet-activating factor. Nature. 1995;377(6548):435-8.

25 Volanakis JE, Kaplan MH. Interaction of C-reactive protein complexes with the complement system. II. Consumption of guinea pig complement by CRP complexes: requirement for human C1q. J Immunol. 1974;113(1):9-17.

26 Eisenhardt SU, Thiele JR, Bannasch H, Stark GB, Peter K. C-reactive protein: how conformational changes influence inflammatory properties. Cell Cycle. 2009;8(23):3885-92.

27 Tuomanen E, Rich R, Zak O. Induction of pulmonary inflammation by components of the pneumococcal cell surface. Am Rev Respir Dis. 1987;135(4):869-74.

28 Tuomanen EI, Austrian R, Masure HR. Pathogenesis of pneumococcal infection. N Engl J Med. 1995;332(19):1280-4.

29 Paterson GK, Orihuela CJ. Pneumococci: immunology of the innate host response. Respirology. 2010;15(7):1057-63.

30 Albiger B, Dahlberg S, Sandgren A, Wartha F, Beiter K, Katsuragi H, et al. Toll-like receptor 9 acts at an early stage in host defence against pneumococcal infection. Cell Microbiol. 2007;9(3):633-44.

31 Wiese KM, Coates BM, Ridge KM. The Role of Nucleotide-Binding Oligomerization Domain-Like Receptors in Pulmonary Infection. Am J Respir Cell Mol Biol. 2017;57(2):151-61.

32 Opitz B, Puschel A, Schmeck B, Hocke AC, Rosseau S, Hammerschmidt S, et al. Nucleotide-binding oligomerization domain proteins are innate immune receptors for internalized Streptococcus pneumoniae. J Biol Chem. 2004;279(35):36426-32.

33 Davis KM, Nakamura S, Weiser JN. Nod2 sensing of lysozyme-digested peptidoglycan promotes macrophage recruitment and clearance of S. pneumoniae colonization in mice. J Clin Invest. 2011;121(9):3666-76.

34 Lee CJ, Lee LH, Frasch CE. Protective immunity of pneumococcal glycoconjugates. Crit Rev Microbiol. 2003;29(4):333-49.

35 Zhu J, Paul WE. CD4 T cells: fates, functions, and faults. Blood. 2008;112(5):1557-69.

36 Mold C, Du Clos TW. C-reactive protein increases cytokine responses to Streptococcus pneumoniae through interactions with Fc gamma receptors. J Immunol. 2006;176(12):7598-604.

37 Malley R. Antibody and cell-mediated immunity to Streptococcus pneumoniae: implications for vaccine development. J Mol Med (Berl). 2010;88(2):135-42.

38 Goldblatt D, Hussain M, Andrews N, Ashton L, Virta C, Melegaro A, et al. Antibody responses to nasopharyngeal carriage of Streptococcus pneumoniae in adults: a longitudinal household study. J Infect Dis. 2005;192(3):387-93.

39 Hoe E, Anderson J, Nathanielsz J, Toh ZQ, Marimla R, Balloch A, et al. The contrasting roles of Th17 immunity in human health and disease. Microbiol Immunol. 2017;61(2):49-56.

40 Tu AH, Fulgham RL, McCrory MA, Briles DE, Szalai AJ. Pneumococcal surface protein A inhibits complement activation by Streptococcus pneumoniae. Infect Immun. 1999;67(9):4720-4.

41 Lu L, Ma Z, Jokiranta TS, Whitney AR, DeLeo FR, Zhang JR. Species-specific interaction of Streptococcus pneumoniae with human complement factor H. J Immunol. 2008;181(10):7138-46.

42 Wartha F, Beiter K, Albiger B, Fernebro J, Zychlinsky A, Normark S, et al. Capsule and D-alanylated lipoteichoic acids protect Streptococcus pneumoniae against neutrophil extracellular traps. Cell Microbiol. 2007;9(5):1162-71.

43 Wright AE, Morgan W, Colebrook L., Dodgson R.W. Observations on prophylactic inoculation against pneumococcus infections, and on the results which have been achieved by it. . The Lancet. 1914(1):1-10, 87-95.

44 Sorensen UB, Henrichsen J. C-polysaccharide in a pneumococcal vaccine. Acta Pathol Microbiol Immunol Scand C. 1984;92(6):351-6.

45 Waight PA, Andrews NJ, Ladhani NJ, Sheppard CL, Slack MP, Miller E. Effect of the 13-valent pneumococcal conjugate vaccine on invasive pneumococcal disease in England and Wales 4 years after its introduction: an observational cohort study. Lancet Infect Dis. 2015;15(6):629.

46 Sjogren A, Lindholm B, Holme T. Availability of reaction with antibodies of the pneumococcal C-polysaccharide on the surface of capsulated pneumococci. Acta Pathol Microbiol Immunol Scand B. 1987;95(6):371-8.

47 Tomasz A. Biological consequences of the replacement of choline by ethanolamine in the cell wall of Pneumococcus: chanin formation, loss of transformability, and loss of autolysis. Proc Natl Acad Sci U S A. 1968;59(1):86-93.

48 Krivan HC, Roberts DD, Ginsburg V. Many pulmonary pathogenic bacteria bind specifically to the carbohydrate sequence GalNAc beta 1-4Gal found in some glycolipids. Proc Natl Acad Sci U S A. 1988;85(16):6157-61.

49 Andersson B, Leffler H, Magnusson G, Svanborg Eden C. Molecular mechanisms of adhesion of Streptococcus pneumoniae to human oropharyngeal epithelial cells. Scand J Infect Dis Suppl. 1983;39:45-7.

50 Andersson B, Porras O, Hanson LA, Lagergard T, Svanborg-Eden C. Inhibition of attachment of Streptococcus pneumoniae and Haemophilus influenzae by human milk and receptor oligosaccharides. J Infect Dis. 1986;153(2):232-7.

51 Jagannatha HM, Sharma UK, Ramaseshan T, Surolia A, Balganesh TS. Identifi-

cation of carbohydrate structures as receptors for localised adherent enteropathogenic Escherichia coli. Microb Pathog. 1991;11(4):259-68.

52 Courtney HS, von Hunolstein C, Dale JB, Bronze MS, Beachey EH, Hasty DL. Lipoteichoic acid and M protein: dual adhesins of group A streptococci. Microb Pathog. 1992;12(3):199-208.

53 Geelen S, Bhattacharyya C, Tuomanen E. The cell wall mediates pneumococcal attachment to and cytopathology in human endothelial cells. Infect Immun. 1993;61(4):1538-43.

54 Paton JC, Andrew PW, Boulnois GJ, Mitchell TJ. Molecular analysis of the pathogenicity of Streptococcus pneumoniae: the role of pneumococcal proteins. Annu Rev Microbiol. 1993;47:89-115.

55 Hietala J, Uhari M, Tuokko H, Leinonen M. Mixed bacterial and viral infections are common in children. Pediatr Infect Dis J. 1989;8(10):683-6.

56 Tong HH, Blue LE, James MA, DeMaria TF. Evaluation of the virulence of a Streptococcus pneumoniae neuraminidase-deficient mutant in nasopharyngeal colonization and development of otitis media in the chinchilla model. Infect Immun. 2000;68(2):921-4.

57 Karlsson KA. Animal glycosphingolipids as membrane attachment sites for bacteria. Annu Rev Biochem. 1989;58:309-50.

58 Radin JN, Orihuela CJ, Murti G, Guglielmo C, Murray PJ, Tuomanen EI. beta-Arrestin 1 participates in platelet-activating factor receptor-mediated endocytosis of Streptococcus pneumoniae. Infect Immun. 2005;73(12):7827-35.

59 Zhang JR, Mostov KE, Lamm ME, Nanno M, Shimida S, Ohwaki M, et al. The polymeric immunoglobulin receptor translocates pneumococci across human nasopharyngeal epithelial cells. Cell. 2000;102(6):827-37.

60 Iovino F, Molema G, Bijlsma JJ. Streptococcus pneumoniae Interacts with pIgR expressed by the brain microvascular endothelium but does not co-localize with PAF receptor. PLoS One. 2014;9(5):e97914.

61 Iovino F, Engelen-Lee JY, Brouwer M, van de Beek D, van der Ende A, Valls Seron M, et al. pIgR and PECAM-1 bind to pneumococcal adhesins RrgA and PspC mediating bacterial brain invasion. J Exp Med. 2017;214(6):1619-30.

62 Iovino F, Brouwer MC, van de Beek D, Molema G, Bijlsma JJ. Signalling or binding: the role of the platelet-activating factor receptor in invasive pneumococcal disease. Cell Microbiol. 2013;15(6):870-81.

63 Sampson JS, O'Connor SP, Stinson AR, Tharpe JA, Russell H. Cloning and nucleotide sequence analysis of psaA, the Streptococcus pneumoniae gene encoding a 37-kilodalton protein homologous to previously reported Streptococcus

sp. adhesins. Infect Immun. 1994;62(1):319-24.

64 Cundell DR, Tuomanen EI. Receptor specificity of adherence of Streptococcus pneumoniae to human type-II pneumocytes and vascular endothelial cells in vitro. Microb Pathog. 1994;17(6):361-74.

65 Sundberg-Kovamees M, Holme T, Sjogren A. Specific binding of Streptococcus pneumoniae to two receptor saccharide structures. Microb Pathog. 1994;17(1):63-8.

66 Sorensen UB, Henrichsen J, Chen HC, Szu SC. Covalent linkage between the capsular polysaccharide and the cell wall peptidoglycan of Streptococcus pneumoniae revealed by immunochemical methods. Microb Pathog. 1990;8(5):325-34.

67 Koenig VL, Perrings JD. Sedimentation and viscosity studies on the capsular and somatic polysaccharides of pneumococcus Type III. J Biophys Biochem Cytol. 1955;1(2):93-8.

68 Fischer H, Tomasz A. Peptidoglycan cross-linking and teichoic acid attachment in Streptococcus pneumoniae. J Bacteriol. 1985;163(1):46-54.

69 Gupta SK, Berk RS, Masinick S, Hazlett LD. Pili and lipopolysaccharide of Pseudomonas aeruginosa bind to the glycolipid asialo GM1. Infect Immun. 1994;62(10):4572-9.

70 Swanson AF, Kuo CC. Binding of the glycan of the major outer membrane protein of Chlamydia trachomatis to HeLa cells. Infect Immun. 1994;62(1):24-8.

71 Thornton JA, Durick-Eder K, Tuomanen EI. Pneumococcal pathogenesis: "innate invasion" yet organ-specific damage. J Mol Med (Berl). 2010;88(2):103-7.

72 Orihuela CJ, Mahdavi J, Thornton J, Mann B, Wooldridge KG, Abouseada N, et al. Laminin receptor initiates bacterial contact with the blood brain barrier in experimental meningitis models. J Clin Invest. 2009;119(6):1638-46.

73 Voss S, Hallstrom T, Saleh M, Burchhardt G, Pribyl T, Singh B, et al. The choline-binding protein PspC of Streptococcus pneumoniae interacts with the C-terminal heparin-binding domain of vitronectin. J Biol Chem. 2013;288(22):15614-27.

74 Gosink KK, Mann ER, Guglielmo C, Tuomanen EI, Masure HR. Role of novel choline binding proteins in virulence of Streptococcus pneumoniae. Infect Immun. 2000;68(10):5690-5.

75 Barocchi MA, Ries J, Zogaj X, Hemsley C, Albiger B, Kanth A, et al. A pneumococcal pilus influences virulence and host inflammatory responses. Proc Natl Acad Sci U S A. 2006;103(8):2857-62.

76 Sanchez CJ, Shivshankar P, Stol K, Trakhtenbroit S, Sullam PM, Sauer K, et al. The pneumococcal serine-rich repeat protein is an intra-species bacterial adhesin that promotes bacterial aggregation in vivo and in biofilms. PLoS Pathog. 2010;6(8):e1001044.

77 Sen G, Khan AQ, Chen Q, Snapper CM. In vivo humoral immune responses to isolated pneumococcal polysaccharides are dependent on the presence of associated TLR ligands. J Immunol. 2005;175(5):3084-91.

78 Malley R, Srivastava A, Lipsitch M, Thompson CM, Watkins C, Tzianabos A, et al. Antibody-independent, interleukin-17A-mediated, cross-serotype immunity to pneumococci in mice immunized intranasally with the cell wall polysaccharide. Infect Immun. 2006;74(4):2187-95.

79 Wack A, Gallorini S. Bacterial polysaccharides with zwitterionic charge motifs: Toll-like receptor 2 agonists, T cell antigens, or both? Immunopharmacol Immunotoxicol. 2008;30(4):761-70.

80 Snapper CM. Differential regulation of protein- and polysaccharide-specific Ig isotype production in vivo in response to intact Streptococcus pneumoniae. Curr Protein Pept Sci. 2006;7(4):295-305.

81 Gray JD, Horwitz DA. Activated human NK cells can stimulate resting B cells to secrete immunoglobulin. J Immunol. 1995;154(11):5656-64.

82 Yuan D, Bibi R, Dang T. The role of adjuvant on the regulatory effects of NK cells on B cell responses as revealed by a new model of NK cell deficiency. Int Immunol. 2004;16(5):707-16.

83 Tian H, Groner A, Boes M, Pirofski LA. Pneumococcal capsular polysaccharide vaccine-mediated protection against serotype 3 Streptococcus pneumoniae in immunodeficient mice. Infect Immun. 2007;75(4):1643-50.

84 Kobrynski LJ, Sousa AO, Nahmias AJ, Lee FK. Cutting edge: antibody production to pneumococcal polysaccharides requires CD1 molecules and CD8+ T cells. J Immunol. 2005;174(4):1787-90.

85 Adib-Conquy M, Scott-Algara D, Cavaillon JM, Souza-Fonseca-Guimaraes F. TLR-mediated activation of NK cells and their role in bacterial/viral immune responses in mammals. Immunol Cell Biol. 2014;92(3):256-62.

86 Saikh KU, Lee JS, Kissner TL, Dyas B, Ulrich RG. Toll-like receptor and cytokine expression patterns of CD56+ T cells are similar to natural killer cells in response to infection with Venezuelan equine encephalitis virus replicons. J Infect Dis. 2003;188(10):1562-70.

87 Xu Q, Abeygunawardana C, Ng AS, Sturgess AW, Harmon BJ, Hennessey JP, Jr. Characterization and quantification of C-polysaccharide in Streptococcus pneumoniae capsular polysaccharide preparations. Anal Biochem.

2005;336(2):262-72.

88 Karlsson C, Jansson PE, Skov Sorensen UB. The pneumococcal common antigen C-polysaccharide occurs in different forms. Mono-substituted or di-substituted with phosphocholine. Eur J Biochem. 1999;265(3):1091-7.

89 Mawas F, Feavers IM, Corbel MJ. Serotype of Streptococcus pneumoniae capsular polysaccharide can modify the Th1/Th2 cytokine profile and IgG subclass response to pneumococal-CRM(197) conjugate vaccines in a murine model. Vaccine. 2000;19(9-10):1159-66.

90 Ortqvist A, Henckaerts I, Hedlund J, Poolman J. Non-response to specific serotypes likely cause for failure to 23-valent pneumococcal polysaccharide vaccine in the elderly. Vaccine. 2007;25(13):2445-50.

91 Sundaram K, Rahman MA, Mitra S, Knoell DL, Woodiga SA, King SJ, et al. IkappaBzeta Regulates Human Monocyte Pro-Inflammatory Responses Induced by Streptococcus pneumoniae. PLoS One. 2016;11(9):e0161931.

92 Sundberg-Kovamees M, Grunewald J, Wahlstrom J. Immune cell activation and cytokine release after stimulation of whole blood with pneumococcal C-polysaccharide and capsular polysaccharides. Int J Infect Dis. 2016;52:1-8.

93 Gaydos J, McNally A, Guo R, Vandivier RW, Simonian PL, Burnham EL. Alcohol abuse and smoking alter inflammatory mediator production by pulmonary and systemic immune cells. Am J Physiol Lung Cell Mol Physiol. 2016;310(6):L507-18.

94 Belge KU, Dayyani F, Horelt A, Siedlar M, Frankenberger M, Frankenberger B, et al. The proinflammatory CD14+CD16+DR++ monocytes are a major source of TNF. J Immunol. 2002;168(7):3536-42.

95 Frankenberger M, Sternsdorf T, Pechumer H, Pforte A, Ziegler-Heitbrock HW. Differential cytokine expression in human blood monocyte subpopulations: a polymerase chain reaction analysis. Blood. 1996;87(1):373-7.

96 Schutt C, Ringel B, Nausch M, Bazil V, Horejsi V, Neels P, et al. Human monocyte activation induced by an anti-CD14 monoclonal antibody. Immunol Lett. 1988;19(4):321-7.

97 Shalova IN, Kajiji T, Lim JY, Gomez-Pina V, Fernandez-Ruiz I, Arnalich F, et al. CD16 regulates TRIF-dependent TLR4 response in human monocytes and their subsets. J Immunol. 2012;188(8):3584-93.

98 Kelley JL, Rozek MM, Suenram CA, Schwartz CJ. Activation of human blood monocytes by adherence to tissue culture plastic surfaces. Exp Mol Pathol. 1987;46(3):266-78.

99 Elkord E, Williams PE, Kynaston H, Rowbottom AW. Human monocyte isola-

tion methods influence cytokine production from in vitro generated dendritic cells. Immunology. 2005;114(2):204-12.

100 Baetz A, Frey M, Heeg K, Dalpke AH. Suppressor of cytokine signaling (SOCS) proteins indirectly regulate toll-like receptor signaling in innate immune cells. J Biol Chem. 2004;279(52):54708-15.

101 Kinjyo I, Hanada T, Inagaki-Ohara K, Mori H, Aki D, Ohishi M, et al. SOCS1/JAB is a negative regulator of LPS-induced macrophage activation. Immunity. 2002;17(5):583-91.

102 Nakagawa R, Naka T, Tsutsui H, Fujimoto M, Kimura A, Abe T, et al. SOCS-1 participates in negative regulation of LPS responses. Immunity. 2002;17(5):677-87.

103 Posselt G, Schwarz H, Duschl A, Horejs-Hoeck J. Suppressor of cytokine signaling 2 is a feedback inhibitor of TLR-induced activation in human monocyte-derived dendritic cells. J Immunol. 2011;187(6):2875-84.

104 Dong R, Xie L, Zhao K, Zhang Q, Zhou M, He P. Cigarette smoke-induced lung inflammation in COPD mediated via LTB4/BLT1/SOCS1 pathway. Int J Chron Obstruct Pulmon Dis. 2016;11:31-41.

105 Fillon S, Soulis K, Rajasekaran S, Benedict-Hamilton H, Radin JN, Orihuela CJ, et al. Platelet-activating factor receptor and innate immunity: uptake of gram-positive bacterial cell wall into host cells and cell-specific pathophysiology. J Immunol. 2006;177(9):6182-91.

106 Takenouchi-Ohkubo N, Moro I, Mukae S, Kaneko Y, Komiyama K. Tumour necrosis factor-alpha-mediated human polymeric immunoglobulin receptor expression is regulated by both mitogen-activated protein kinase and phosphatidylinositol-3-kinase in HT-29 cell line. Immunology. 2008;123(4):500-7.

107 Bruno ME, Frantz AL, Rogier EW, Johansen FE, Kaetzel CS. Regulation of the polymeric immunoglobulin receptor by the classical and alternative NF-kappaB pathways in intestinal epithelial cells. Mucosal Immunol. 2011;4(4):468-78.

108 Goonetilleke UR, Ward SA, Gordon SB. Could proteomic research deliver the next generation of treatments for pneumococcal meningitis? Interdiscip Perspect Infect Dis. 2009;2009:214216.

109 McCullers JA, Tuomanen EI. Molecular pathogenesis of pneumococcal pneumonia. Front Biosci. 2001;6:D877-89.